LONDON'S GLOBAL UNIVERSITY

Why do eggs fail?

An investigation into hatching failure in wild and managed bird populations

A thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

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Declaration

I, Ashleigh Fleming Marshall, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

A 2022 assessment by *BirdLife International* found that around 13% of bird species are threatened with extinction, with an additional 9% considered Near Threatened. Conservation programmes worldwide work to protect threatened species, often establishing managed populations undergoing a range of conservation interventions as part of strategies to bring species “back from the brink”. However, an issue facing many managed threatened populations is a high incidence of hatching failure. Despite birds being among the most well-studied taxa, and improving reproductive success often being of major interest to conservation programmes, there are gaps in our understanding of the causes and underlying drivers of hatching failure in both wild and managed populations of threatened species. In my PhD, I aim to improve our understanding of why eggs fail to hatch and explore how this knowledge can be used to inform management of the species most at risk from extinction.

First, I conduct a comprehensive meta-analysis examining the associations between threat status, management interventions, and avian hatching failure, finding an updated estimate of the mean overall hatching failure rate and conclusively showing that threatened and managed bird populations experience elevated rates of hatching failure. Next, by applying methods of accurately determining the fertility of unhatched eggs to several different managed populations I demonstrate how fertilisation failure rates can be overestimated, potentially leading to the application of non-optimal mitigation strategies. Following this, I characterise and compare the eggshell microbiomes of two threatened species, finding the first evidence of an effect of captivity, as well as several influential parental and environmental variables. Finally, I analyse the relationship between these threatened species’ eggshell microbiomes and hatching failure, showing that trans-shell infection may not be a major driver of hatching failure in these species and demonstrating the importance of investigating species- and population-specific drivers of hatching failure to best inform management efforts.

Impact Statement

Hatching failure is a well-known but poorly understood problem for many bird populations. Through this thesis I have increased our understanding of avian reproductive failure, particularly in threatened and non-model species, and have produced an updated estimate of the mean avian hatching failure rate showing it is higher than previously estimated. I have shown that global rates of avian reproductive failure vary with threat status, indicating that hatching success declines with increasing risk of extinction and highlighting the need to investigate the drivers underlying hatching failure in declining populations. I have also revealed that some techniques widely used in avian research may impact early stages of development, with a need to take these into account during studies of reproduction. I have demonstrated inconsistencies in hatching failure terminology throughout the scientific literature, leading to issues when comparing across studies, and have proposed the use of a standardised definition. My findings are not only relevant to conservation science and to birds, but also to other fields such as population biology, behavioural ecology, and evolutionary ecology, and to non-avian, oviparous, taxa.

Beyond academia, I have demonstrated that differentiation between fertilisation failure and embryo mortality can be incredibly valuable in conservation settings to inform best practice and support practitioners making decisions regarding appropriate management interventions to mitigate hatching failure. The mean failure rates for populations under different management and threat levels established in my systematic review of hatching failure could be used by conservation practitioners to assess the potential effectiveness of different management scenarios and aid in conservation decision-making. I have added support to growing evidence that true infertility in managed populations is likely to be overestimated and have shown that early embryo mortality is likely a bigger issue in managed populations than previously recognised, hence should be a greater focus of efforts to reduce reproductive failures. I have helped to create guidelines and protocols to be used by practitioners to apply the techniques used in this thesis more generally, and have reported my egg fertility diagnoses back to the relevant conservation programmes where they have already been used to support breeding management decisions.

To bring about impact from my thesis I have disseminated my research as two peer-reviewed publications, (Assersohn *et al.*, 2021b) and (Marshall *et al.*, 2023), the latter of which was covered in several online news outlets. I have helped to create online resources increasing accessibility to the fluorescence microscopy methods and have helped to deliver training directly. I have presented various aspects of my research at in-person and online student, national, and international academic and conservation conferences and public outreach events, and have spoken about my research to schools. My work has featured in two ZSL newsletters, and I also wrote a blog about unhatched eggs for the ZSL website to accompany a public avian hatching failure event. I have also communicated about my research on Twitter, posting threads about my publications and to accompany conference presentations, plus taking part in online science communication campaigns and Twitter takeovers.

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  AFM, FB, and PB conceived the initial idea for the study. AFM carried out the literature search, data extraction, and all analyses with support and advice from NH and PB. AFM, NH, and PB interpreted the results. AFM wrote the manuscript and prepared all figures, tables, and supplementary material with support from NH, PB, and FB. All authors contributed to responding to reviewer comments and AFM completed the manuscript revisions.

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Glossary

Abbreviations

AIC	Akaike's information criterion
ASV	Amplicon sequence variants
DOC	[New Zealand] Department of Conservation
ICWT	Isaac Conservation and Wildlife Trust
IUCN	International Union for Conservation of Nature
NZTCS	New Zealand Threat Classification System
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PCoA	Principal coordinate analysis
PVL	Perivitelline layer
UC	University of Canterbury
ZIMS	Zoological Information Management System
ZSL	Zoological Society of London

Te Reo Māori Terms

Aotearoa	New Zealand
Hapū	Subtribe
Iwi	Tribe
Māori	Indigenous people of New Zealand
Taonga	Treasure, culturally significant
Te Reo Māori	The Māori language

Where applicable, Te Reo Māori names for endemic Aotearoa New Zealand species are used throughout this thesis, with the English name and scientific name used only on the first instance.

Chapter 1: Introduction

Hatching failure is recognised as one of the most prevalent issues affecting bird populations worldwide, with clear implications for threatened species in which early reproductive losses can severely limit the effectiveness of conservation management. However, despite the long history of research into avian hatching failure, there are substantial gaps in our understanding of why eggs fail in different populations, and subsequently how best to address any problems through conservation actions. In my thesis, I begin by systematically reviewing existing research on avian hatching failure, before answering several unresolved questions using empirical data. Throughout the thesis, I also identify important avenues for further research. In this first chapter, I introduce my thesis and put my research in context by discussing the background of avian hatching failure in wild and managed populations. I then outline my research focus and its relevance to the field, and provide a brief overview of the thesis structure.

Background

The importance of birds

There are approximately 11,000 species of birds on Earth, found on every continent and across a massive range of habitats. Their ubiquity and phenomenal variety in appearance, ecology, and behaviour makes them incredibly prominent in scientific research, with more known about birds than any other comparable group of organisms (BirdLife International, 2022a). Birds also have a long history of importance in culture, art, and recreation, and provide key ecosystem services including pollination, food security and pest control (Whelan *et al.*, 2015; BirdLife International, 2022a). However, birds are in trouble. Since the start of the 16th century, at least 187 bird species are estimated to have been lost, with one in eight bird species worldwide currently threatened with extinction (BirdLife International, 2022a). The IUCN Red List Index indicates an overall increase in bird extinction risk over the last three decades (BirdLife International, 2022a; IUCN, 2022). Extinctions not only impact biodiversity, but can also have devastating social, cultural, and economic consequences due to effects on birdwatching, ‘avitourism’, and other bird-

mediated ecosystem services (Şekercioğlu *et al.*, 2004; Steven *et al.*, 2015; Whelan *et al.*, 2015; BirdLife International, 2022a). The majority of the threats birds are facing are anthropogenic in origin, including over-hunting, habitat loss, the introduction of invasive species, and climate change (Duncan *et al.*, 2002; Davies *et al.*, 2006; Jetz *et al.*, 2007; Aronson *et al.*, 2014; Benítez-López *et al.*, 2017; BirdLife International, 2022a), with a high likelihood that a growing number of species will become threatened in their natural habitats over the coming years and decades.

Conservation programmes

The plight of birds has led to the establishment of conservation programmes worldwide that are focused on protecting threatened species and increasing their population sizes. These programmes range in intensity from monitoring and *in situ* management of wild populations, through to *ex situ* practices such as captive-breeding, often considered to be more invasive and usually reserved for species at the most immediate risk of extinction (Williams *et al.*, 2013). Throughout this thesis, I classify populations under different levels of management as: (a) ‘wild’, i.e. populations in which the adults live in a natural setting with no management interventions applied, with the exception of monitoring methods such as nest checks; (b) ‘wild managed’, i.e. populations in which adults live in a natural setting with management interventions applied such as artificial nestbox provision, supplementary feeding, and egg manipulations such as the removal of eggs for artificial incubation; and (c) ‘captive’, i.e. populations in which adults are maintained in captivity and eggs are laid, hatched, and reared in captivity, inclusive of any other management interventions such as artificial insemination or artificial incubation.

The main goal of captive-breeding is often to establish and maintain populations in captivity without allowing domestication, so that captive-bred individuals can be released into the wild to augment existing populations, or establish new ones, either within the species’ former home range or in a new suitable area (Jones, 2004; Seddon, 2010; Williams *et al.*, 2013). Such translocations – a term which also encompasses the capture, movement, and release of wild birds to augment or establish populations – have been used with varying degrees of success for several high-profile threatened birds (e.g. Bunin *et al.*, 1997;

Aikman *et al.*, 2001; Cristinacce *et al.*, 2009). However, the life history or mating systems of some species make them unsuitable for captivity, hence interventions may be restricted to *in situ* management and translocations of wild birds even for highly threatened species, for example the Critically Endangered lek-breeding parrot, the kākāpō (*Strigops habroptilus*) (Lloyd & Powlesland, 1994; Clout & Merton, 1998) and the Vulnerable polygynandrous passerine, the hihi (stitchbird; *Notiomystis cincta*) (Ewen *et al.*, 2011), one of my main study species.

The most successful conservation programmes may be those that implement long-term strategies targeting the ultimate causes of population declines, alongside strategies aimed at increasing populations as quickly as possible by addressing the proximate demographic factors, i.e. poor survival and reproduction (Jones, 2004). Manipulating the productivity of non-commercial breeding birds is a relatively recent practice, with methods still being developed, tested, and implemented (Jones, 2004). Some of the pioneers of these intensive management interventions, particularly with wild birds, are conservation programmes in Aotearoa New Zealand, where two of my study species are located.

The problem of hatching failure

A major issue often affecting the overall reproductive success of threatened bird species is hatching failure, i.e. the proportion of eggs present at the end of the incubation period that fail to hatch, excluding eggs lost due to predation, desertion, accident, extreme weather, or that disappeared during the incubation period due to unknown factors. While hatching failure is common across avian species, it can be devastating for threatened species, with rates of failure of over 60% documented in some populations (Jamieson & Ryan, 2000; Briskie & Mackintosh, 2004; Harvey *et al.*, 2004; Ferreira *et al.*, 2005; Aourir *et al.*, 2008; Heber & Briskie, 2010; Burnett *et al.*, 2013; Taylor *et al.*, 2017).

Despite the long history of research into avian hatching failure in both wild and managed populations, better estimates are still needed and there is a lack of empirical evidence into the underlying drivers (Assersohn *et al.*, 2021b). As part of a systematic review of hatching failure (**Chapter 2**; Marshall *et al.*, 2023), I

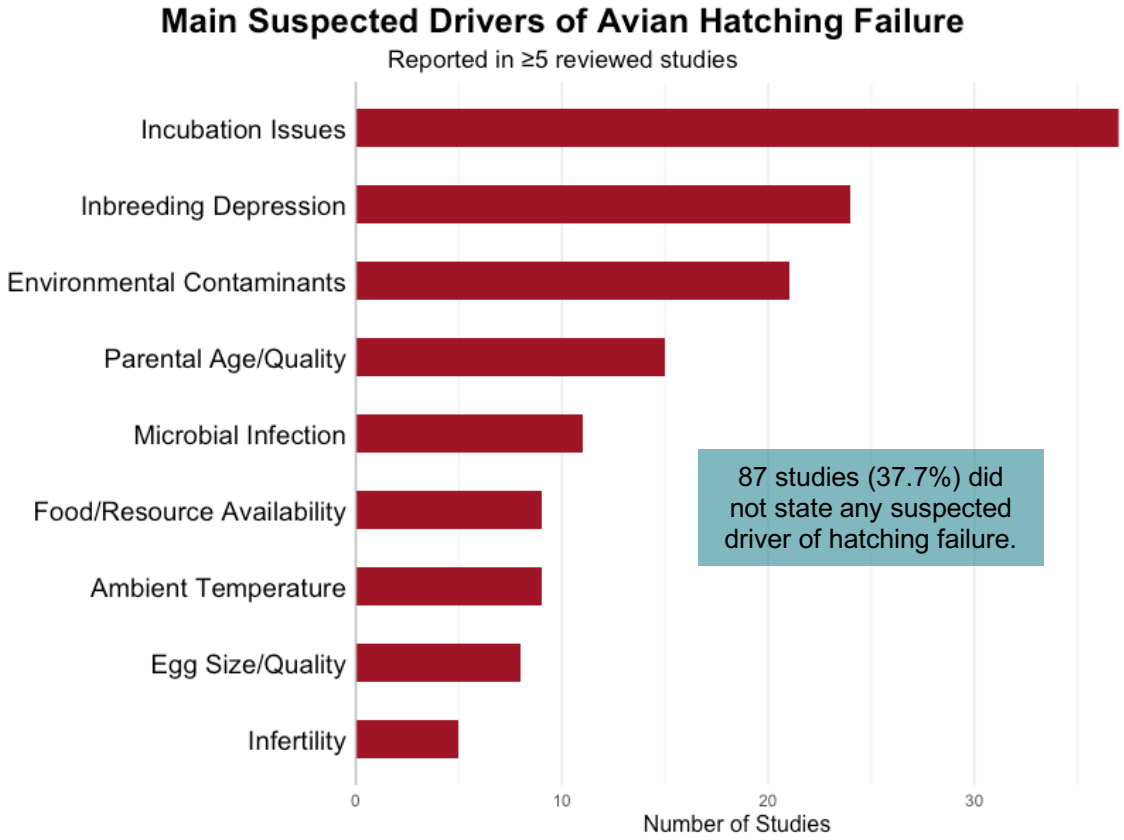

Figure 1-1. A summary of the most frequently reported suspected drivers of hatching failure across 231 studies included in a systematic review and meta-analysis of hatching failure (**Chapter 2**). Drivers were not always actually tested within the study, and if they were tested, their effect was not always supported by the results.

recorded and categorised the main suspected drivers of hatching failure as stated by the study authors (**Figure 1-1**). In many cases, the study either did not test for a relationship between the suspected driver and hatching failure within the context of the study, or, when tested, results did not provide support for the hypothesised effect. This implies that studies may often make assumptions about the underlying drivers of hatching failure within their study population based on findings from other populations or species, without any further investigation into potential population- or species-specific factors.

The elevated rates of hatching failure reported for threatened populations, which are often under some degree of conservation management, have also received surprisingly little investigation, with a particular paucity of research into the potential influence of common management interventions on hatching failure. Most current literature quantitatively assessing the effectiveness of different management interventions focuses primarily on effects on adult and nestling survival, often using fledgling success and recruitment into the

population as primary measures of effectiveness (Jones, 2004; Williams *et al.*, 2013; Sutherland *et al.*, 2018). When studies do assess hatching failure under different management strategies, the sample sizes are often too small to draw any significant conclusions. By focusing primarily on post-hatch individuals, management programmes are omitting a key stage of life and reproduction, and may therefore be limiting the efficacy of interventions designed to improve the productivity of threatened species (Grueber *et al.*, 2015; Farquharson *et al.*, 2017).

Fertilisation failure versus *embryo mortality*

Hatching failure results from either fertilisation failure (whereby sperm do not successfully fertilise ova), or embryo mortality (whereby a fertilised ovum fails to develop into a hatched chick) (Birkhead *et al.*, 2008). While embryo mortality can occur at any stage between fertilisation and hatching, studies from domesticated birds have indicated that embryo deaths typically occur either very early or late in the incubation period (Lodge *et al.*, 1971; Christensen, 2001; Sellier *et al.*, 2005; Birkhead *et al.*, 2008). Although later embryo deaths are usually easy to detect during a macroscopic visual examination, it can be difficult to discriminate between eggs that have failed due to fertilisation failure and those that have experienced early embryo mortality, i.e. death before signs

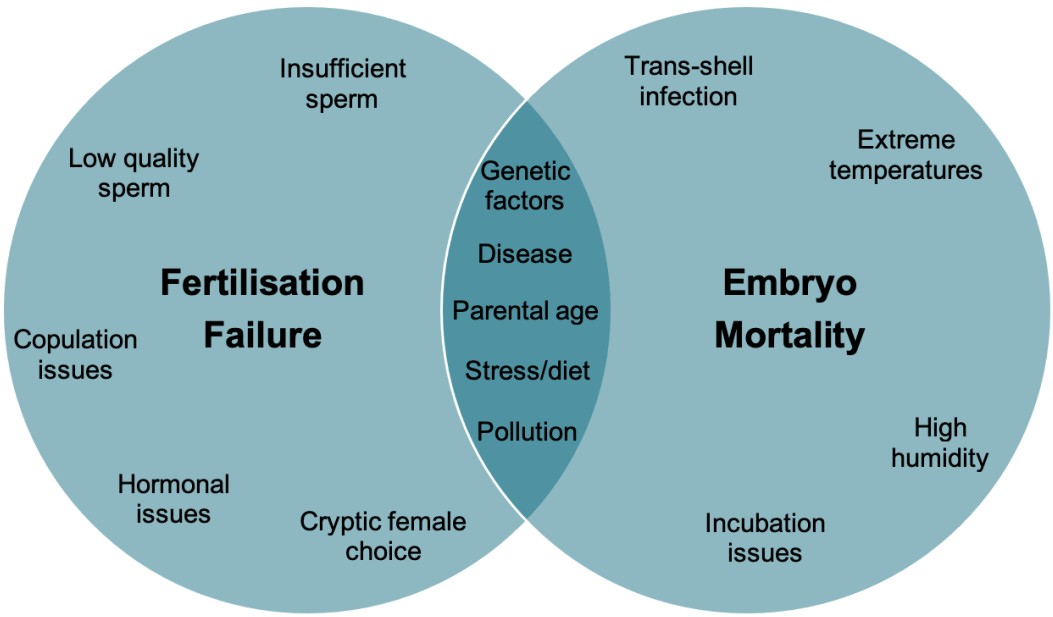

Figure 1-2. A summary of some of the factors believed to lead to failure of eggs to hatch due to fertilisation failure or embryo mortality. Please see Assersohn *et al.* (2021b) for a thorough review and summary of these factors.

of embryo development are apparent (Birkhead *et al.*, 2008). This is made even more challenging when managing threatened populations, particularly in the wild, as suspected failed eggs will often be left to be incubated to full-term to prevent the accidental removal of viable eggs, meaning their contents begin to decompose (Birkhead *et al.*, 2008). This ambiguity may result in fertilised eggs being misclassified as unfertilised, and has also led some ornithologists to refer to all undeveloped, or even all unhatched, eggs as 'infertile'. It is likely that fertilisation failure and embryo mortality are influenced by different combinations of ecological, behavioural, genetic, and environmental factors (**Figure 1-2**; Assersohn *et al.* (2021b)), so establishing which cause is at play when examining unhatched eggs can help to determine the factors leading to hatching failure (Birkhead *et al.*, 2008; Hemmings *et al.*, 2012b).

A potential solution to this issue lies in a fluorescence microscopy protocol developed by Birkhead *et al.* (2008) which enables accurate determination of the fertilisation status of undeveloped, and even heavily degraded, eggs. These methods present an opportunity to differentiate between fertilisation failure and early embryo mortality in unhatched eggs lacking signs of embryo development collected from managed threatened populations, which would most likely be classified as 'infertile' based on external or macroscopic examination. Combination of microscopic assessments with other data, such as environmental measures and behavioural observations, facilitates the identification of the underlying drivers leading to hatching failure, which can then be used to increase the efficacy of conservation strategies (Hemmings *et al.*, 2012b; Assersohn *et al.*, 2021b). However, the application of such techniques has so far been extremely limited, and in fact many studies do not attempt to differentiate between fertilisation failure and embryo mortality at all (**Figure 3-1**, **Chapter 3**). The findings from the relatively few studies that have employed fluorescence microscopy techniques consistently show that fertilisation failure is overestimated when using macroscopic examination alone. This indicates that infertility issues are also overestimated within both individuals and populations, and that conservation interventions overall should perhaps be targeted more towards the putative drivers of embryo mortality.

One outcome of accurate determination of the fertility of undeveloped eggs has been the suggestion that captive populations of threatened species may

experience a higher proportion of egg losses due to fertilisation failure than populations of threatened species managed in the wild, where the majority of hatching failure is instead attributed to embryo mortality (Hemmings *et al.*, 2012b). While several features of captive and wild managed threatened populations are likely to be shared, for example typically small population sizes and reduced genetic diversity, the different challenges and opportunities presented by *ex situ* and *in situ* conservation may lead to the implementation of different interventions, which could explain this difference. By advancing our understanding of the underlying drivers of hatching failure and the potential impacts, both negative and positive, of various management interventions, we have the opportunity to improve how we mitigate hatching failure in managed populations, ultimately helping to reduce their risk of extinction.

Research Focus and Value

The ultimate goal of my PhD is to improve our understanding of why eggs fail to hatch, and in particular to examine why managed populations of threatened birds experience very high rates of hatching failure. Throughout this thesis I aim to critically evaluate previous findings surrounding hatching failure, identify its most important drivers, and explore the influence of different management interventions to provide evidence-based information which is of practical use for both researchers and conservation practitioners.

Despite the widespread acknowledgement that managed populations and threatened species of birds often experience elevated levels of hatching failure relative to wild and common species, these differences have not been quantified or explored on a broad scale. Previous interspecific analyses of hatching failure have typically focused on only threatened or only non-threatened species, and have generally excluded managed populations altogether. By providing a comprehensive picture of how avian hatching failure rates differ across populations as part of my thesis, I aim to highlight how an improved understanding of the drivers of hatching failure could be beneficial for both academic research and, vitally, conservation of the species most at risk from extinction.

The oviparous nature of birds presents an opportunity to include even the earliest of embryo deaths in population sampling, allowing us to truly

understand the causes of differential survival. This is not possible in another well-studied taxa, mammals, wherein the earliest embryo deaths occur internally, prior to sampling observations, and therefore remain largely undetected (Grueber et al., 2015). Incorporating this 'invisible fraction', i.e. the demographic group composed of individuals that died before sampling age, would have important consequences for all fields in which life history data is key to analyses (Hemmings & Evans, 2020), but could have particularly critical implications for conservation biology, in which a better understanding of embryo mortality would enable conservation practitioners to adapt their approaches and provide more tailored solutions to reproductive failure.

For example, in cases of proven fertilisation failure in captivity, practitioners can be more confident in removing a likely infertile male to allow a female to form a new pair bond with another male, or applying alternative techniques such as artificial insemination using sperm from a male with proven fertility. The identification of a potentially infertile male could also provide crucial information for translocation decisions, as inclusion of such a male could potentially threaten the successful establishment of a founder population. Conversely, if eggs are found to have experienced embryo mortality, management interventions can shift focus to issues such as maternal health or incubation conditions. Key to this decision-making is the ability to accurately determine whether an unhatched egg has been fertilised. The Birkhead *et al.* (2008) fluorescence microscopy method has been used successfully to inform the management of a small number of captive and managed bird populations (e.g. Hemmings *et al.*, 2012b; Croyle *et al.*, 2015). However, these methods have not yet been widely adopted (**Table B1**, **Appendix B**). By applying these methods to unhatched eggs laid by a range of different bird species in captivity and a wild managed population, I aim to further explore the relative importance of embryo mortality and fertilisation failure as causes of hatching failure and provide data on egg fertility which can be used to support evidence-based conservation decision-making within these threatened and managed populations.

Finally, I aim to investigate one of the most commonly suspected drivers of hatching failure and one of the mostly poorly understood causes of embryo mortality in non-commercial bird populations: microbial infection. Disease in general is an important limiting factor for population growth in many birds

(Newton, 1998), and is often a crucial concern in conservation programmes, particularly those incorporating translocations between wild and captive populations, due to the potential risks of introducing pathogens into naïve populations (Jones, 2004). Hatching failure has been linked to poor health of parents, whether due to infection (e.g. Losdat *et al.*, 2011), inbreeding depression (e.g. Opatová *et al.*, 2016), stress (e.g. Mazaro & Lamano-Carvalho, 2006), advanced age (Reed *et al.*, 2008), or contamination with toxic substances (e.g. Ackerman *et al.*, 1999). These factors could reduce fertilisation success due to unsuccessful copulations or insufficient sperm quantity/quality, or increase embryo mortality due to poor maternal condition influencing egg and incubation investment (e.g. Blount *et al.*, 2002; Parker, 2002; Rutkowska & Cichoń, 2002).

However, as well as influencing hatching success by reducing parental condition, pathogens may also cause hatching failure directly by invading egg contents through pores in the eggshell, inducing embryo mortality (Cook *et al.*, 2003, 2005b, 2005a; Beissinger *et al.*, 2005; Godard *et al.*, 2007; Soler *et al.*, 2010; Hansen *et al.*, 2015). Molecular techniques present an opportunity to explore the relationship between the avian egg and the microbial communities present on the eggshell (i.e. the eggshell microbiome) to better understand their role in trans-shell infection, and consequent hatching failure. While this has been extensively investigated for poultry species, particularly in relation to the contamination of eggs used as food (Baggott & Graeme-Cook, 2002; Fardows & Shamsuzzaman, 2015; Olsen *et al.*, 2017; Shi *et al.*, 2020; Chousalkar *et al.*, 2021; Rychlik *et al.*, 2023), the application of such molecular techniques to non-commercial, and in particular threatened, bird species has so far been relatively sparse. By using 16S rRNA sequencing to characterise the eggshell microbiomes of two threatened species and examining how they relate to hatching failure and management interventions, I make a novel contribution to this limited area of knowledge, and explore the potential opportunities to consider eggshell microbiomes as part of conservation management.

Thesis Structure Overview

Throughout this thesis I aim to thoroughly review the current body of knowledge surrounding avian hatching failure and make use of modern techniques to

investigate the patterns and drivers of hatching failure in managed populations of threatened species.

In **Chapter 2,** I conduct a systematic review and phylogenetically controlled multilevel meta-analysis of the relationships between threat status, management interventions, and avian hatching failure. I provide an updated estimate of the overall mean rate of hatching failure across birds, comprehensively assessing both wild and managed populations, and both non-threatened and threatened species, for the first time. An improved understanding of how management interventions may impact hatching failure has the potential to help support conservation decision-making around the best management scenarios for struggling bird populations.

In **Chapter 3**, I apply fluorescence microscopy methods to accurately assess the fertility of unhatched eggs from several captive zoo populations in the UK and a managed population of kakī (black stilt; *Himantopus novaezelandiae*) in Aotearoa New Zealand, and determine the relative importance of fertilisation failure and embryo mortality as causes of hatching failure in these populations. This chapter illustrates how overestimation of fertilisation failure rates in a population, which can limit our ability to identify and mitigate underlying problems, can be prevented through accurate diagnoses of egg fertility.

In **Chapter 4**, I use 16S rRNA gene sequencing methods to characterise the eggshell microbiomes of two threatened species with elevated rates of hatching failure, the kakī and the hihi (stitchbird; *Notiomystis cincta*), for the first time. By drawing comparisons between these very different species and exploring the influence of various parental, environmental, and management-related drivers on their eggshell microbiomes, some of the factors playing a role in shaping this so far understudied, but potentially important, component of avian reproduction can be identified, opening the door for their consideration in conservation management.

Building on the previous chapter, in **Chapter 5**, I again use 16S rRNA gene sequencing methods along with statistical modelling methods to investigate whether eggshell microbiome diversity or composition predicts hatching failure in hihi or kakī. By exploring the potential links between the eggshell microbiome and the elevated rates of hatching failure seen in these two threatened species,

I demonstrate the importance of investigating the causes of hatching failure in individual species, and show how this can inform and focus conservation management efforts to reduce hatching failure most effectively.

Finally, in **Chapter 6**, I summarise my overall findings, discussing the main results and conclusions from the preceding chapters and their implications for our understanding of hatching failure and conservation management, as well as making recommendations for future work.

Chapter 2: Systematic review of avian hatching failure and implications for conservation

Abstract

Avian hatching failure is a widespread phenomenon, affecting around 10% of all eggs that are laid and not lost to predation, damage, or desertion. Our understanding of hatching failure is limited in terms of both its underpinning mechanisms and its occurrence across different populations. It is widely acknowledged that rates of hatching failure are higher in threatened species and in populations maintained in captivity compared to wild, non-threatened species, but these differences have rarely been quantified and any broader patterns remain unexplored. To examine the associations between threat status, management interventions, and hatching failure across populations I conducted a phylogenetically controlled multilevel meta-analysis across 231 studies and 241 species of birds. My dataset included both threatened (Critically Endangered, Endangered, and Vulnerable) and non-threatened (Near Threatened and Least Concern) species across wild and captive populations, as well as 'wild managed' ('free-living') populations. I found the mean overall rate of hatching failure across all populations to be 16.79%, with the hatching failure rate of wild, non-threatened species being 12.40%. I found that populations of threatened species experienced significantly higher mean hatching failure than populations of non-threatened species. Different levels of management were also associated with different rates of hatching failure, with wild populations experiencing the lowest rate of hatching failure, followed by wild managed populations, and populations in captivity experiencing the highest rate. Similarly, populations that were subject to the specific management interventions of artificial incubation, supplementary feeding, and artificial nest provision displayed significantly higher rates of hatching failure than populations without these interventions. The driver of this correlation between hatching failure and management remains unclear, but could be an indirect result of threatened species being more likely to have lower hatching success and also being more likely to be subject to management, indicating that conservation efforts are fittingly being focused towards the species potentially most at risk from extinction. This is the most comprehensive comparative analysis of avian hatching failure that has been conducted to date, and the first to quantify explicitly how threat status and management are associated with the rate of hatching failure in a population. I discuss the implications of these results, focusing on their potential applications to conservation. Although I identified several factors clearly associated with variation in hatching failure, a significant amount of heterogeneity was not explained by the meta-analytical model, indicating that other factors influencing hatching failure were not included here. I discuss what these factors might be and suggest avenues for further research. Finally, I discuss the inconsistency in how hatching failure is defined and reported within the literature, and propose a

standardised definition to be used in future studies which will enable better comparison across populations and ensure that the most accurate information is used to support management decisions.

Introduction

Evolutionary theory suggests that selection should act strongly against traits that lead to reproductive failure. Despite this, hatching failure (i.e. the failure of eggs to hatch due to fertilisation failure or embryo mortality) is ubiquitous across birds. While mean hatching failure rates of around 8–12% (Koenig, 1982; Morrow *et al.*, 2002; Spottiswoode & Møller, 2004; Møller *et al.*, 2010) have been reported across species, there can be large intra- and interspecific variation (Rothstein, 1973). Importantly, much higher rates have been described in some threatened species (e.g. Heber & Briskie, 2010). Currently, 13–14% of bird species are threatened with extinction (IUCN, 2022), with more species expected to become threatened in their natural habitats in coming years due to climate change, habitat loss, and invasive species (BirdLife International, 2018a). Birds are amongst the most well-studied taxa, but the limited amount of research into the causes of hatching failure and infertility in non-model, non-domestic species relative to poultry has resulted in substantial gaps in our understanding of why eggs fail (Assersohn *et al.*, 2021b, 2021a). In particular, there is a lack of understanding of how conservation management impacts hatching rates across bird populations, despite evidence that hatching failure can be higher in captive populations relative to wild counterparts (e.g. Burnham, 1983; Saint Jalme *et al.*, 1996). Given the growing importance of managed populations to the conservation of threatened bird species, a systematic review of the influence of management on such a key reproductive measure as hatching failure seems timely.

Several comparative reviews of hatching failure across bird species have been performed previously, with findings linking hatching rates to (*a*) genetic effects such as past population bottlenecks and high genetic similarity (Briskie & Mackintosh, 2004; Spottiswoode & Møller, 2004; Heber & Briskie, 2010), and (*b*) environmental effects, including latitude, nest type, diet, and breeding/social system (Koenig, 1982; Spottiswoode & Møller, 2004) (see **Table A1** & **Table A2**, **Appendix A.1**). However, previous reviews have included minimal comparison of threatened *versus* non-threatened species and have generally

excluded managed populations. While hatching failure occurs across all birds and there are likely to be common drivers, the very high rates reported for some threatened species indicate that they are either more strongly affected by these shared drivers, or that they are subject to additional drivers compared to non-threatened species. Similarly, captivity has been shown to depress species' reproductive success relative to wild counterparts (Farquharson *et al.*, 2018), and there is some evidence that hatching failure in captive populations is primarily caused by fertilisation failure, while embryo mortality is the more common cause of failure in the wild (Hemmings *et al.*, 2012b). This could indicate that captive and wild populations may be differentially affected by certain drivers. Failing to account for variation in threat status and management in comparisons of hatching failure could therefore lead to key drivers of hatching failure being missed or underestimated in the species and populations that experience hatching failure at the highest rates, and in which reproductive failure could have a larger impact on extinction risk.

The causes of elevated hatching failure in threatened species remain under-explored, potentially due to the widespread assumption that because threatened species are often present in small, isolated populations, hatching failure results predominantly from inbreeding depression (e.g. Heber & Briskie, 2010). However, the range of hatching failure rates exhibited by threatened species, and the variation among populations of the same species, suggests that inbreeding depression is not solely responsible (Hemmings *et al.*, 2012b; Assersohn *et al.*, 2021b). Threatened populations may possess characteristics that could partially explain their higher failure rates, for example, their inherently small population sizes can disrupt social dynamics and breeding systems in cooperative breeders, while demographic stochasticity in the sex ratio can impair reproduction in monogamous species (Lacy, 2000; Lee *et al.*, 2011). Also, the factors that led a population to become threatened in the first place may continue to impact reproduction negatively. For example, high predation levels can indirectly affect hatching failure in non-predated nests due to perceived predation risk disturbing parents during incubation (e.g. Zanette *et al.*, 2011). Habitat loss or deterioration could also increase hatching failure by creating reliance on sub-optimal nesting sites (e.g. Perlut *et al.*, 2016) and/or prompting competition for resources leading to reduced nest attendance (e.g.

Koski *et al.*, 2020) and parental condition (e.g. Ardia & Clotfelter, 2007). Finally, some traits that increase a species' vulnerability to extinction, such as high levels of endemism, living in extreme environments, and complex life histories (Mckinney, 1997; Owens & Bennett, 2000; Purvis *et al.*, 2000) could also make them more sensitive to environmental change, negatively impacting their hatching success.

It is estimated that nearly a quarter of all extant bird species are currently held in zoos (Conde *et al.*, 2011; Biega *et al.*, 2019) and captive-breeding programmes have played important roles in saving several bird species from extinction (Bolam *et al.*, 2021), despite the often high rates of hatching failure reported (Farquharson *et al.*, 2018). Regardless of whether or not a species is threatened, there are conditions typical of captivity that could explain relatively higher rates of hatching failure, including: limited mate choice (Asa *et al.*, 2011) resulting in atypical breeding behaviour (e.g. Driscoll, 2008), postcopulatory selection (e.g. Pizzari & Birkhead, 2000), and reduced extra-pair copulation opportunities (e.g. Wetton & Parkin, 1991); inclusion of older individuals and resultant senescence effects (e.g. Ricklefs *et al.*, 2003; Rabier *et al.*, 2021); poor parental condition related to diet (e.g. Sancha *et al.*, 2004), stress (Monaghan *et al.*, 2009; Selman *et al.*, 2012), or disease/parasitic infection (Snyder *et al.*, 1996); and adaptation to captivity (Frankham, 2008; Williams & Hoffman, 2009).

Since it is difficult to recreate optimal breeding environments in captivity, populations under management might be expected to have lower hatching success rates compared to wild populations of the same species. However, this has not been thoroughly investigated or quantified. Understanding how management interventions influence hatching outcomes is essential when developing management guidelines and assessing the effectiveness of different interventions. Conservation managers are often operating under a number of constraints, and may need to weigh up the effectiveness of interventions with any trade-offs in terms of financial costs, time commitment, and stakeholder motivations (e.g. Pritchard *et al.*, 2022). Hatching failure is a relatively straightforward parameter to utilise for this (e.g. Martins *et al.*, 2021; Edwards *et al.*, 2022). For example, a population in the wild might have a low rate of intrinsic hatching failure but loses a large number of eggs to predation. Moving

this population into captivity and hence eliminating the effect of predation will likely increase the number of eggs surviving to hatching, but may also increase the rate of hatching failure in non-predated eggs above natural levels. If this increase in hatching failure cancels out the reduction in egg loss due to predation, it may be more effective to leave the population in the wild and instead employ predator exclusion or removal strategies. Models such as population viability analyses are increasingly being used to estimate the likelihood that a population will go extinct under different scenarios (e.g. Bustamante, 1996; Dolman *et al.*, 2015; Heinrichs *et al.*, 2019). Incorporating 'baseline' measures of hatching failure in a wild, unmanaged population, along with the expected effect of different management interventions, into such models could help increase their accuracy and support decision-making. This may be particularly useful for species that become candidates for management for the first time due to degradation of their natural habitats (BirdLife International, 2018a; IUCN, 2022).

To address some of the knowledge gaps surrounding hatching failure and management and to establish average baseline measures of hatching failure across different threat classifications and management levels, I conducted a phylogenetically corrected meta-analysis to investigate: (1) how hatching failure rate varies with a species' threat status; (2) how hatching failure rate varies across different levels of management; (3) how hatching failure rate varies under specific management interventions (artificial incubation, artificial nest site provision, and supplementary feeding); and (4) how threat status and management level or specific management interventions interact with respect to their association with hatching failure. This analysis represents the most up-to-date and comprehensive systematic review of avian hatching failure conducted so far, and the first to consider directly the potential influence of management interventions and threat status, with a focus on the implications for conservation. Performing this analysis highlighted the absence of consistent hatching failure terminology in the literature and the consequent constraints upon the scale of comparison among studies, hence I also suggest a framework for defining and reporting hatching failure which could be adopted more broadly. Finally, I identify a number of remaining questions and knowledge gaps and suggest how they could be addressed in future research.

Materials and Methods

Koenig (1982) defined ‘hatchability’ as the percentage of eggs surviving to the time of hatching that produce a chick, thus excluding eggs lost to predation, abandonment, accidental breakage, or any other unknown factor. However, ‘hatchability’ is commonly used within poultry studies to describe only the proportion of fertile eggs that hatch (King’ori, 2011), and other studies that follow Koenig’s definition have instead used ‘hatching success’ (e.g. Spottiswoode & Møller, 2004) or ‘hatching failure’ (e.g. Briskie & Mackintosh, 2004). The literature contains examples of ‘hatchability’ and ‘hatching success’ being used both interchangeably (e.g. Cichoń *et al.*, 2005) and distinctly (e.g. Schwarzbach *et al.*, 2006), as well as usage of alternative terms (e.g. ‘embryo survival’; Aldredge, 2017). Often, studies lack an explanation of how a term is defined, with further complexity arising when studies do not state whether eggs lost to external factors (e.g. predation) are included within reported hatching failure estimates.

In this review, hatching failure is defined as the proportion of eggs present at the end of the incubation period that fail to hatch, excluding eggs lost due to predation, desertion, accident, extreme weather, or that disappeared during the incubation period due to unknown factors. This definition is used under the assumption that intrinsic and extrinsic factors are independent. For example, it is assumed that unfertilised eggs are not more likely to be abandoned by parents or predated than fertilised eggs, since there is currently no evidence for this in the literature.

(1) Data compilation

Studies included in the meta-analyses were compiled from three main sources:

(*i*) The electronic database *Web of Science* was chosen as the primary search system due to its functionality, use in similar comparative reviews, and suitability as a principal search system for a systematic review (Gusenbauer & Haddaway, 2020). I included the most commonly used terms for hatching failure identified in an initial survey of the literature in the search, i.e. “hatching failure”, “hatching success”, “hatchability”, and “hatching rate”. Multiple search sets with different terms and restrictions were trialled on 23/01/2020, with the final search

set chosen to maximise the likelihood of locating relevant studies while keeping the total number of studies to a manageable level for closer manual inspection (Foo *et al.*, 2021) (see **Table A3**, **Appendix A.2**). The final database search using the chosen search set (**Appendix A.2**) was conducted on 16/02/2020. A *Web of Science* alert for the final search set was in place until 01/09/2020 and a small number (n = 6) of eligible records were identified and included in the dataset during this period.

(*ii*) The datasets of previous comparative analyses that either investigated hatching failure directly or included it as a key variable were searched and the original sources of the data were examined where possible to verify data eligibility and extract additional information. The comparative analyses considered were: Briskie & Mackintosh (2004), Spottiswoode & Møller (2004), Heber & Briskie (2010), Møller *et al*. (2010), Galván & Sanz (2011), Soler *et al*. (2012a), Reding (2015) and Johnsen (2019) (**Table A1**, **Appendix A.1**). In a number of cases the source of the data was cited as 'personal communication' and hence could not be independently verified and additional information around the population could not be obtained.

(*iii*) Papers encountered during reading of the general literature on hatching failure and reproductive success in birds that contained information relevant to this analysis but were not identified by the *Web of Science* search were considered for inclusion, as were some partial datasets of hatching failure literature previously compiled by my supervisors and their colleagues. A small number of articles found in February 2020 *via* the search engine *Google Dataset Search* using the search terms "hatching failure" and "egg hatching success" which were not identified in other searches were also considered for inclusion. A final source of additional studies was *Google Scholar* alerts for the terms "hatching success", "hatching failure", "hatchability", and "captive breeding" + "bird". The alerts were originally set up in January 2019 and relevant papers from alerts were included up until 01/09/2020.

(2) Inclusion criteria

The criteria for inclusion of a study in the final dataset were as follows:

(1) The study was published in a peer-reviewed journal or master's and PhD theses/dissertations published online. Articles published in 'predatory' journals or from 'predatory' publishers as listed on Beall's List (https://beallslist.net/) were excluded.

(2) The study was accessible through reasonable effort on the publisher's website using institutional access or available in institutional libraries.

(3) The study was conducted on wild or captive birds, but not domesticated species kept for commercial purposes such as poultry or gamebirds, or birds bred intensively for the pet trade.

(4) The study did not include *in-ovo* injection of compounds or physical manipulation of egg components or eggshell structure such as 'windowing' of eggs. The control dataset from experimental studies was included if appropriate, for example if control eggs were handled, but not if they were injected with a non-active compound.

(5) The study's definition of hatching failure matched that of this review. In some cases, checking the original sources of data included in previous comparative analyses revealed that the hatching success rate cited by the comparative analysis did not appear to fit my definition. However, as authors of previous analyses reported validating rates through personal communication with researchers, cited rates were accepted as reliable.

(6) The study contained sufficient information in the text or supplementary material to calculate hatching failure/success even if the study itself did not report a hatching failure/success percentage, or the definition used did not match my definition.

(7) The study contained information on the sample size (total number of eggs present at the end of the incubation) and/or the total number of eggs hatched/unhatched along with hatching failure/success proportion (as in criterion 6). A minimum sample size of 10 was required for inclusion.

The processes of literature searching, data eligibility assessment, and ultimately data inclusion are summarised in a Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) diagram (**Figure 2-1**) and a PRISMA-

EcoEvo checklist provided in **Appendix A.3** (Moher *et al.*, 2009; O'Dea *et al.*, 2021). Further details are included in **Appendix A.2** & **Appendix A.4**, and the final dataset is also available online (https://github.com/ashleigh-f-marshall/hatch-fail-meta). After the initial pooling of all articles, I removed all studies prior to 2004 that were not included in the previous comparative analyses as it was assumed that the majority of eligible studies from this period would have been captured in these previous analyses.

The final dataset consisted of 483 records (contained within 233 articles; see **Appendix A.5** for the full bibliography of the final dataset), of which 422 records had been identified in the *Web of Science* search, 13 in *Google Scholar* alerts, 4 from *Google Dataset Search*, 5 from partial datasets previously compiled by my supervisors and former colleagues, 28 from backward searches within these previously mentioned articles, and 11 from general reading of the literature. The main reasons that eligible records were not identified in the main *Web of Science* search was due to their absence from the *Web of Science* Core Collection, or due to the lack of any of the key terms "hatching failure", "hatching success", "hatchability", or "hatching rate" in their title, abstract, or key words, primarily due to hatching failure not being a main focus of the paper and hence only being reported within the main body of the text. While including records found during general reading of the literature could potentially introduce bias due to the influence of personal research interests, the hatching failure data from this small number of studies reflected the trends seen in the overall dataset, and I am confident that their inclusion did not influence the results of the meta-analysis.

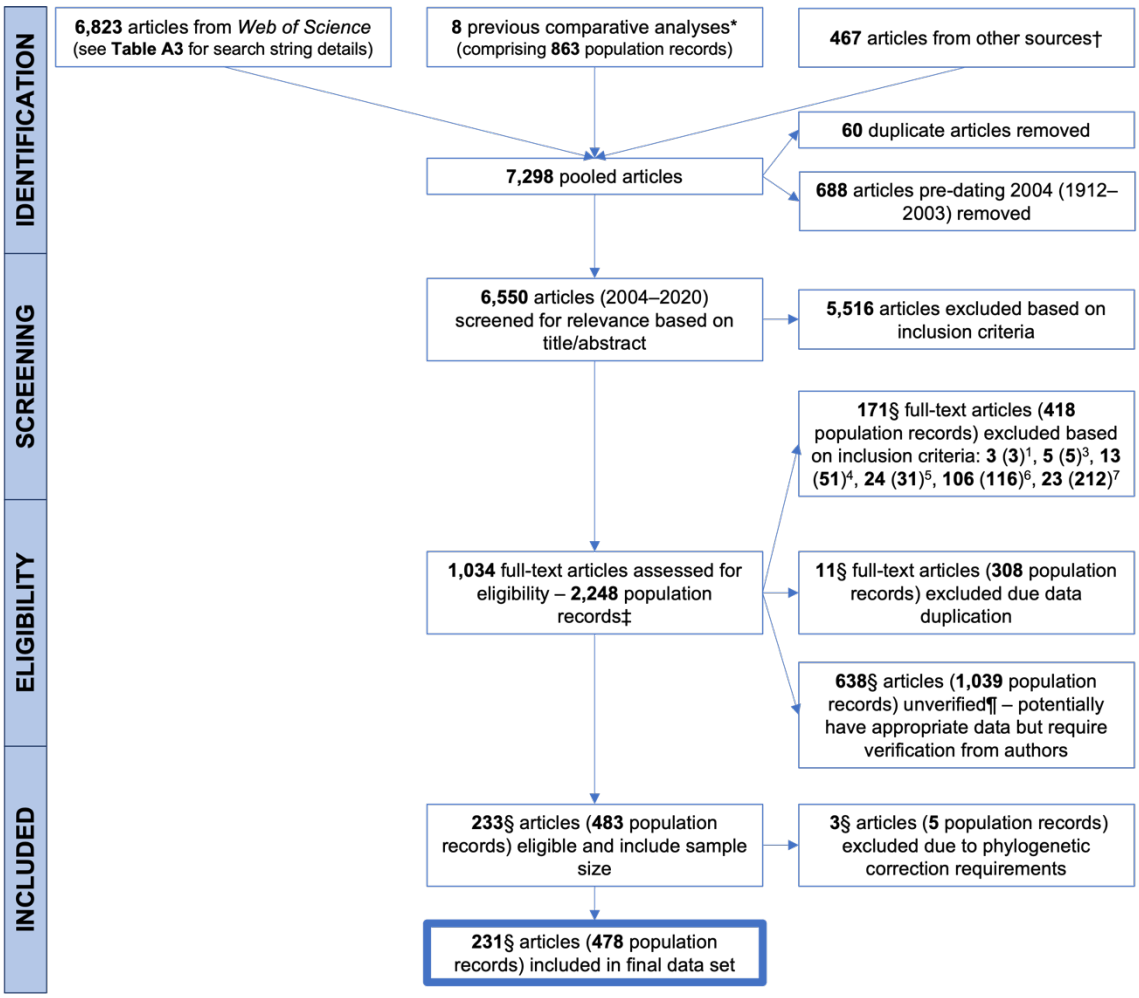

Figure 2-1. Depiction of the literature search and screening process following Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher *et al.,* 2009; O'Dea *et al.*, 2021). *Previous comparative analyses: Briskie & Mackintosh (2004) [22 records]; Spottiswoode & Møller (2004) [99 records]; Heber & Briskie (2010) [51 records]; Møller *et al*. (2010) [212 records]; Galván & Sanz (2011) [170 records]; Soler *et al*. (2012) [22 records]; Reding (2015) [113 records]; and Johnsen (2019) [174 records]. †Other sources: articles passively encountered during reading; articles taken from partially completed databases; studies captured in *Google Scholar* alerts, studied captured in *Google Dataset Search* searches. ‡When a study contained multiple population records these were assessed for inclusion individually. §Some articles contained a mixture of excluded, unverified, and included population records and are counted in multiple record totals. ¶'Unverified' articles were potentially eligible for inclusion but their data could not be confirmed without individual author verification. Superscript numbers indicate the inclusion criteria used to exclude articles/records.

(3) Effect size extraction and calculation

The chosen effect size was the proportion of hatching failure across the population, i.e. the proportion of eggs present at the end of the incubation period that fail to hatch, excluding eggs lost due to predation, desertion, accident, extreme weather, or that disappeared during the incubation period due to unknown factors. For 286 (59.2%) of the 483 records the absolute sample size (total number of eggs present at the end of the incubation) was taken or calculated directly from the information given in the study or

supplementary material. Where this information was not available an estimation for the sample size was performed. In 125 records (25.9%) the sample size was estimated as the product of the total number of successfully hatched clutches/nests (i.e. excluding any whole clutches lost to predation, desertion etc.) and the mean clutch size reported in the study. In the absence of a mean clutch size value within the study, for 60 records (12.4%) the mean clutch size was taken from Cooney *et al*. (2020), for 10 records (2.1%) the mean clutch size was taken from *New Zealand Birds Online* (www.nzbirdsonline.org.nz), and for two records (0.4%) the mean clutch size was taken from *Birds of the World* (www.birdsoftheworld.org). For subspecies without a reported mean clutch size the available clutch size for the parent species was used. The appropriateness of the two main proxies used was assessed by performing a series of statistical tests on 125 records for which a sample size could be directly obtained from the study and estimated using the study's mean clutch size and 101 records for which a sample size could be directly obtained from the study and estimated using the mean clutch size from Cooney *et al*. (2020). A test for association using Pearson's correlation coefficient showed that in both cases there was a significantly strong relationship between the directly obtained and estimated sample sizes ($p < 0.001$), with correlations of 0.9998 and 0.9973 for the estimations from the study's mean clutch size and the mean clutch size from Cooney *et al*. (2020) respectively. A Bartlett's test for homogeneity of variances indicated that in both cases the variances were homogenous, with no significant difference in variances between direct and estimated values ($p > 0.05$). The results of a paired sample *t*-test indicated that overall the directly obtained values and those estimated from the study's mean clutch size were not significantly different ($p = 0.273$; the estimation could therefore be considered an appropriate proxy. For the directly obtained values and those estimated using the mean clutch size from Cooney *et al*. (2020), the results of a paired sample *t*-test indicated that overall the estimated values were significantly smaller than the direct values ($p = 0.012$). This indicates that records for which the sample size was estimated using the mean clutch size from Cooney *et al*. (2020) may have generally been underweighted in the model. A sensitivity analysis was conducted with these estimated values removed to assess the effect this had on the model. All calculations and estimations are detailed in the final dataset.

For each effect size a number of key variables were also coded for use in analyses, including: order, species name, common name, *IUCN Red List* classification [Least Concern (LC), Near Threatened (NT), Vulnerable (VU), Endangered (EN), or Critically Endangered (CR)], threat status (threatened or non-threatened), management level (wild, wild managed, or captive), incubation type (artificial or natural), supplementary feeding (fed or not fed), and artificial nest provision (provided or not provided). For the *IUCN Red List* classification I used the current classification (IUCN, 2021) at the time of this meta-analysis to avoid having to exclude records which were not classified at the time of the original study, and I used global assessments where both global and local assessments were available. For populations of subspecies the classification of the parent species was used unless a classification for the subspecies was available from a regional or national *Red List* (www.nationalredlist.org), *Federal Endangered Species List* (www.fws.gov/endangered), or *European Red List* (https://eunis.eea.europa.eu/species.jsp). Management level classifications were based on the management applied to both the breeding population and the eggs. Populations were classified as 'wild' if the adults lived in the wild with no management applied, and as 'captive' if the adults were maintained in captivity and the eggs were laid, hatched, and reared in captivity. Populations were classified as 'wild managed' if the adults lived in the wild with management interventions applied, including nestbox provision, supplementary feeding, and/or egg manipulations such as removal for artificial incubation. See **Appendix A.3** for additional details of the classification of variables.

(4) Data analyses

Analyses were completed in RStudio v1.4.1090 (RStudio Team, 2020) running R v4.0.3 (R Core Team, 2020) and were conducted using the final dataset of included studies unless stated otherwise. The full R code is available online at: *https://github.com/ashleigh-f-marshall/hatch-fail-meta*.

(a) Hatching success/failure terminology

To investigate the usage of different definitions and terminology for hatching failure within each study I noted whether "hatching success", "hatching failure", "hatchability", or an alternative term was used within the text, tables, figures, or

supplementary material and extracted any definition exactly as written. If a term was used but not defined, the definition was recorded as ‘not stated’. To account for syntax differences that did not reflect actual differences in meaning, definitions were homogenised to use consistent phrasing, abbreviations were expanded, and calculations were expressed in words. All definitions of hatching failure were inverted to hatching success as the latter was used substantially more frequently, and where alternative terms were defined but were being used interchangeably with hatching success the definitions were reassigned to define hatching success. See **Table A4** & **Table A5** in **Appendix A.6** for further details.

(b) Meta-analysis

I conducted a meta-analysis with the goal of obtaining a more precise estimate of the overall hatching failure rate and identifying and characterising the factors that have an impact on between-study heterogeneity and the overall effect estimate. The studies included were generally observational and non-comparative, with each contributing a number of failures (eggs present at the end of the incubation period that failed to hatch) and a sample size (total number of eggs present at the end of the incubation period).

(i) Model choice and transformation of proportions

Due to a hierarchical structure resulting from several studies contributing multiple effect size estimates, and the need to account for phylogenetic dependence across the multiple included species, a multilevel meta-analytic model structure was required (Konstantopoulos, 2011; Nakagawa & Schielzeth, 2013; Cinar *et al.*, 2022).

The meta-analytic models were run using the ‘rma.mv’ function in the package *metafor* v3.8.1 (Viechtbauer, 2010). The function ‘escalc’ from *metafor* was used to estimate the individual effect sizes and their sample variances with a Freeman–Tukey double arcsine transformation (Freeman & Tukey, 1950; Miller, 1978) and the meta-analysis model was fitted using a restricted maximum-likelihood (REML) estimation (Viechtbauer, 2005). The Knapp–Hartung adjustment (Knapp & Hartung, 2003) was applied to all meta-analytical models

as often recommended within the literature (Assink & Wibbelink, 2016; Harrer *et al.*, 2021). **Appendix A.7** provides additional information on model selection.

(ii) Phylogeny

A phylogenetic framework was constructed using the tool available on www.birdtree.org, which is based on the taxonomy and phylogenies of Jetz *et al*. (2012). I downloaded 1000 trees based on the full 'Hackett' backbone [10,000 trees with 9993 operational taxonomic units (OTUs) each] with a subset of species matching my dataset. This number has been shown to result in very precise model parameters (Rubolini *et al.*, 2015). Three species from my dataset (five records in total) were not available on BirdTree and were excluded from the phylogenetic tree and all subsequent analysis. This reduced the final dataset to 478 records across 231 studies (**Figure 2-1**). Where my dataset contained subspecies the parent species was used for construction of the phylogenetic tree and any taxonomical name differences between my dataset and BirdTree were resolved as described in **Table A6** in **Appendix A.8**. The R packages *ape* v5.6.2 (Paradis & Schliep, 2019) and *phangorn* v2.10.0 (Schliep, 2011; Schliep *et al.*, 2017) were used to remove any excluded species and compute the maximum clade credibility tree, the branch lengths, and the correlation matrix. The final ultrametric tree used to compute the correlation matrix contained 241 unique species (**Figure A1**, **Appendix A.8**).

(iii) Controlling for non-independence of data

Random effects were incorporated into the meta-analytic model to control for various sources of non-independence. To account for between-study variability and the inclusion of multiple effect sizes per study, a random effect was included at the study level, and to capture variability in the true effects within studies a random effect was included at the effect size level (Nakagawa & Santos, 2012; Assink & Wibbelink, 2016; Harrer *et al.*, 2021). As the final dataset included multiple populations of the same species, which can lead to overestimation of the actual degrees of freedom, this was controlled for by including the species name as a random effect intercept (Benítez-López *et al.*, 2021; Cinar *et al.*, 2022). The 'phylosig' function from *phytools* v1.2.0 (Revell, 2012) was used to compute the phylogenetic signal in the effect size and conduct hypothesis tests for its significance. There was a significant

phylogenetic signal ($p < 0.05$) in the effect size from the final dataset using both the *K* and λ methods, and the phylogenetic relationships were relatively strong based on the mean correlation of the phylogenetic correlation matrix (see Cinar *et al.*, 2022). Hence, a phylogenetic relatedness correlation matrix was also included as a random effect (Hadfield & Nakagawa, 2010; Chamberlain *et al.*, 2012; Cinar *et al.*, 2022).

To ensure that all four random effects (effect size ID, study ID, species name, and phylogeny) were identifiable in the multilevel meta-analytical model, profile likelihood plots were plotted for each using the 'profile' function within *metafor* (**Figure A2**, **Appendix A.9**).

(iv) Outliers and influential studies

The functions 'rstudent' and 'cooks.distance' from *metafor* were used to identify potential outliers and influential cases through calculation of the externally studentised residuals and the Cook's Distance (Cook, 1977). While there is some discussion over interpretation of both of these measures, according to Viechtbauer & Cheung (2010) finding more than *k*/10 studentised residuals larger than ±1.96 in a set of *k* studies would be considered unusual. Twelve studies in my dataset of 478 records were found to have absolute externally studentised residuals larger than ±1.96, which is well within this threshold. The criteria commonly used to interpret Cook's Distance are to investigate records with a Cook's Distance value above 0.5, more than three times the mean of all distances, or equal to more than 4/*N* where *N* is the total number of records (Glen, 2016). No records had Cook's Distances above 0.5, 11 records had values more than three times the mean, and three of these also had values more than 4/*N* (**Table A7**, **Appendix A.9**). Investigating all potential outliers showed no reasons to suspect errors in the data or other justification for removal of these studies from the meta-analysis. Many of the potential outliers displayed very high hatching failure proportions, with 12 out of 20 having rates of >50%, but these records also generally represented captive species, threatened species, or populations undergoing artificial incubation. As these are suspected to have moderating effects on hatching failure which will be accounted for once these variables are included in the meta-analytical model (**Table A8**, **Appendix A.9**), the outliers were not removed for the main

analyses. However, sensitivity analyses were conducted with the outliers removed to validate findings (**Table A9** & **Table A10**, **Appendix A.9**).

(v) Identifying and quantifying heterogeneity

The overall mean hatching failure was calculated by running the multilevel meta-analytical model with only the previously described random effects (effect size ID, study ID, species name, and phylogeny). The level of heterogeneity across all effect sizes was calculated using the I^2 statistic (Higgins & Thompson, 2002; Higgins *et al.*, 2003) and partitioned heterogeneity with respect to the random factors following Nakagawa & Santos (2012) (**Table A9**, **Appendix A.9**). The I^2 statistic is defined as the percentage of variability in the effect sizes that is not caused by sampling error, and is a common method used to quantify the between-study heterogeneity. A high degree of heterogeneity (I^2 = 99.23%) (**Table A9**, **Appendix A.9**) was found which could not be attributed to outliers or to sampling variance alone, hence moderator analyses were conducted to identify the sources of heterogeneity, extending the model to become a mixed-effects multilevel meta-analytic model.

(vi) Explaining heterogeneity with moderator analyses

The potential moderators considered for this analysis were threat status (threatened or non-threatened), management level (wild, wild managed, or captive), incubation type (natural or artificial), supplementary feeding (fed or not fed), and artificial nest provision (provided or not provided). All moderators were binary variables with the exception of management level which was coded as a categorical variable. While information on some other management interventions was available for several populations (for example, the use of artificial insemination, predator control, and fostering of eggs), in general there were not enough observations per category within the dataset to estimate the moderator effects accurately. An additional moderator of *IUCN Red List* classification (LC, NT, VU, EN, CR) was also tested in certain models as an alternative to threat status to assess finer level hatching failure differences between threat categories. As with management level, this moderator was coded as a categorical variable. Finally, to examine whether there was a trend in mean hatching failure over time, I ran a meta-regression with publication year as a fixed effect.

Variables considered for inclusion as moderators in a meta-regression are often correlated, which can lead to multicollinearity (Hox *et al.*, 2010; Assink & Wibbelink, 2016; Harrer *et al.*, 2021). Correlations between categorical variables were assessed using a series of pairwise chi-squared tests of independence using the 'chisq.test' function from the package *stats* v4.2.1 (R Core Team, 2020) and by computing the Cramér's V (Cramér, 1946) for each relationship (**Table A11**, **Appendix A.9**). This revealed weak significant correlations between threat status and management level and between threat status and artificial nest provision, strong significant correlations between management level and incubation type, supplementary feeding, and artificial nest provision, a moderate significant correlation between incubation type and supplementary feeding, and a weak significant correlation between supplementary feeding and artificial nest provision. No significant correlations were found between threat status and incubation type or supplementary feeding, nor between incubation type and artificial nest provision. The variance inflation factor (VIF) was also calculated for each two-level moderator using the 'vif' function in the *metafor* package, with a high VIF value indicating high collinearity with other moderators in the model (**Table A12**, **Appendix A.9**). All moderators had VIF values between 1 and 4, indicating low to moderate multicollinearity (although the appropriate VIF threshold is subject to debate; Kock & Lynn, 2012). For the three-level moderator management level the generalised variance inflation factor (GVIF) was calculated (Fox & Monette, 1992) and the transformation $(GVIF^{1/(2\times df)})^2$ applied to enable comparison with VIF values, also indicating low to moderate multicollinearity (**Table A12**, **Appendix A.9**).

To avoid potentially obscuring significant effects due to collinearity if moderators were combined into a single model, all proposed moderators were tested individually in separate univariate models to check for significance. However, I acknowledge that this approach could increase the chances of false-positive results due to multiple testing (Davies *et al.*, 2020). The univariate models for incubation type, supplementary feeding, and artificial nest provision were repeated excluding captive populations to attempt to account for the likeliness that captive populations were subjected to multiple concurrent management interventions that could mask the effect of the individual intervention included in

the model. Thus, I also repeated the univariate models for the specific management interventions where each named intervention was the only one applied to the population. Given that meta-regression is able to handle low levels of collinearity (Harrer *et al.*, 2021), multivariate mixed-effects multilevel meta-analytical models were used to test for evidence of interactions between both threat status and *IUCN Red List* classification with management level, artificial incubation, supplementary feeding, and artificial nest provision. These models were also run based on an additive model to obtain estimates of mean hatching failure for each combination of factors for these moderators (**Table A13–Table A20**, **Appendix A.9**). Significance tests and likelihood ratio tests were used to access whether a significant interaction was present between any of the pairs of moderators.

As the Knapp–Hartung adjustment was applied to all meta-analytical models, the *F* distribution was used to determine whether the mean effect size (hatching failure proportion) significantly differed across moderator categories, and the mean effect size and 95% confidence intervals were estimated for each moderator category.

(c) Publication bias

Publication bias is a major threat to the validity of meta-analysis (Nakagawa *et al.*, 2022). However, as this meta-analysis includes studies that are generally observational and non-comparative, and hence do not calculate significance levels, the results are unlikely to be strongly coloured by outcome reporting bias or time-lag bias (Nakagawa *et al.*, 2022). As the data were primarily obtained from a search of the title, abstract, and key words of articles, it is possible that my choice of search terms (“hatching success”, “hatching failure”, “hatchability”, and “hatching rate”) could have resulted in a search bias due to authors being more likely to include these terms in a prominent part of the article if they found an unusually high or low level of hatching failure. While this is difficult to detect with any certainty, comparing the mean hatching failure rate across studies with the search terms in more prominent (title and key words) *versus* less prominent (abstract) parts of the article did not indicate any consistent bias. It is possible that as I searched for both “hatching success” and “hatching failure” this may

have helped to ensure a balance in the literature between strong findings in either direction.

I considered a number of other sources of research biases (Gurevitch & Hedges, 1999; O'Dea *et al.*, 2021) and attempted to mitigate these where possible. First, studies were generally limited to those written in the English language. Efforts were made to access translations of non-English language studies, but these were often limited to the title and/or abstract only, which did not allow for thorough examination of the text to verify the study's eligibility, ultimately leading to their exclusion. Excluding non-English language studies could under-represent study populations from certain geographic regions, which can be further exacerbated by unequal output of published research globally, particularly in English language journals. To assess the extent of this, each record's study location was plotted using *ggplot2* v3.4.0 (Wickham, 2016) to visualise their distribution (**Figure 2-2**). This revealed an apparent over-representation of records from English-speaking countries and an under-representation of records from much of the African continent, but in general the final dataset included a wide geographic distribution of populations. Visualising the geographic distribution also highlighted the frequent occurrence of island-dwelling threatened species in the final dataset, but this is perhaps expected given the overall trend of island species being more threatened than mainland species (Tershy *et al.*, 2015) and does not necessarily represent a bias in the dataset itself. Another consideration in this meta-analysis are potential taxonomic biases due to some species, families, or orders being subject to substantially more research effort than others. To check if the final dataset was representative of the global distribution of birds, I used a chi-squared goodness of fit test to compare my dataset with the total number of species per order according to the *IUCN Red List* (www.iucnredlist.org/statistics) (**Table A21**, **Appendix A.9**). The results showed a significant *P* value ($p < 0.001$), indicating that the final dataset was not taxonomically representative of the global distribution of birds. Certain orders such as Accipitriformes, Caprimulgiformes, and Columbiformes were underrepresented in my dataset relative to the overall global distribution, while other orders including Charadriiformes, Falconiformes, and Sphenisciformes were overrepresented relative to the global distribution. Finally, the threat status of a species could cause bias in the dataset either due

to non-threatened species being studied more due to being more common or widespread, or threatened species being studied more due to being of greater research interest. I used a chi-squared goodness of fit test to compare the final dataset with the threat statuses of the global distribution of birds (www.iucnredlist.org/statistics) (**Table A22**, **Appendix A.9**), and again found a significant *P* value ($p < 0.001$), indicating that my dataset is not representative of the overall global distribution of birds. This appears to be due to a slight bias towards threatened species in my dataset relative to the overall global distribution.

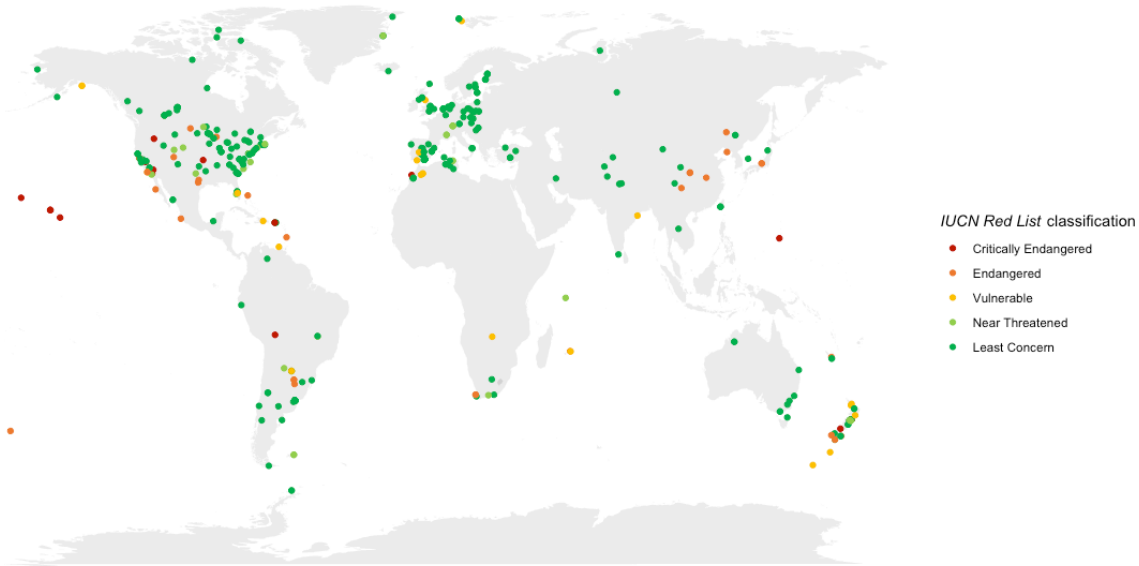

Figure 2-2. The study locations of the final dataset where latitude and longitude measurements were available or could be estimated. Points are coloured according to the *IUCN Red List* classification of the study species.

Results

While the effect size used for all analyses was the proportion of hatching failure across the population, for ease of interpretation and comparison with findings from other studies, all results in the text, tables, and figures are reported as percentage values. The alpha level used for statistical significance was 0.05.

(1) Description of dataset

The PRISMA diagram depicting the literature search and screening process is shown in **Figure 2-1**. A total of 231 articles comprising 478 population records were included in the final dataset. An additional 638 articles reporting 1039 population records were initially considered for inclusion pending verification of

data based on contacting authors directly, but as this number became insurmountable, they were ultimately excluded from the quantitative analyses. Information on the taxonomy, threat status, and management level distribution in the final dataset can be found in **Table 2-1**.

Table 2-1. Information on the taxonomy, *IUCN Red List* classification, and management level for each species or population record across included records.

	Final dataset for meta-analysis 231 studies, 478 records	
Taxonomy		
Orders	26	
Families	79	
Species	241	
IUCN Red List classification	*Number of unique species*	*Total number of records*
Least Concern	158	347
Near Threatened	23	35
Vulnerable	25	37
Endangered	25	39
Critically Endangered	13	20
Management level	*Number of unique species*	*Total number of records*
Wild	178	265
Wild managed	74	172
Captive	16	41

(2) Hatching success/failure terminology

After homogenising the language used in definitions by revising the wording to use consistent phrasing and terminology, inverting definitions of "hatching failure" to "hatching success", and reassigning definitions of alternative terms to define "hatching success" where appropriate (e.g. a study used "hatching success" and the alternative term synonymously), 51 different definitions of hatching success and 12 definitions of hatchability remained across the final dataset (**Table A4** & **Table A5**, **Appendix A.6**). In terms of usage, 166 records used both terms, with 11 of these records defining hatching success and hatchability to describe different results, while 155 either defined only one of the terms or neither term, hence it was assumed that they were using them interchangeably. In total, 149/483 (30.8%) records in the final dataset did not include any definition of hatching success, hatchability, or an alternative term.

(3) Meta-analysis

There was significant within- and between-study variance in hatching failure amongst the studies included in the meta-analysis. Compared to a two-level (i.e. traditional random effects model) and hierarchical (random effects at effect

size level and study level) meta-analytical model, the multilevel meta-analytical model (random effects for effect size, study, species, and phylogeny) was found to be the best fit for the data based on the Akaike (AIC) and Bayesian Information Criterion (BIC) values, and the significance of the likelihood ratio test. The profile likelihood plots of the random effects peaked at the respective parameter estimates and the restricted log-likelihood values decreased as the component values moved away from the REML estimates, providing confidence that all four random effects were identifiable (**Figure A2**, **Appendix A.9**).

The results of the final multilevel meta-analytical model showed an overall hatching failure percentage of 16.79% (95% CI: 8.28–27.40%; n = 478) across all bird populations (**Figure 2-3**). The total amount of heterogeneity (I^2) across effect sizes was 99.23% (**Table A9**, **Appendix A.9**), which is considered high (Higgins & Thompson, 2002; Higgins *et al.*, 2003). The highest amount of variance was attributed to phylogenetic history, with an I^2 of 39.70%. The within-study, or observation-level, variance was found to be 24.02%, between-study

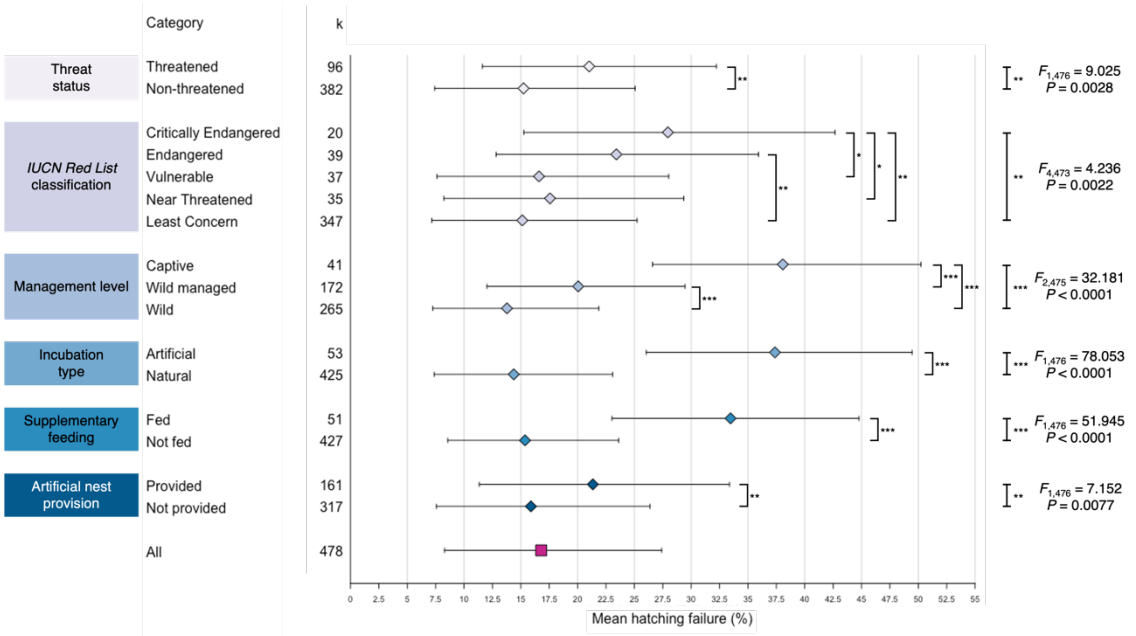

Figure 2-3. Forest plot of the mean effect size (hatching failure percentage) estimates and 95% confidence intervals for each factor level for six moderators: threat status, *IUCN Red List* classification, management level, incubation type, supplementary feeding, and artificial nest provision. Random effects for each model were effect size ID, study ID, species name, and phylogenetic history. Proportions were transformed using a Freeman–Tukey double arcsine transformation, and all models were fitted using a restricted maximum likelihood (REML) estimation with a Knapp–Hartung adjustment. A significant F statistic indicates that at least one of the regression coefficients of the moderator categories significantly deviates from zero, indicating that that moderator influences hatching failure. Significant differences between levels are also shown. The mean hatching failure percentage for the whole final dataset is shown for comparison (red square). Estimates were back-transformed with the inverse of the Freeman–Tukey transformation using the harmonic mean of the sample sizes and are presented here in the natural scale. k = number of effect sizes. Levels of significance across categories and levels: ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

variance was 15.04%, and between-species variance was 20.46% (**Table A9**, **Appendix A.9**). According to the 'rule of thumb' that I^2 values of 25%, 50%, and 75% correspond to low, moderate, and high heterogeneity, these random effects can all be seen to have low to moderate heterogeneity.

The results of the univariate models for each moderator are presented in **Table A8** (**Appendix A.9**) and **Figure 2-3**. Hatching failure was significantly (p = 0.0028) influenced by threat status, with threatened species experiencing a mean hatching failure percentage of 21.02% (95% CI: 11.62–32.22%; n = 96) and non-threatened species experiencing a mean hatching failure percentage of 15.24% (95% CI: 7.42–25.07%; n = 382). Examining the influence of the *IUCN Red List* classification levels also showed a significant (p = 0.0022) overall effect on mean hatching failure, but the differences between threat categories were not all significant. Populations classified as Critically Endangered had significantly higher mean hatching failure than those classified as Vulnerable, Near Threatened, or Least Concern, and populations classified as Endangered had significantly higher mean hatching failure than those classified as Least Concern. Populations classified as Vulnerable experienced a lower mean hatching failure than those classified as Near Threatened but this difference was not significant, and the general trend of populations at higher risk of extinction having lower hatching success is still clearly apparent (**Figure 2-3**). There was no significant relationship (p = 0.9897) between hatching failure and publication year (**Figure A3**, **Appendix A.9**). However, when I used the publication year of the original source (i.e. for data included in previous comparative analyses) I did find a weakly positive, significant relationship (p = 0.0037) (**Figure A4**, **Appendix A.9**).

The management level of a population was significantly associated with its hatching failure percentage (p < 0.0001), with wild populations experiencing the lowest mean hatching failure of 13.78% (95% CI: 7.25–21.87%; n = 265), wild managed populations experiencing a higher mean hatching failure of 20.05% (95% CI: 12.02–29.46%; n = 172) and captive populations experiencing the highest mean hatching failure of 38.07% (95% CI: 26.60–50.23%; n = 41) (**Table A8**, **Appendix A.9**; **Figure 2-3**). The three specific management interventions investigated were also significantly associated with mean hatching failure, with populations undergoing artificial incubation displaying higher mean

hatching failure than those practicing natural incubation, supplementary fed populations experiencing higher mean hatching failure than non-supplementary fed populations, and populations provided with artificial nests experiencing higher mean hatching failure than populations without nests (**Table A8**, **Appendix A.9**; **Figure 2-3**). Repeating the univariate models for the specific management interventions excluding captive populations showed consistent results for the association between hatching failure and both incubation type and supplementary feeding, however, the relationship between artificial nest provision and hatching failure was no longer significant (**Table A23**, **Appendix A.9**). Similarly, repeating the univariate models for each specific management intervention where the named intervention was the only one applied to the population showed higher hatching failure rates in populations undergoing artificial incubation, supplementary fed populations, and populations provided with artificial nests compared to unmanaged populations, but only the relationships for artificial incubation and supplementary feeding were significant (**Table A24**, **Appendix A.9**).

The results of both the significance tests and likelihood ratio tests showed that there were no significant interactions ($p > 0.05$) between either threat status or *IUCN Red List* classification with management level, or with any of the three included management interventions, indicating that the relationship between management and hatching failure is similar regardless of a species' risk of extinction (**Table A13–Table A20**, **Appendix A.9**). As there was no evidence of interactions between the moderators, the models were also run based on an additive model to obtain mean hatching failure percentage estimates and 95% confidence intervals for each combination of factors of these moderators, with the results for *IUCN Red List* classification presented in **Table 2-2**.

Three sensitivity analyses were run to assess the robustness of the results. The first removed all previously identified potential outliers, the second removed all records for which the sample size was estimated based on the mean clutch size from Cooney *et al*. (2020), while the last removed all records from Briskie & Mackintosh (2004) due to ambiguity about whether or not records should be classified as wild or wild managed based on information in the paper (**Appendix A.4**). Results were consistent across all sensitivity analyses and the analysis with the final dataset (**Table A9**, **Table A10**, **Table A25** & **Table A26**,

Appendix A.9). One result of note was a substantial decrease in the proportion of heterogeneity attributed to phylogenetic history (dropping from 39.70% to 18.81%) in the analysis without outliers compared to the full analysis (**Table A9**, **Appendix A.9**).

Table 2-2. The mean hatching failure percentage estimates with 95% confidence intervals for each level of IUCN Red List classification with all other significant moderators. Random effects included were effect size ID, study ID, species name, and phylogenetic history. Proportions were transformed using a Freeman–Tukey double arcsine transformation, and all models were fitted using a restricted maximum likelihood (REML) estimation with a Knapp–Hartung adjustment. Estimates are based on an additive model as there was no evidence for significant interactions between the moderators (**Table A13–Table A20**, **Appendix A.9**). Estimates were back-transformed with the inverse of the Freeman–Tukey transformation using the harmonic mean of the sample sizes and are presented here in the natural scale. LC, Least Concern; NT, Near Threatened; VU, Vulnerable; EN, Endangered; CR, Critically Endangered.

Hatching failure %		***IUCN Red List* classification**				
		Non-threatened			*Threatened*	
		LC	*NT*	*VU*	*EN*	*CR*
Management level	*Wild*	**12.40** (6.59–19.60)			**17.39** (10.26–25.85)	
		12.07 (6.23–19.37)	**15.20** (7.90–24.22)	**12.53** (5.95–20.94)	**20.41** (12.14–30.09)	**23.46** (13.35–35.31)
	Wild managed	**18.36** (11.10–26.89)			**24.06** (15.58–33.67)	
		18.34 (10.98–27.01)	**21.98** (12.97–32.50)	**18.88** (10.73–28.61)	**27.83** (17.99–38.85)	**31.19** (19.82–43.80)
	Captive	**36.06** (25.35–47.51)			**42.84** (31.28–54.80)	
		36.12 (25.31–47.68)	**40.50** (28.16–53.46)	**36.79** (25.08–49.31)	**47.19** (34.60–59.96)	**50.86** (36.62–65.02)
Incubation type	*Artificial*	**35.51** (24.62–47.19)			**41.46** (29.80–53.61)	
		34.86 (23.91–46.65)	**37.20** (25.09–50.15)	**36.49** (24.52–49.34)	**43.96** (31.34–56.97)	**45.65** (31.84–59.81)
	Natural	**13.24** (6.71–21.42)			**17.71** (9.94–27.07)	
		13.15 (6.58–21.42)	**14.87** (7.20–24.54)	**14.34** (6.90–23.76)	**20.14** (11.36–30.58)	**21.54** (11.33–33.83)
Supplementary feeding	*Fed*	**31.37** (21.51–42.13)			**37.16** (26.50–48.48)	
		32.00 (22.03–42.84)	**35.39** (23.95–47.71)	**31.65** (21.04–43.29)	**41.05** (29.33–53.30)	**47.63** (34.05–61.39)
	Not fed	**14.15** (7.90–21.76)			**18.72** (11.27–27.46)	
		14.00 (7.72–21.67)	**16.61** (8.98–25.90)	**13.74** (6.86–22.38)	**21.21** (12.77–31.05)	**26.89** (16.29–38.98)
Artificial nest provision	*Provided*	**19.78** (10.55–30.94)			**26.39** (15.55–38.84)	
		20.07 (10.58–31.54)	**23.58** (12.38–36.96)	**21.57** (11.07–34.27)	**30.16** (17.87–44.04)	**34.43** (20.25–50.14)
	Not provided	**14.22** (6.71–23.78)			**20.16** (10.99–31.15)	
		13.93 (6.32–23.72)	**17.02** (7.87–28.61)	**15.24** (6.67–26.33)	**22.98** (12.56–35.34)	**26.94** (14.52–41.46)

Discussion

Here I performed a multilevel meta-analysis of 478 measures of hatching failure in bird populations from 231 studies across 241 species, finding a mean overall hatching failure percentage of 16.79% (95% CI: 8.28–27.40%). Hatching failure was significantly lower for non-threatened species than for threatened species, and wild populations had significantly lower hatching failure than both wild managed and captive populations, with wild managed populations also having significantly lower hatching failure than captive populations. In addition, populations undergoing the specific management interventions of artificial incubation, supplementary feeding, and artificial nest provision all displayed significantly higher hatching failure than populations without these interventions. I did not find any significant interactions between threat status and overall management level of a population, nor with any specific management interventions. I also examined the terminology and definitions of hatching success and failure currently used throughout the literature and found a lack of consistency among studies.

As far as I am aware, this meta-analysis represents the most comprehensive analysis of avian hatching failure conducted to date. My mean overall rate of hatching failure (16.79%) exceeds the widely accepted value of 9.4% (Koenig, 1982), as well as the mean hatching failure rates found by a number of other comparative analyses [12.35% (Morrow *et al.*, 2002); 10.9% (Spottiswoode & Møller, 2004); and 8.27% (Møller *et al.*, 2010)]. I found a weak but significant relationship between original publication year and hatching failure, which indicates a slight increase in hatching failure over time (**Figure A4**, **Appendix A.9**). Given that the current global trend is generally for species decline, even for many populations currently classified as Least Concern, it may be expected that an assessment including the most recent data on hatching failure would report slightly higher rates of hatching failure overall. However, I believe my higher value likely mainly results from the explicit inclusion of threatened and managed populations (and their general exclusion from other studies). The mean rate of hatching failure for wild, non-threatened species in my study was 12.40%, which is more consistent with the rates found by previous comparative analyses which primarily focused on natural or free-living populations and included mainly (>90%) non-threatened species (**Table 2-2**; **Table A1**,

Appendix A.1). By contrast, the mean rate of hatching failure for captive, threatened species was 42.84% (**Table 2-2**), showing the degree to which variation in failure rate depends on a population's threat status and management level and explaining my higher overall hatching failure rate relative to other analyses.

My finding that threatened species had significantly higher rates of hatching failure than non-threatened species is consistent with findings from other studies (e.g. Briskie & Mackintosh, 2004). The link between hatching failure rate and a population's risk of extinction suggests that hatching failure rate could be used as an indicator of a population's risk of extinction, and similarly that threat status could be used to identify populations that may be experiencing high rates of hatching failure. This further highlights the need for an improved understanding of the drivers of hatching failure, which will enable the development and implementation of appropriate management interventions to hopefully mitigate reproductive losses.

The significantly higher rates of hatching failure in captive populations compared to wild populations is perhaps unsurprising given existing evidence of poor reproductive success in populations in captivity (Asa *et al.*, 2011; Farquharson *et al.*, 2018). As previously mentioned, there are particular features of captivity which could explain relatively higher rates of hatching failure such as limited mate choice and inclusion of older individuals in breeding events, but the finding that wild managed populations also have significantly higher hatching failure than wild populations may indicate that breeding conditions in captivity are not solely responsible for lower hatching success in managed populations. It is possible that populations are more likely to be managed if they are already experiencing a high rate of hatching failure, with the exception of artificial nest provision which is often applied to common species with low risk of extinction. This is partly supported by my finding of a weak but significant correlation between threat status and management level (**Table A11**, **Appendix A.9**), with threatened species slightly more likely to be managed compared to non-threatened species. As I have also shown that threatened species exhibit significantly higher hatching failure than non-threatened species, this could therefore partially explain the higher rates of hatching failure in managed populations. Overall, this finding shows that it is

important to consider both threat status and the extent of management a population is experiencing when measuring hatching failure and to compare different populations of the same species, and also indicates that conservation efforts are being focused on the species with the lowest rates of hatching success and hence potentially most vulnerable to extinction.

The lack of an interaction between threat status and management level indicates that threatened species do not experience a greater deficit in hatching failure under management compared to non-threatened species. However, based on an additive model the mean hatching failure of threatened species in captivity (42.84%) is much higher than the mean hatching failure of threatened species in the wild (17.39%) (**Table 2-2**). Again, it is possible that populations that are already experiencing high rates of hatching failure are more likely to be taken into captivity or otherwise managed. However, a similar increase in hatching failure is also seen in a comparison of non-threatened species in captivity (36.06%) *versus* the wild (12.40%) (**Table 2-2**). While the reasons behind higher rates of hatching failure in managed populations urgently need further investigation, the quantification of mean rates of hatching failure across different management levels established here can be used by conservation managers and other decision-makers to estimate the expected hatching failure rate of populations under different management scenarios, given their threat classification. This can be used alongside other information such as predicted survivability of hatched chicks, which is typically higher in carefully managed captive populations compared to wild populations, when determining the best conservation strategy for a species. To illustrate, if a wild threatened population lays 100 eggs in a breeding season but loses 40 eggs to predators and 10 to hatching failure (a hatching failure rate of 16.67%), only 50 chicks would have the chance to reach fledging. That same population laying 100 eggs in captivity without the threat of predation could lose 42 eggs to hatching failure, i.e. more than twice the rate of hatching failure in the wild, and still result in more chicks having the chance to reach fledging.

I found that populations in which eggs are artificially incubated have significantly higher hatching failure compared to those where eggs are naturally incubated, supporting evidence from other studies (Page *et al.*, 1989; Hamilton *et al.*, 1999; Amar *et al.*, 2000; Sancha *et al.*, 2004). This difference is generally considered

to be a consequence of the difficulty in simulating natural incubation conditions (Deeming, 2002; Klimstra *et al.*, 2009), as well as the possibly increased risk of trans-shell infections in artificially incubated eggs compared to parentally incubated eggs (Cook *et al.*, 2005b; Rideout, 2012; Assersohn *et al.*, 2021b). In addition, the eggs of different species may require particular artificial incubation conditions (Kuehler & Good, 1990; Klimstra *et al.*, 2009) and information on these optimal conditions, if known, may not be easily or openly available, resulting in the use of unsuitable incubation parameters and subsequent higher rates of hatching failure. There is some evidence that captive-laid eggs have lower hatching success than wild-laid eggs under artificial incubation (Burnham, 1983; van Heezik *et al.*, 2005) which could be contributing to my results, but a repeated analysis excluding captive populations still showed a significantly higher rate of hatching failure for eggs undergoing artificial incubation (**Table A23**, **Appendix A.9**). The removal of eggs from the wild for artificial incubation has been used successfully in a number of well-known species' conservation programs, for example the California condor (*Gymnogyps californianus*) (Kuehler & Witman, 1988), the North Island brown kiwi (*Apteryx mantelli*) and rowi (Okarito kiwi; *Apteryx rowi*) (Colbourne *et al.*, 2005), and the Mauritius kestrel (*Falco punctatus*) (Cade & Jones, 1993; Jones *et al.*, 1994). Consequently, this technique may be considered a relatively safe option by conservation managers hoping to improve the number of chicks hatching in a population, particularly if a population is experiencing external risks to eggs such as predation or extreme weather conditions (Williams *et al.*, 2013). However, my finding that populations of threatened species undergoing artificial incubation have a mean hatching failure rate of 41.46% compared to 17.71% for those practicing natural incubation demonstrates that this potential reduction in hatching success should be taken into consideration when deciding whether to remove eggs (**Table 2-2**). Overall, this finding further indicates that focusing efforts on improving artificial incubation in a wide range of species could be critically important for improving hatching success in managed populations.

Supplementary feeding is often considered to be a passive, non-invasive management intervention, so the finding that supplementary fed populations experience significantly higher rates of hatching failure than non-supplementary fed populations may appear somewhat unexpected. Several previous studies

have found no effect of supplementary feeding on hatching success (Newton & Marquiss, 1981; Sanz, 1996; Peach *et al.*, 2014; Ruffino *et al.*, 2014; Vafidis *et al.*, 2016), while evidence of both positive (Nilsson & Smith, 1988; Korpimäki, 1989) and negative (Harrison *et al.*, 2010) effects has also been found. One explanation for higher hatching failure in supplementary fed populations may be that supplementary feeding is often applied to populations suspected to be living in depleted environments, meaning that they may also be experiencing other problems such as habitat degradation, lack of nest sites, and disturbance, all of which may increase hatching failure. Supplementary feeding is also often used to support reintroduced and translocated populations while they become established in an unfamiliar habitat (Ewen *et al.*, 2015), with such populations likely to be of small population size which could negatively impact their hatching success (Heber & Briskie, 2010). Supplementary feeding has been linked to the spread of disease (Wilson & Macdonald, 1967) which could potentially impact parental condition or increase the risk of trans-shell infection, increasing hatching failure. The presence of food may also attract competitors or predators (Arcese & Smith, 1988; Carrete *et al.*, 2006), which could lead to higher hatching failure due to limitation of other resources or non-consumptive predator effects respectively. Finally, it may be difficult to provide supplementary food which adequately meets the nutritional needs of a species, with a risk that if a population is highly dependent on the supplementary food due to unavailability of natural food sources (e.g. Edmunds *et al.*, 2008) this could have consequences for hatching success. The majority of the supplementary fed populations in my dataset were also captive, as by default populations in captivity are not dependent upon natural food sources, and since captive populations were found to experience significantly higher hatching failure than wild managed populations, this could be a driving factor of the higher rate seen in supplementary fed populations. However, a repeated analysis that excluded captive species still showed significantly higher rates of hatching failure for supplementary fed populations, indicating that this is not the case (**Table A23**, **Appendix A.9**). The relatively low financial cost, effort, and perceived risk of disturbance of supplementary feeding contribute to it often being one of the first interventions applied to a struggling population, but as with other interventions any potential negative effects on hatching success should also be taken into consideration during decision-making.

Artificial nest provision, particularly in the form of nestboxes, is another widely practiced intervention generally considered to be relatively passive, and is often used when suitable natural nest sites are limited and as a rudimentary form of protection from predators (Sutherland *et al.*, 2021). Nestboxes are also often used to facilitate population monitoring for various conservation-related and non-conservation-related research purposes. My finding that populations provided with artificial nests have significantly higher hatching failure than those without artificial nests may therefore seem surprising. However, while appropriate artificial nest sites have been shown to have positive effects on the number of eggs hatching, this is often driven by a reduction in egg losses due to predation, brood parasitism, or adverse weather conditions (Semel *et al.*, 1988; Piper *et al.*, 2002; Shealer *et al.*, 2006) rather than an increase in hatching success. Very few studies have compared hatching failure rates between natural and artificial nests; instead, clutch size, nesting success, and fledging success appear to be much more frequently used as measures of reproductive performance (Sutherland *et al.*, 2021). There is some evidence that the orientation of artificial nestboxes can impact hatching failure due to creating a non-optimal microclimate (Butler *et al.*, 2009), and as nestboxes can provide a favourable environment for micro-organisms (Baggott & Graeme-Cook, 2002; Goodenough & Hart, 2011; González-Braojos *et al.*, 2012; Devaynes *et al.*, 2018) this may increase the risk of egg infection and subsequently hatching failure. To my knowledge there has been almost no investigation to date into the microbial loads of artificial *versus* natural nests, but several studies have shown artificial nests to contain significantly higher loads of ectoparasites (Wesolowski & Stanska, 2001; Hebda & Wesołowski, 2012; Espinaze *et al.*, 2020). As with supplementary feeding, a large proportion of my sample of populations provided with artificial nests were also captive, potentially contributing to the higher rate of failure. When the analysis was repeated excluding captive populations the hatching failure of populations provided with artificial nests was still higher, but the relationship was no longer significant, adding support to this possibility (**Table A23**, **Appendix A.9**). Similarly, a repeated analysis limited to populations where artificial nest provision was the only intervention applied also showed a higher hatching failure rate for populations provided with artificial nests, but again the relationship was no longer significant (**Table A24**, **Appendix A.9**). Given the long history and widespread use of artificial nest

provision, there is remarkably little published information on the interaction with hatching failure. The results of this analysis indicate that the potential for negative effects on hatching success should not be overlooked when deciding whether to provide artificial nests as part of a conservation strategy.

While I found several moderators that influenced the rate of hatching failure across my dataset, a significant amount of heterogeneity remains unexplained, indicating that there are other factors not included in my analysis that influence hatching failure. I only had sufficient data for three management interventions to be included in this meta-analysis, but there are several other interventions that could have consequences for hatching failure. Firstly, artificial insemination has been applied in a number of different species to improve fertility and hatching success, with evidence of positive effects (Wiemeyer, 1981; Saint Jalme *et al.*, 1994, 1996; Gee *et al.*, 2004; Blanco *et al.*, 2009) and hence would be a useful addition to future analyses. Secondly, while artificial incubation was included as a moderator in this analysis, an often-associated management intervention is forced re-clutching, where eggs are removed from a population and artificially incubated (or fostered) to encourage the breeding pair to lay a replacement clutch, increasing the overall number of eggs laid in the population (Wood & Collopy, 1993; Seddon *et al.*, 1995; Ellis *et al.*, 1996; Jones, 2004). Egg fertility, hatching success, and egg quality have been shown to decline in replacement clutches (Cade & Jones, 1993; Jones *et al.*, 1994) and forced re-clutching can also impact other aspects of current and future reproductive success (Wood & Collopy, 1993; Parmley *et al.*, 2015). A comparison of hatching failure across multiple clutches would therefore be a useful inclusion in future analyses. Another aspect of breeding management which can influence hatching success is ‘forced’ pairing of individuals and maintenance in captivity as a single pair due to genetic or logistical reasons, eliminating the opportunity for mate choice and extra-pair copulations, both of which are gaining recognition in their potential influence on hatching success (Wetton & Parkin, 1991; Pizzari & Birkhead, 2000; Hemmings & Birkhead, 2015, 2017). While it can be difficult to facilitate mate choice in captivity, practices such as ‘flock-mating’ may help (Asa *et al.*, 2011; Ihle *et al.*, 2015). Other management interventions not included here which may account for some of the unexplained heterogeneity include predator exclusion, egg cleaning, egg fostering, and egg warming or cooling, while other

aspects of management potentially affecting breeding or incubation such as levels of researcher disturbance or use of radio transmitters on birds could also be worth investigating in future analyses, but are currently limited in their frequency of utilisation or in how often or well they are reported in studies.

Grouping mean hatching failure rates by taxonomic order appeared to show some variation across orders, along with differing levels of intra-order variation (**Figure A5**, **Appendix A.9**). However, these apparent differences may have been due to some orders being represented by only a small number of effect sizes. For example, Accipitriformes, Otidiformes, and Pterocliformes appeared to have much higher rates of hatching failure relative to the other orders, but all contained less than five effect sizes taken primarily from captive and/or threatened populations, likely explaining the high hatching failure for the order overall. Greater consistency in hatching failure rates was seen across orders represented by at least 10 effect sizes, although some still appeared to show higher intra-order variation than others, potentially due to the inclusion of populations from across a wide range of threat and management levels (**Figure A5** & **Figure A6**, **Appendix A.9**). Another possible explanation for high variation within taxonomic orders, which was not explored in this analysis, is the influence of species-specific life-history variables. Life-history variables including diet, nest type, breeding system, incubation pattern, and latitude have been found to influence hatching failure significantly in other comparative analyses (Koenig, 1982; Spottiswoode & Møller, 2004), although not consistently (**Table A2**, **Appendix A.1**). While the meta-analysis was phylogenetically controlled, I found that phylogenetic history accounted for a large proportion of overall heterogeneity, and that the removal of potential outliers dramatically reduced this proportion (**Table A9**, **Appendix A.9**). As more closely related species tend to share similar life-history traits this could indicate that certain traits influence hatching failure in different ways, and that these could also interact with the applicability or efficacy of management interventions. For example, Koenig (1982) found that hole-nesting species exhibited higher hatching failure than open-nesters, speculating that this was an indirect result of correlations between breeding inexperience, predation, and hatching success. I support this possibility, and also suggest a link between microclimate and microbial conditions of cavities and related temperature

fluctuations or trans-shell infection impacting hatching failure. Hole-nesting species may be more likely to be managed in the wild due to their often-widespread acceptance of nestboxes. Further research is needed to assess if certain life-history variables significantly affect hatching failure, in particular those that may influence or interact with any management interventions which are applied to a population.

An additional dimension which would have been interesting to examine in this study is the variability in hatching failure between nests within studies, i.e. whether hatching failure rate was similar across all nests in a population or concentrated within a few nests. It seems likely that a population where the mean hatching failure rate is due to high, or total, failure in a small number of nests *versus* a population with the same mean hatching failure rate but resulting from lower failure across a higher proportion of the nests may be experiencing different drivers of hatching failure. Unfortunately, the overwhelming majority of studies included only hatching failure averaged across all nests in the population, so I was unable to include information on the level of within-population variance in hatching failure in this analysis. I suggest this would be a useful inclusion in future studies.

It is clear that a very wide variety of definitions have been used throughout the existing body of literature on hatching success and failure, and it is reasonable to assume are still being used by researchers currently assessing and reporting levels of hatching failure in different bird species. The lack of consistency in terminology and definitions, and in particular the frequent absence of any definition of the terms used or description of data collection, resulted in the exclusion from this meta-analysis of a large proportion of studies (which would have likely otherwise been eligible for inclusion) due to uncertainty around the reported value of hatching failure. I propose that the definition used here – i.e. hatching failure is the proportion of eggs present at the end of the incubation period that fail to hatch, excluding eggs lost due to predation, desertion, accident, extreme weather, or that disappeared during the incubation period due to unknown factors – which is consistent with that used in several previous comparative analyses of hatching failure, should be considered as the standardised definition of hatching failure for future studies. A standardised definition will not only enable better comparison of hatching failure across

multiple studies, allowing for more robust comparative analyses in future, but will also help to ensure that studies are reporting the most accurate information from the population which can be used to draw more meaningful conclusions and apply more appropriate management interventions. While this definition was developed for birds it can also be applied to oviparous species in other taxa such as amphibians, reptiles, and fish. Additionally, the lack of consistent terminology and methodology for reporting reproductive failure in populations is likely to be a problem in viviparous taxa, and it may be possible to adapt this definition of hatching failure to be applicable for measuring reproductive success in viviparous species.

Conclusions and future directions

Overall, this review demonstrates the importance of gaining a better understanding of the occurrence of hatching failure, its drivers, and the effects of management interventions, in order to optimise the outcomes of conservation efforts. Conservation programmes worldwide are focused on protecting and recovering threatened species (Ebenhard, 1995; Mallinson, 1995; Bolam *et al.*, 2021), with interventions ranging in intensity from *in-situ* monitoring and 'non-invasive' management (e.g. supplementary feeding), through to practices such as captive-breeding. Faced with a struggling wild population, conservation managers may decide when and how to intervene using evidence-based conservation (Sutherland *et al.*, 2004, 2021). When multiple alternative interventions are available it is especially important to be able to compare their potential impacts accurately and systematically (e.g. Ruiz *et al.*, 2021). Reproductive parameters such as clutch size, fledging success, and recruitment success are already commonly used for such assessments. Incorporating baseline measures of hatching failure in the wild and in captivity, alongside an understanding of how interventions can impact hatching failure, will add another dimension to facilitating decisions on the best strategy to reduce early reproductive stage losses. Furthermore, is has been found that threatened species are less likely to be held in zoos than non-threatened species, with one possible explanation being that some species are difficult to breed in captivity (Conde *et al.*, 2013; Martin *et al.*, 2014; Biega *et al.*, 2019). By improving our understanding of hatching failure under management it may become possible to apply captive breeding practices to taxa where this has not previously been

feasible, hence providing more options to protect some of the most vulnerable species from extinction. While this review focuses exclusively on birds it is likely that similar knowledge gaps and assessment issues occur across other taxa (Grueber *et al.*, 2015; Kaumanns *et al.*, 2020), and hence some of the lessons learned from this review may be applicable beyond birds alone.

Chapter 3: Fertilisation failure *versus* embryo mortality: distinguishing the main causes of hatching failure in managed populations

Abstract

Reproduction is a fundamental process of life, and yet many species experience issues which impact their ability to produce viable offspring. In birds, hatching failure appears to affect almost every species to some degree, with particularly high rates experienced by threatened species and managed populations. Despite the vast literature on avian reproduction, substantial gaps remain in our understanding of reproductive failure at the earliest stages of development. In particular, there has been minimal focus on accurately differentiating between eggs that fail to hatch due to fertilisation failure and those that fail due to embryo mortality, despite the likelihood that these outcomes are the result of different processes and problems within an individual, breeding pair, or population. The aim of this study was to determine the relative importance of fertilisation failure and embryo mortality as causes of hatching failure across a range of different bird species. By examining the contents of unhatched eggs from several captive populations in the UK and a managed population of kakī (black stilt; *Himantopus novaezelandiae*) in Aotearoa New Zealand for evidence of fertilisation using fluorescence microscopy methods, I show that the majority of eggs classified as 'infertile' based on macroscopic examination have actually experienced early embryo mortality, revealing that rates of fertilisation failure are much lower than had previously been thought. In kakī, for which I analysed a large intraspecific sample of unhatched eggs from both captive and free-living breeding pairs, eggs from larger clutches were more likely to hatch than eggs from smaller clutches, as were heavier eggs and those that contained older embryos at the time of collection for artificial incubation. The cause of hatching failure (fertilisation failure *versus* embryo mortality) also varied with clutch size and egg weight: failed eggs that were lighter, or that were from larger clutches, were more likely to be unfertilised, whereas heavier eggs and those from smaller clutches were more likely to have suffered embryo mortality. Kakī embryo mortality tended to occur early rather than late in the incubation period, particularly in captive-laid eggs, but there was little evidence of differences in the overall rate of hatching failure or the proportion of hatching failure attributed to embryo mortality *versus* fertilisation failure between captive- and wild-laid kakī eggs. These findings add to the increasing evidence that fertilisation failure plays a relatively minor role in determining hatching outcomes in birds, and provides some insights into the influence of certain management practices on embryo survival that can inform conservation management decisions.

Introduction

For several decades, the mean rate of avian hatching failure has been assumed to be 8-12% based on the results of several comparative analyses (Koenig, 1982; Morrow *et al.*, 2002; Spottiswoode & Møller, 2004; Møller *et al.*, 2010). However, recent work has shown that the mean hatching failure rate across birds is closer to 17%, with populations of threatened species and those undergoing management experiencing much higher rates (**Chapter 2**; Marshall *et al.*, 2023). High rates of hatching failure can reduce the effectiveness of conservation actions and may increase a species' risk of extinction. However, the underlying drivers of hatching failure in threatened populations often receive remarkably little attention, and there are gaps in our understanding of why hatching failure occurs and how best to mitigate it through conservation interventions (Assersohn *et al.*, 2021b).

Hatching failure can result from either fertilisation failure (whereby sperm do not successfully fertilise ova) or embryo mortality (failure of a fertilised ovum to develop into a hatched chick) (Birkhead *et al.*, 2008). Fertilisation failure can occur when the amount of sperm reaching the ovum is insufficient, for example due to unsuccessful copulations resulting from poor paternal condition (Blanco *et al.*, 2009) or behavioural incompatibilities between a breeding pair (e.g. Spoon *et al.*, 2006). Alternatively, fertilisation failure can be the result of the sperm reaching the ovum being of poor quality, which could be due to males experiencing factors such as extreme temperatures (e.g. Hurley *et al.*, 2018), infection (e.g. Losdat *et al.*, 2011), inbreeding depression (e.g. Opatová *et al.*, 2016), stress (e.g. Mazaro & Lamano-Carvalho, 2006), or contamination with toxic substances (e.g. Ackerman *et al.*, 1999). While female fertility traits have generally been understudied relative to male fertility traits, there is evidence that fertilisation failure can also be female-mediated, for example through post-copulatory female choice (e.g. Pizzari & Birkhead, 2000) or via hormonal, environmental, behavioural, and physiological factors affecting female fertility (reviewed in Assersohn *et al.*, 2021a). Embryo mortality can occur at any time during incubation, although different developmental stages are typically associated with different risk factors (Assersohn *et al.*, 2021b). Early embryo deaths are often attributed to genetic factors (Shook *et al.*, 1971; Hemmings *et al.*, 2012a), and eggs prior to the onset of incubation are thought to be

particularly vulnerable to the negative effects of elevated ambient temperatures and trans-shell infection (Meijerhof, 1992), although eggs can become infected by pathogens throughout incubation (Berrang *et al.*, 1999). Early embryo mortality has also been associated with female age (e.g. Fairchild *et al.*, 2002), female body weight (e.g. Coleman & Siegel, 1966), and poor egg quality (e.g. Lerner *et al.*, 1993). Mid-incubation embryo mortality is thought to be relatively uncommon, but has been linked to extreme temperatures (Christensen, 2001) and maternal diet (Romanoff, 1949). Late embryo deaths can result from genetically related morphological abnormalities (e.g. Romanoff, 1949), malpositioning due to extreme temperatures or insufficient egg turning (Romanoff, 1972), or inadequate water loss during incubation resulting in weak, oedematous chicks which may fail to hatch (Kuehler, 1993; Rideout, 2012). Given the variety of different causes that can result in hatching failure, identifying an egg's stage of failure is critical to be able to identify any underlying issues affecting the breeding pair or population.

To differentiate between fertilisation failure and embryo mortality, an unhatched egg may be candled to detect signs of development, or undergo a necropsy examination during which an investigator macroscopically inspects the egg contents to identify an embryo or signs of development such as blood vessel formation. While embryo mortality can occur at any stage between fertilisation and hatching, studies from domesticated birds indicate that the majority of embryo deaths occur either very early or late in the incubation period (Lodge *et al.*, 1971; Christensen, 2001; Sellier *et al.*, 2005). Later embryo deaths are usually easily distinguishable, but it can be difficult to discriminate between eggs that have failed due to fertilisation failure and those that have experienced early embryo mortality (EEM), i.e. before signs of development are apparent. This difficulty can lead to fertilised eggs being mistakenly classified as unfertilised (Birkhead *et al.*, 2008). The situation can become further complicated if the contents of eggs have begun to degrade due to infection or natural decay over time, which makes visual signs of development even harder to detect. Degraded eggs may therefore also be mistaken as unfertilised or simply classified as 'addled' and excluded from any estimations of fertility rates (Birkhead *et al.*, 2008). Egg degradation is a particular issue in the management of wild/threatened species, as unhatched eggs are typically left to be incubated

for some time beyond the expected hatch date to avoid accidentally removing a viable egg or disturbing hatched nestmates (Birkhead *et al.*, 2008; Hemmings *et al.*, 2012b). As fertilisation failure and embryo mortality are likely to be driven by different reproductive issues, determining which of these causes is most prevalent in a managed population may help to identify the most pertinent reproductive problems and support the effective design of mitigation strategies.

To accurately differentiate between fertilisation failure and EEM in unhatched eggs, including those that have become addled, Birkhead *et al.* (2008) developed a microscopy method employing Hoechst 33342 fluorescent DNA staining methods. Application of this, and similar methods, has shown that a large number of apparently undeveloped eggs, which would likely have been classified as 'infertile', have actually experienced EEM (Birkhead *et al.*, 1995; Kempenaers *et al.*, 1999; Hemmings *et al.*, 2012b; Kato *et al.*, 2017; Hemmings & Evans, 2020; Savage *et al.*, 2021), suggesting that infertility issues may generally be overestimated when using macroscopic examination alone to determine egg fertility status (**Table B1**, **Appendix B**). However, an assessment of 478 records included in a systematic review of hatching failure (**Chapter 2**; Marshall *et al.*, 2023) shows that the majority of studies did not attempt to differentiate between fertilisation failure and embryo mortality of unhatched eggs at all, and of those that did, very few applied fluorescence microscopy methods, with most studies determining egg fertility using necropsy examinations or external methods such as candling, shaking, or floating of eggs (**Figure 3-1**; **Table B2**, **Appendix B**). Many investigations of hatching failure do not, therefore, accurately identify embryo mortality during the early stages of development and may subsequently overestimate infertility.

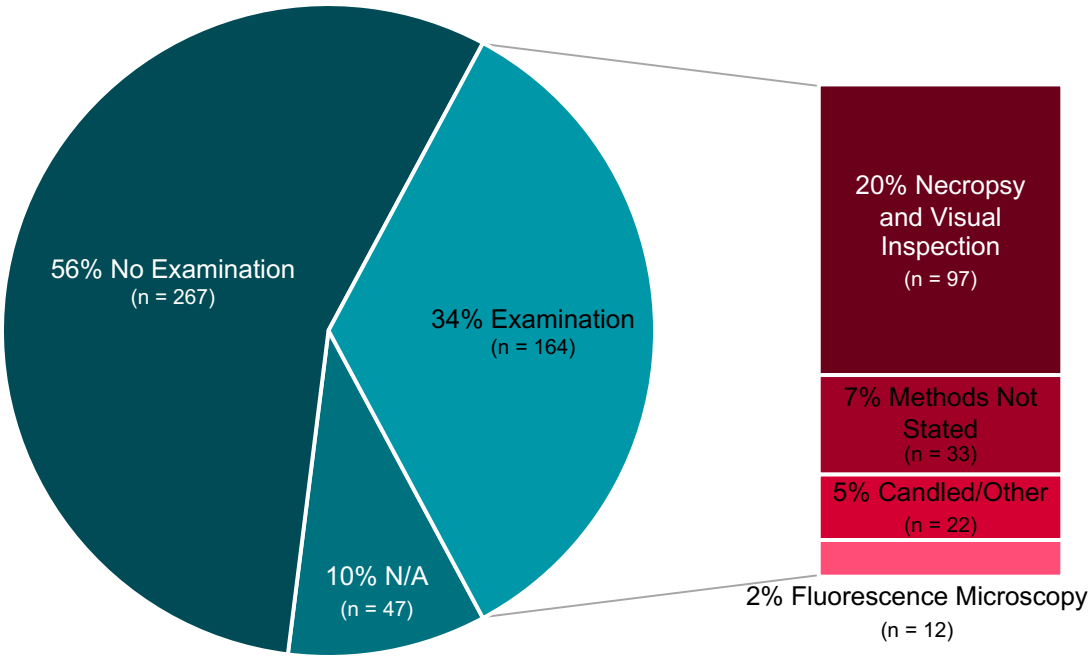

Figure 3-1. Breakdown of how each of the 478 records included in a systematic review and meta-analysis of hatching failure (**Chapter 2**; Marshall *et al.*, 2023) described differentiating between unhatched eggs that failed due to embryo mortality and those that failed due to fertilisation failure.

Understanding the causes of hatching failure is especially important for conservation programmes aiming to improve threatened species' productivity both in the wild and captivity, as this knowledge can help managers to tailor mitigation strategies towards reducing early reproductive stage losses. Captive populations typically experience higher rates of hatching failure than wild populations (**Chapter 2**; Marshall *et al.*, 2023), and there is some evidence that hatching failure in threatened populations in captivity is primarily caused by fertilisation failure, while embryo mortality is more common in the wild (Hemmings *et al.*, 2012b). Hemmings *et al.* (2012b) examined undeveloped eggs from five threatened species, finding that while most eggs were actually fertilised, eggs laid in captivity were more likely to be unfertilised than those laid in the wild. This indicates that although populations of threatened species typically share features such as small population sizes and high levels of inbreeding, other aspects which differ between captive and wild populations may lead to different factors driving hatching failure in these populations, hence requiring different management interventions. For example, typical reproductive behaviour may be suppressed in captivity (Saint Jalme *et al.*, 1996; Driscoll, 2008; Farquharson *et al.*, 2018), and there is often a lack of mate choice and reduced opportunity for extra-pair copulations, both of which may be important

for fertilisation success (Wetton & Parkin, 1991; Zeh & Zeh, 1997; Yuta *et al.*, 2018). Despite this, there has been surprisingly little investigation into the underlying drivers of hatching failure in threatened species, perhaps due to the common belief that failures are predominantly due to the negative effects of inbreeding depression in species which have experienced population bottlenecks (Briskie & Mackintosh, 2004; Spottiswoode & Møller, 2004; Heber & Briskie, 2010). However, as all five of the threatened species included in the Hemmings *et al.* (2012b) study are known, or suspected, to be inbred, the differences in the causes of hatching failure indicate that there may be other factors at play in addition to, or in combination with, inbreeding depression.

In this study, I test the hypotheses that (a) rates of fertilisation failure are overestimated in many managed bird populations, but (b) these rates are higher in captive populations than wild populations. To do this, I apply microscopic methods for accurately determining the fertility status of unhatched bird eggs developed by Birkhead *et al.* (2008), hereafter referred to as 'fluorescence microscopy methods', to unhatched eggs from free-living and captive kakī (black stilt; *Himantopus novaezelandiae*) in Aotearoa New Zealand, and captive populations of a range of different bird species held in the Zoological Society of London (ZSL) Living Bird Collection, in order to identify the rate of fertilisation failure among eggs that fail to hatch. I also determine the developmental stages of kakī embryos that died during the incubation period and use these to describe the timing of embryo mortality during incubation, identify vulnerable stages, and consider the potential drivers. Finally, I use long-term kakī breeding records to identify factors associated with variation in rates of hatching failure, fertilisation failure, and embryo mortality, to provide useful information to support evidence-based conservation management.

Materials and Methods

Study populations

ZSL Living Bird Collection

As part of ZSL bird management protocols, almost all eggs that fail to hatch across its two sites, London Zoo and Whipsnade Zoo, are collected and examined macroscopically as part of a necropsy by wildlife veterinary

pathologists to determine fertility and cause of death where possible. Between January 2019 and May 2021, a total of 177 eggs examined in this way, from 27 different species, were found to either (a) be addled or (b) lack an embryo or other sign of development (**Table B3**, **Appendix B)**. These eggs were fixed in 10% formalin and transferred to me for examination using fluorescence microscopy methods. Of these 177 eggs, 111 had been removed after failing to hatch by the end of the estimated incubation period, and 66 had been electively removed as part of existing management protocols. Electively removed eggs were generally collected within 24 hours of being laid, hence had received little or no parental incubation, and were euthanised through exposure to isoflurane gas to humanely halt any development that had begun, making them analogous to eggs which have naturally experienced early embryo mortality. Electively removed eggs aren't truly unhatched eggs and are therefore excluded from my estimations of fertilisation failure rates. However, they are still useful for testing the degree to which macroscopic inspection alone may misidentify fertilised eggs as unfertilised in the absence of visual indicators of development, and are hence included in analyses of the accuracy of fertility diagnosis via necropsy examination *versus* fluorescence microscopy.

Managed kakī population

Kakī are a Critically Endangered non-migratory wader endemic to Aotearoa New Zealand which have been monitored and managed by the New Zealand Department of Conservation (DOC) since 1979 (BirdLife International, 2018b). They have a reported mean rate of hatching failure of 28.4% (Heber & Briskie, 2010). The population is considered to be wild managed, with ~150 free-living adults located within Te Manahuna/Mackenzie Basin of South Canterbury and North Otago, and three captive pairs maintained in the DOC Kakī Recovery Centre, Twizel, and the Isaac Conservation and Wildlife Centre, Canterbury. Almost every egg laid by both free-living and captive pairs is collected for artificial incubation to protect it from external threats such as predation and flooding, and encourage breeding pairs to lay a replacement clutch (Reed *et al.*, 1993; van Heezik *et al.*, 2005; Pierce, 2013).

During artificial incubation, eggs are candled at least twice a week to check developmental progress. Suspected embryo mortality is confirmed using a

Buddy® digital egg monitor (Avitronics, Truro, Cornwall) that can detect the heartbeat of an embryo from relatively early in incubation. When eggs are confidently confirmed as having ceased developing, they are removed from incubation and refrigerated prior to necropsy examination. In the 2019/20 breeding season a total of 166 eggs (44 captive, 122 wild) were laid, of which 123 (36 captive, 87 wild) hatched. I performed necropsy examinations on the 43 unhatched eggs, except for one captive-laid egg which was accidentally crushed in the nest soon after laying. Any eggs that were addled or otherwise lacked an embryo or other unambiguous signs of development were further examined using fluorescence microscopy.

Kakī breeding records from 2006/07–2018/19, in which eggs were examined using necropsy alone, were combined with the results from 2019/20 for some analyses to further investigate the occurrence and drivers of hatching failure in this population. While records dating from 1979 onwards are available, naturally occurring hybrids of kakī and poaka (pied stilt; *Himantopus leucocephalus*) were actively included in conservation activities until the 2005/06 breeding season (Greene, 1999; Wallis, 1999; Maloney & Murray, 2001; Steeves *et al.*, 2010), so these have been excluded, as have any individual records of hybrid eggs from 2006/07 onwards.

Egg necropsy examinations and embryo staging

Necropsy examinations of the ZSL eggs were performed by the wildlife veterinary pathology team following their own standard protocols. I examined the formalin-fixed egg contents using a Leica S6 D stereomicroscope to detect any small embryos which may have been missed, and any embryos detected were staged (assigned to one of the 46 identifiable phases of embryonic development) based on Hamburger & Hamilton's (1951) normal stages of chick development, with support from Hemmings & Birkhead (2016) and Araújo *et al.* (2019).

Unhatched kakī eggs were opened using small scissors or blunt forceps and emptied into a petri dish containing sterile phosphate-buffered saline (PBS) solution. Any embryos were separated from the remainder of the contents, cleaned in PBS and examined under a stereomicroscope to be staged as described above. Embryos could typically be assigned to one specific stage or

within a range of 2-3 stages, but where degradation was advanced a wider range was assigned. Stages were grouped into classifications of ‘died pre-incubation’ (<4), ‘died early’ (4-25), ‘died mid’ (26-37), ‘died late’ (38-43), and ‘died hatching’ (≥44) based on an approximate 25-day incubation period and integration of the 46 identifiable phases with broad-scale staging performed in previous breeding seasons for kakī (Brown, 2017). The main biological changes marking the transition into each successive classification were: died early = the primitive streak has reached its maximum length (definitive streak); died mid = initial demarcation of digits is visible; died late = extensive feather germ coverage including upper eyelid, and opening between eyelids much reduced; died hatching = chick has internally pipped, yolk-sac has started to retract within body cavity. A kakī embryo staging guide was produced as part of this work and is included in **Appendix E**. If no embryo was found, the egg contents were fixed in a solution of 5% formalin prior to fluorescence microscopy examination.

Fertility examinations using fluorescence microscopy

Following Birkhead *et al.* (2008), the fertility of unhatched eggs was determined by staining the germinal disc and the perivitelline layer (PVL) of the ovum with Hoechst 33342 fluorescent DNA stain (0.05 mg ml^{-1}) and then examining them using a fluorescence microscope to detect: (1) presence of nuclei from embryonic cells in the germinal disc, (2) sperm trapped in the PVL, (3) penetration holes in the PVL made by sperm entering the ovum. Eggs from ZSL were examined at the University of Sheffield using a Leica DMLB fluorescence microscope, and kakī eggs were examined at the University of Auckland using a Leica DMR fluorescence microscope. Both microscopes had a BP 340-380 excitation filter, LP 425 suppression filter, and darkfield filter. Following Hemmings and Evans (2020), fertilisation success was determined based on the presence of nuclei from embryonic cells, with trapped sperm or penetration holes as supporting evidence only. Likewise, eggs were only assumed unfertilised if the germinal disc was located and examined, and both embryonic and sperm cells were absent. In other circumstances, such as in cases where I was not confident that the germinal disc was located and stained (and hence the lack of embryonic nuclei may not have reflected their true absence from the egg), the fertility status was designated as 'inconclusive'. In cases where the egg contents had degraded to the point where no examinable material (either

germinal disc or PVL) was available, the fertility status was classified as 'unknown'. For the purposes of the analyses these final two categories were combined into a single category of ‘undetermined’. This is a conservative method chosen to minimise incorrect diagnoses. A full description of the criteria used to diagnose the fertility of eggs can be found in **Table B4, Appendix B**.

Statistical analyses

All statistical analyses were completed in RStudio v 2022.7.0.548 (RStudio Team, 2022) running R v4.2.1 (R Core Team, 2022). The alpha level used for statistical significance was 0.05 throughout the Results section.

The function 'prop.test' from the R package *stats* v4.2.1 was used to apply two-proportion z-tests to compare overall hatching failure rates, assumed infertility rates, and proportion of hatching failure attributed to embryo mortality *versus* fertilisation failure of wild- and captive-laid kakī eggs from 2006/07 to 2018/19 or 2019/20. For seasons prior to 2019/20, assumed infertility rates and proportions of embryo mortality *versus* fertilisation failure were calculated with addled eggs excluded, assumed to be unfertilised, and assumed to be fertilised, all of which may be used in studies to address the uncertainty surrounding addled egg fertility.

Generalised Linear Mixed Models (GLMMs) were applied with the function ‘glmer’ from the R package *lme4* v1.1.31 (Bates et al., 2015) to investigate the relationships between parental, clutch, and egg characteristics with hatching failure and with fertilisation failure *versus* embryo mortality using all kakī breeding data from the 2006/07 to 2019/20 breeding seasons. Separate sets of GLMMs were constructed with a binary unhatched *versus* hatched status for each egg and with a binary fertilisation failure *versus* embryo mortality for each failed egg as the response variables. The model of embryo mortality *versus* fertilisation failure was run on datasets with all addled eggs excluded, with addled eggs assumed to be unfertilised, and with addled eggs assumed to be fertilised. All GLMMs were binomial with a logit link function, and both dam ID and breeding season were included as random factors. To select the best models, each predictor variable was tested individually and compared to the null model with the Akaike’s information criterion (AIC) (Akaike, 1981; Burnham & Anderson, 2002). Variables in models with a significantly lower AIC value than

the null model were included in an additive full model, which was then compared to models with each variable removed one by one, and to a model with interactions between the variables. The ‘aictab’ function from the package *AICcmodavg* v2.3.1 (Mazerolle, 2020) was used to compare all models by the AIC corrected for small sample size (AIC_c) (Akaike, 1973; Hurvich & Tsai, 1989).

I used a Cumulative Link Mixed Model (CLMM) applied with the function ‘clmm’ from the R package *ordinal* v2022.11.16 (Christensen, 2022) to investigate the relationships between parental, clutch, and egg characteristics and the stage of embryo mortality. While embryos from the 2019/20 season were staged according to Hamburger and Hamilton’s 46 identifiable stages of chick development (Hamburger & Hamilton, 1951), in previous seasons embryos were assigned to broader categories. Therefore, the response variable was the stage of embryo mortality with levels: 'died pre-incubation', 'died early', 'died mid', 'died late', and 'died hatching'. Breeding season was set as a random factor. Dam ID was trialled as a second random factor but did not improve the fit of the model. Model selection was performed based on the AIC_c as described above.

The predictor variables tested in each set of GLMMs and CLMMs were chosen based on their previous identification as potential drivers of hatching failure in kakī or other species: dam age (linear and quadratic) (Croxall *et al.*, 1992; Bourke *et al.*, 2023); sire age (linear and quadratic) (Preston *et al.*, 2015; Bourke *et al.*, 2023); clutch number (Nager *et al.*, 2000); clutch size (Reid *et al.*, 2000); estimated embryo age (i.e. the length of parental incubation) at time of collection for artificial incubation (Smith *et al.*, 2011); egg weight at time of collection for artificial incubation (Bourke *et al.*, 2023); collection location (Bourke *et al.*, 2023); and whether the egg was wild- or captive-laid (van Heezik *et al.*, 2005). This data was all available as part of the long-term kakī breeding records, with the embryo age at collection estimated based on candling. All continuous variables were scaled and centred. To check for correlations between the variables which could lead to multicollinearity, correlations between continuous variables were assessed by calculating Pearson correlation coefficient values, correlations between categorical variables were assessed by performing pairwise chi-squared tests of independence and calculating

Cramér's V (Cramér, 1946) for each relationship, and correlations between continuous and dichotomous categorical variables were assessed by calculating point biserial correlation coefficient values (Tate, 1954, 1955) (**Table B5–Table B7**, **Appendix B)**. All correlations were found to be of low, very low, or negligible strength, hence it was considered appropriate to include all variables in the models. The only exception was a strong significant correlation between nest location and whether an egg was captive- or wild-laid, but the methods of model selection meant that these two factors were never combined in the same model, hence avoiding multicollinearity. The estimated embryo age at time of collection for artificial incubation was not included as a variable in the GLMM of embryo mortality *versus* fertilisation failure as eggs with failure later attributed to a lack of fertilisation had, as expected, almost all been estimated as zero days old (i.e. unincubated) during candling, severely skewing the data. Similarly, estimated embryo age was not included in the CLMM of mortality stage as embryos could only fail after the point they have reached in development, also skewing the data.

Across the 14 breeding seasons 2006/07 through to 2019/20, incompleteness in the records meant there were no datapoints from 2006/07, 2007/08, 2010/11, and 2012/13 for any of the GLMMs or CLMMs.

Results

ZSL Living Bird Collection

Fertilisation failure *versus* early embryo mortality

Of the 177 ZSL eggs preserved for further investigation, 15 contained small embryos. Six of these were found in eggs described as addled, disintegrated, or autolysed, and were all estimated to be at around chick development stage 21-26 (**Table B8**, **Appendix B**). A review of the necropsy reports from the wildlife veterinary pathology team indicated that three of these embryos had been detected during the initial necropsy exam and these eggs were therefore excluded from further analyses. A further two eggs which were not found to contain embryos but also did not have any diagnosis of fertility based on the necropsy exam were also excluded from further analyses.

Of the remaining 172 eggs for which an embryo had not been detected during necropsy examination and an initial fertility diagnosis (based on macroscopic examination) was available, 6.4% (11/172) were diagnosed as fertilised, 75% (129/172) were diagnosed as unfertilised, and 18.6% (32/172) had undetermined fertility status (**Table B8**, **Appendix B**). Fluorescence microscopy examination revealed 69.8% (120/172) of these eggs to be fertilised, 6.4% (11/172) to be unfertilised, and for 23.8% (41/172) fertility status was undetermined (**Table B8**, **Appendix B**). Of eggs classified as addled, 66.7% (22/33) were diagnosed as fertilised based on fluorescence microscopy, with the remaining 33.3% (11/33) having undetermined fertility status (**Table B8**, **Appendix B**).

Necropsy frequently led to mistaken diagnoses of fertilisation failure (**Figure 3-2**). Of 129 eggs suspected to be unfertilised based on necropsy, 71.3% (92/129) were diagnosed as fertilised based on fluorescence microscopy, 3.1% (4/129) were diagnosed as unfertilised, and 25.6% (33/129) had undetermined fertility status after fluorescence microscopy examination (**Figure 3-2**; **Table B8**, **Appendix B**). Of the eggs with undetermined fertility status based on

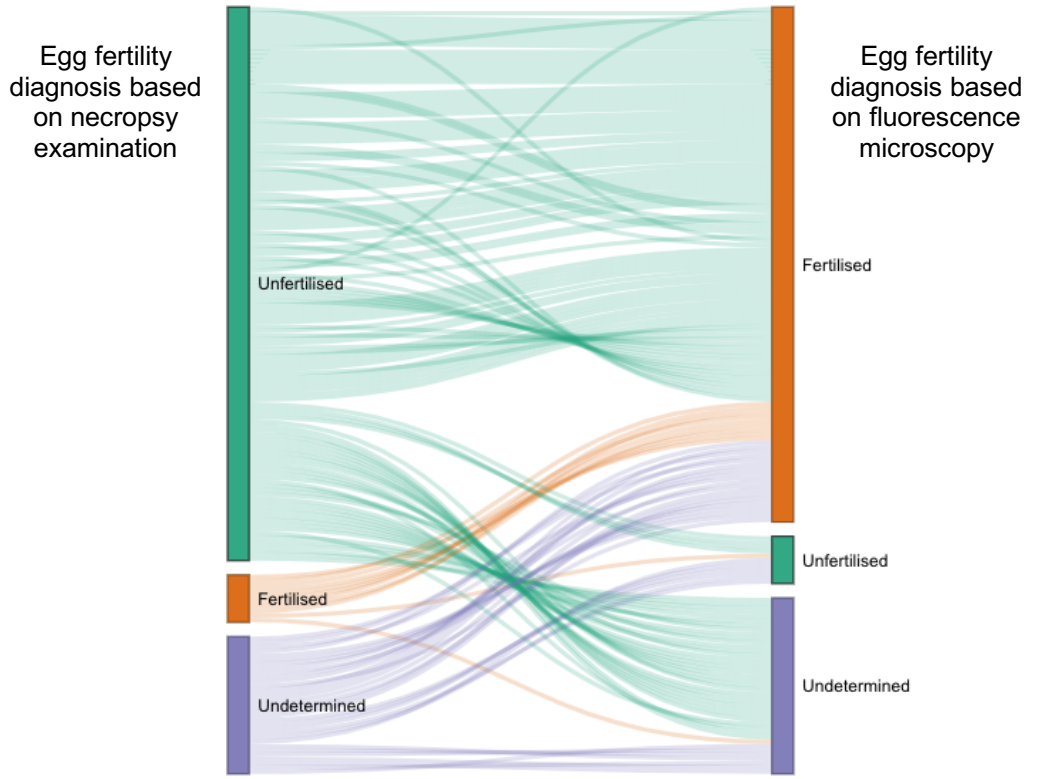

Figure 3-2. Visualisation of the suspected fertility status of examined eggs from the ZSL Living Collection (*n* = 172) based on necropsy examination and fluorescence microscopy examination. Two eggs which did not have a stated fertility status based on necropsy and three eggs for which an embryo had been detected during necropsy were excluded. Each line represents one egg.

necropsy alone, 78.1% (25/32) could be diagnosed using fluorescence microscopy, with 59.4% (19/32) diagnosed as fertilised, 18.8% (6/32) diagnosed as unfertilised, and the remaining 21.9% (7/32) having undetermined results (**Figure 3-2**; **Table B8**, **Appendix B**).

When limited to truly unhatched eggs (i.e. excluding those electively removed for management reasons), necropsy diagnoses resulted in 84.9% (90/106) eggs being categorised as unfertilised, 6.6% (7/106) categorised as fertilised, and 8.5% (9/106) having an undetermined fertility status. However, following fluorescence microscopy, this changed to just 2.8% (3/106) being categorised as unfertilised, 69.8% (74/106) as fertilised, and 27.4% (29/106) as undetermined. Of the unhatched eggs originally classified as unfertilised, only 3.3% (3/90) were confirmed as unfertilised using fluorescence microscopy, while 73.3% (66/90) were found to be fertilised. Therefore, the proportion of all undeveloped eggs from the ZSL captive populations that experienced fertilisation failure was in the range of 2.8% to 30.2%, with the latter figure making the conservative assumption that all eggs with undetermined fertility status were in fact unfertilised.

For threatened (Critically Endangered, Endangered, or Vulnerable) species only, fluorescence microscopy showed that 73.5% (36/49) of undeveloped eggs were fertilised while 26.5% (13/49) of eggs had undetermined fertility status and no eggs were confirmed to be unfertilised. For non-threatened (Near Threatened and Least Concern) species, 66.7% (38/57) of undeveloped eggs were fertilised, 5.3% (3/57) of eggs were unfertilised, and 28.1% (16/57) of eggs had undetermined fertility status. Therefore, for threatened species the proportion of all undeveloped eggs that experienced fertilisation failure was in the range 0.0% to 26.5%, while for non-threatened species the proportion experiencing fertilisation failure was 5.3% to 33.3%, with the maximum value in each range estimated assuming all eggs with an undetermined fertility status were unfertilised.

Managed kakī population

Necropsy and fluorescence microscopy results

Of 42 unhatched kakī eggs which were examined via necropsy, nine died hatching (after chick had internally pipped, stage ≥44), seven died late in incubation (stage 38-43), eight died mid-incubation (stage 26-37), ten died early in incubation (stage ≤25), three were addled, and five showed no signs of development, of which two had been damaged prior to collection. Of eggs that died late in incubation, five displayed severe embryonic malpositioning. Of eggs that died hatching, one had been damaged late in incubation and six displayed minor to severe embryonic malpositioning, with three of these malpositioned chicks also displaying external deformities (**Table B9**, **Appendix B**).

The three addled eggs (two captive-laid, one wild-laid) and five undeveloped eggs (all wild-laid) were examined using fluorescence microscopy. All three addled eggs and four of the undeveloped eggs were found to be fertilised, hence died “pre-incubation” (stage <4), with the remaining undeveloped, wild-laid egg having undetermined fertility (**Table B10**, **Appendix B**). Hence, the proportion of apparently undeveloped eggs, which would have likely been classified as ‘infertile’ based on necropsy alone, found to be unfertilised was either 0% or 12.5% depending on whether the egg with undetermined fertility status was assumed to be fertilised or unfertilised, and either 0% or 16.7% when considering wild-laid eggs only. For all unhatched eggs, the proportion found to be unfertilised was 0% for captive-laid eggs (*n* = 8), 0-2.9% for wild-laid eggs (*n* = 35), and 0-2.3% for all eggs (*n* = 43).

The fate of all eggs from the 2006/07 breeding season onwards, which formed the basis for calculations of hatching failure and assumed infertility rates, can be found in **Table B11**, **Appendix B**. Damaged eggs were excluded from calculations of hatching failure, but where their fertilisation status was known they were included in calculations of fertility.

Hatching failure

The overall rate of hatching failure (proportion of eggs present at the end of the incubation period that failed to hatch, excluding damaged eggs and eggs with

unknown fates) of all managed kakī eggs from 2006/07 to 2019/20 (*n* = 2383) was 23.4% (17.9-34.2%). For captive-laid eggs only (*n* = 825), the overall rate of hatching failure was 23.4% (9.43-39.4%), and for wild-laid eggs only (*n* = 1558), the overall rate of hatching failure was also 23.4% (17.3-43.5%) (**Table 3-1**). There was no significant difference between the overall hatching failure rate of wild- and captive-laid eggs (z = 7.72e-30, $p > 0.05$).

Table 3-1. Hatching failure rates of kakī eggs for breeding seasons 2006/07 to 2019/20. Calculations of hatching failure exclude damaged eggs and eggs with unknown hatching fate (e.g. that were fostered to the wild as pipping eggs).

Breeding Season	Total Eggs Laid	Captive-laid	Wild-laid	Hatching Failure %		
				Captive	Wild	Overall
2006/07	143	35.7%	64.3%	23.5%	35.9%	31.5%
2007/08	179	39.7%	60.3%	39.4%	17.3%	26.2%
2008/09	146	52.7%	47.3%	26.0%	43.5%	34.2%
2009/10	173	43.4%	56.6%	28.0%	20.6%	23.8%
2010/11	150	46.7%	53.3%	27.1%	27.3%	27.2%
2011/12	104	58.7%	41.3%	21.3%	21.4%	21.4%
2012/13	172	34.9%	65.1%	23.3%	21.4%	22.1%
2013/14	172	36.6%	63.4%	14.3%	20.2%	18.0%
2014/15	219	35.6%	64.4%	29.5%	18.4%	22.4%
2015/16	137	31.4%	68.6%	19.0%	26.6%	24.3%
2016/17	206	22.8%	77.2%	19.6%	18.8%	19.0%
2017/18	264	20.5%	79.5%	9.43%	20.1%	17.9%
2018/19	185	21.6%	78.4%	17.5%	24.1%	22.7%
2019/20	166	26.5%	73.5%	16.3%	26.9%	24.1%
Overall	2416	34.5%	65.5%	23.4%	23.4%	23.4%

The best model to predict hatching failure was an additive model with predictor variables of clutch size, estimated embryo age at time of collection for artificial incubation, and egg weight at time of collection for artificial incubation (**Table 3-2**). Eggs from larger clutches were less likely to experience hatching failure (effect ± SE = -0.201 ± 0.087, $z = -2.309$, $p = 0.021$), as were eggs that contained older embryos (effect ± SE = -1.233 ± 0.171, $z = -7.202$, $p < 0.001$) and were heavier (effect ± SE = -0.658 ± 0.114, $z = -5.783$, $p < 0.001$) when they were collected for artificial incubation.

Table 3-2. Summary of results from the best GLMM model for hatching failure. Breeding season and dam ID were included as random factors. continuous variables were scaled and centred. SE = adjusted standard error; CI = confidence intervals. Variables in bold indicate significant p-values ($p < 0.05$) and where the 95% confidence interval did not overlap zero.

Response variable	Estimate	95% CI		SE	*z*	*p*
Unhatched (*n* = 234) vs. hatched (*n* = 907)						
(Intercept)	**-1.965**	**-2.339**	**-1.592**	**0.191**	**-10.311**	**< 0.001**
Clutch size	**-0.201**	**-0.371**	**-0.030**	**0.087**	**-2.309**	**0.021**
Estimated embryo age	**-1.233**	**-1.568**	**-0.897**	**0.171**	**-7.202**	**< 0.001**
Egg weight at collection	**-0.658**	**-0.880**	**-0.435**	**0.114**	**-5.783**	**< 0.001**

Fertilisation failure *versus* embryo mortality

In the 2019/20 breeding season, 164 of 165 examined eggs were diagnosed as fertilised, with the remaining egg having undetermined fertility, giving the proportion of unfertilised eggs (i.e. the overall population fertilisation failure rate) as 0-0.61% overall (0% for captive-laid eggs (*n* = 43), 0-0.82% for wild-laid eggs (*n* = 122)). The overall rate of assumed infertility based on necropsy alone for the 2006/07–2018/19 breeding seasons was 7.43% when addled eggs were excluded from calculations, 11.9% when addled eggs were assumed to be unfertilised, and 7.07% when addled eggs were assumed to be fertilised (**Table B12**, **Appendix B**). When addled eggs were assumed to be unfertilised, the difference between captive-laid (11.7%) and wild-laid (12.1%) eggs was not statistically significant ($z = 0.061$, $p = 0.805$), and when addled eggs were excluded from calculations the difference between captive-laid (8.97%) and wild-laid eggs (6.58%) was close to significance ($z = 3.72$, $p = 0.054$). However, when addled eggs were assumed to be fertilised (the most likely scenario based on the 2019/20 data) the difference between captive-laid (8.71%) and wild-laid (6.19%) eggs was significant ($z = 4.52$, $p = 0.0334$) (**Table B12**, **Appendix B**).

For the 2019/20 breeding season, either 2.3% or 0% of hatching failure could be attributed to fertilisation failure depending on whether the egg with an undetermined fertility diagnosis was taken as unfertilised or fertilised. For the previous seasons, 2006/07 to 2018/19, the overall proportion of hatching failure that could be attributed to fertilisation failure was 38.7% if addled eggs were excluded from calculations, 51.6% if addled eggs were assumed to be unfertilised, or 30.6% if addled eggs were assumed to be fertilised (**Figure 3-3**; **Table B13**, **Appendix B**). There was no significant difference between the overall proportion of hatching failure attributed to fertilisation failure in captive-laid eggs compared to wild-laid eggs when addled eggs were assumed to be unfertilised (49.2% *vs.* 53.0%; $z = 0.551$, $p = 0.458$) or when addled eggs were excluded (42.0% *vs.* 36.6%; $z = 0.980$, $p = 0.322$), but when addled eggs were assumed to be fertilised, which is the most likely scenario based on the 2019/20 data, significantly fewer wild-laid (27.1%) eggs failed due to fertilisation failure than captive-laid (36.8%) eggs ($z = 4.77$, $p = 0.0290$) (**Table B13**, **Appendix B**).

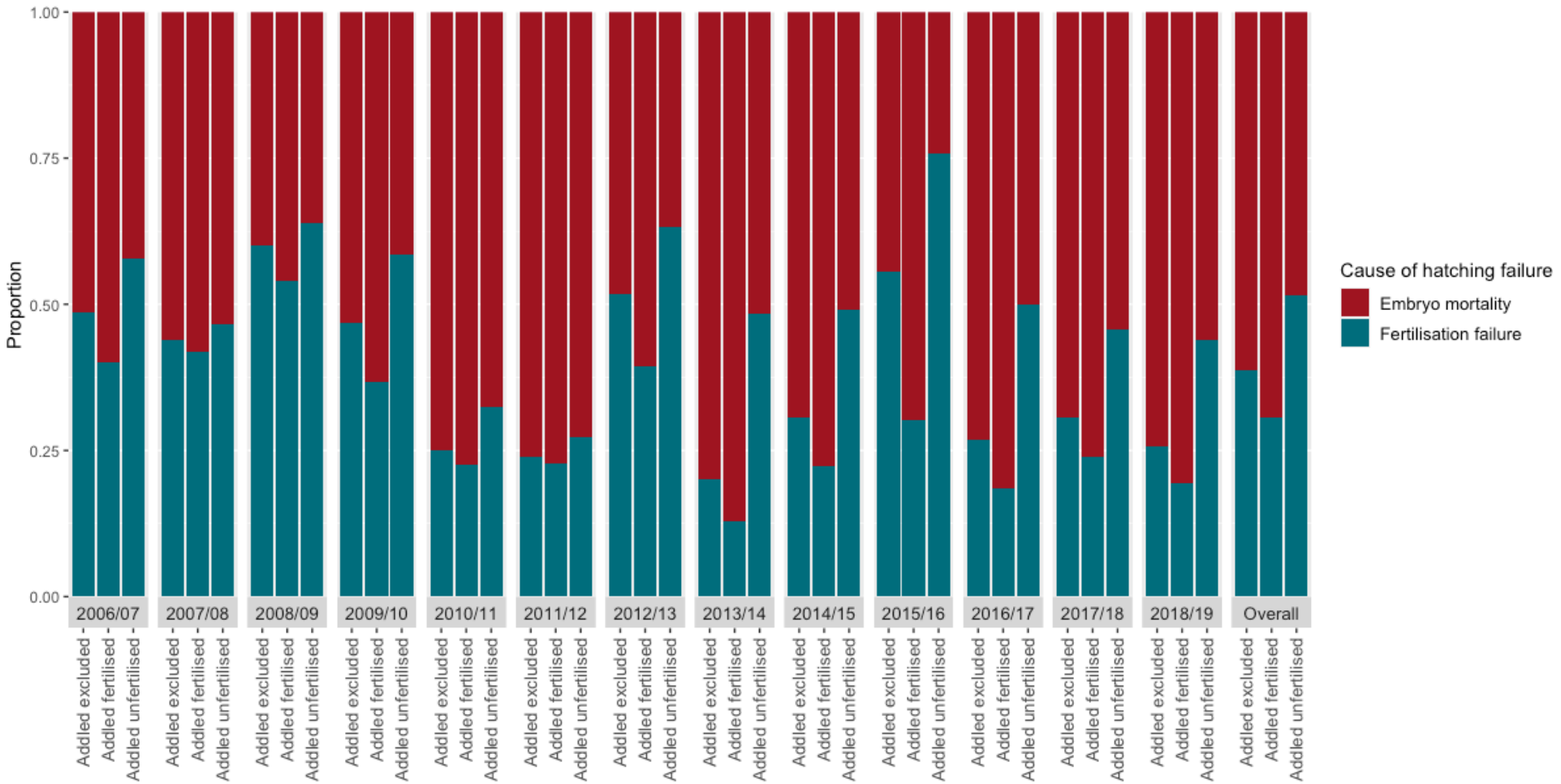

Figure 3-3. The proportion of hatching failure attributed to embryo mortality and fertilisation failure in each breeding season 2006/17-2018/19 and overall, showing the difference depending on whether addled eggs are excluded, assumed to be fertilised, or assumed to be unfertilised.

The best model to predict whether eggs failed due to fertilisation failure when addled eggs were excluded was an additive model with the predictors clutch size and egg weight at collection for artificial incubation (**Table 3-3**). Clutch size had a significant positive coefficient (effect ± SE = 0.776 ± 0.264, z = 2.946, p = 0.002), indicating that failed eggs from larger clutches were more likely to have experienced fertilisation failure. The significant negative coefficient for egg weight (effect ± SE = -0.791 ± 0.282, z = -2.804, p = 0.005) indicates that failed eggs that were heavier at collection were more likely to have failed due to embryo mortality (**Table 3-3**). When addled eggs were included in the models and assumed to be fertilised or unfertilised the results were very similar, with the exception that the best model when addled eggs were assumed to be unfertilised did not include clutch size as a predictor (**Table 3-3**).

Table 3-3. Summary of results from the best GLMM model for fertilisation failure. Breeding season and dam ID were included as random factors. continuous variables were scaled and centred. SE = adjusted standard error; CI = confidence intervals. Variables in bold indicate significant p-values ($p < 0.05$) and where the 95% confidence interval did not overlap zero.

Response variable	Estimate	95% CI		SE	*z*	*p*
Fertilisation failure (*n* = 57) vs. embryo mortality (*n* = 129) – addled eggs excluded						
(Intercept)	**-1.458**	**-2.394**	**-0.523**	**0.477**	**-3.056**	**0.002**
Clutch size	**0.776**	**0.260**	**1.292**	**0.264**	**2.946**	**0.003**
Egg weight at collection	**-0.791**	**-1.343**	**-0.238**	**0.282**	**-2.804**	**0.005**
Fertilisation failure (*n* = 105) vs. embryo mortality (*n* = 129) – addled eggs = unfertilised						
(Intercept)	-0.348	-1.077	0.381	0.372	-0.935	0.3500
Egg weight at collection	-0.372	-0.746	0.003	0.191	-1.946	0.0516
Fertilisation failure (*n* = 57) vs. embryo mortality (*n* = 177) – addled eggs = fertilised						
(Intercept)	**-1.834**	**-2.607**	**-1.061**	**0.394**	**-4.653**	**< 0.001**
Clutch size	**0.664**	**0.216**	**1.112**	**0.229**	**2.904**	**0.004**
Egg weight at collection	**-0.630**	**-1.096**	**-0.164**	**0.238**	**-2.647**	**0.008**

Timing of embryo mortality during incubation

There is considerable variation in the timing of embryo mortality during incubation between breeding seasons, although in general there appears to be more failure early in the incubation period than mid-incubation or later (**Figure 3-4**). The timing of embryo mortality between captive- and wild-laid eggs also appears to be broadly similar, although captive-laid eggs may experience a slightly higher proportion of deaths during early incubation (**Figure 3-4**).

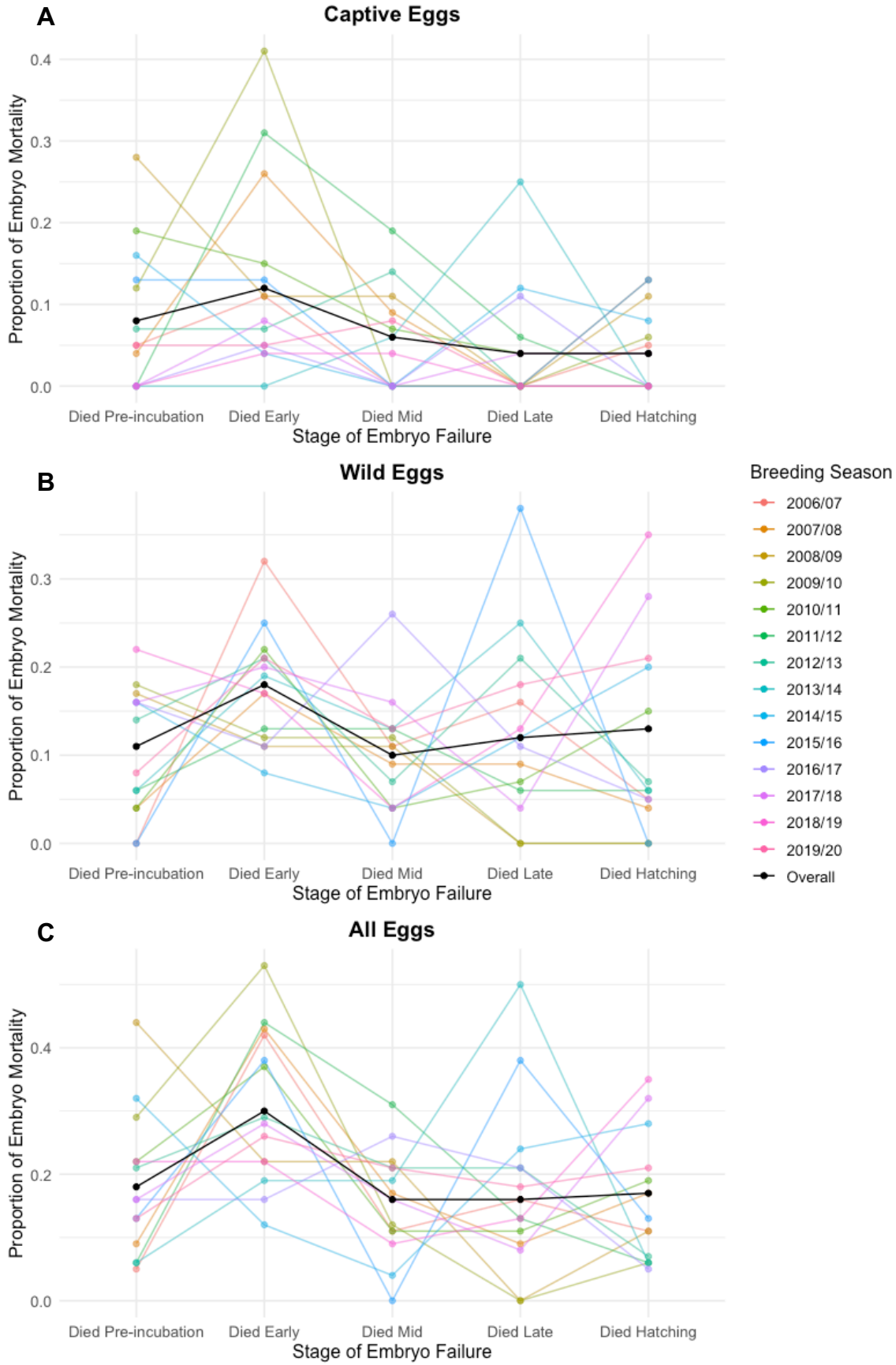

Figure 3-4. The proportion of A) captive, B) wild, and C) all kakī eggs experiencing embryo mortality at each stage of incubation: died pre-incubation (stage <4), died early (stage 4-25), died mid (stage 26-37), died late (stage 38-43), and died hatching (≥44). Addled eggs, eggs diagnosed as being unfertilised based on fluorescence microscopy, and eggs reported as dying during incubation without a specified stage are excluded.

Table 3-4. Summary of results from the best CLMM model. Breeding season was included as a random factor. SE = adjusted standard error; CI = confidence intervals. Variables in bold indicate significant p-values ($p < 0.05$) and where the 95% confidence interval did not overlap zero.

Response variable	Estimate	95% CI		SE	*z*	*p*
Stage of embryo mortality (*n* = 129) *[ordinal levels: died pre-incubation (n = 28), died early (n = 32), died mid (n = 18), died late (n =26), died hatching (n = 25)]*						
Clutch size	**0.610**	**0.174**	**1.046**	**0.222**	**2.742**	**0.006**

The best model to predict the stage of embryo mortality was a model with the predictor variable clutch size. Clutch size had a significant positive coefficient (effect ± SE = 0.610 ± 0.222, $z = 2.742$, $p = 0.006$), indicating that eggs from larger clutches were more likely to fail later in incubation than early (**Table 3-4**).

Discussion

By applying fluorescence microscopy methods to accurately differentiate between fertilisation failure and early embryo mortality in unhatched eggs from both captive zoo populations and a wild managed population of a threatened species, I have found support for the hypothesis that rates of true infertility (i.e. the proportion of unfertilised eggs) may be overestimated in many managed bird populations. My results indicate that necropsy examinations alone are unlikely to be sufficient to accurately determine whether an unhatched egg has experienced fertilisation failure or embryo mortality, which could have consequences for management decisions. Additionally, I found that kakī eggs from larger clutches were more likely to hatch, but if they failed to hatch, this was more likely to be due to fertilisation failure than embryo mortality. Kakī eggs containing older embryos at the time of collection for artificial incubation also had a higher probability of hatching than those collected sooner after laying. Heavier eggs were also more likely to hatch, but if they failed to hatch, this was more likely to be due to embryo mortality than fertilisation failure. Both the inter- and intraspecific findings of my analyses, and the potential implications for management, are discussed below.

Interspecific patterns

Overall, I found that necropsy frequently misdiagnosed fertility, or lack of, in undeveloped eggs, highlighting the extent to which reproductive problems may be misinterpreted when unhatched eggs are subject to macroscopic examination alone. More than two thirds of suspected ‘infertile’ eggs from the ZSL Living Bird Collection were found to be fertilised using the more accurate

fluorescence microscopy methods, and in the 2019/20 breeding season a much lower proportion of kakī eggs were found to be unfertilised compared to previous seasons where only necropsy methods were used. It is worth noting, however, that while this is at least partially due to the application of fluorescence microscopy methods, if these eggs had all been classified as unfertilised based on necropsy examination alone, the fertilisation failure rate of this season would still have been relatively low compared to previous seasons. Regardless, the strong indication that necropsy examination alone is likely to overestimate the rates of fertilisation failure in a population is likely to have implications for conservation management: faced with a breeding pair consistently laying eggs that fail to hatch, conservation practitioners may apply management interventions aimed at reducing the rate of failure. If failed eggs appear to be normal in appearance and are believed to be unfertilised, this may be interpreted as a problem with sperm production or transfer, so one potential intervention may be to separate the pair and provide the female with a new male. However, if the eggs are in fact fertilised, it is unlikely that male sperm production or sperm function is solely to blame, and instead the problem may be related to something else, such as a physiological female problem (Assersohn *et al.*, 2021a), incubation issues (Wang & Beissinger, 2009), environmental conditions such as high ambient temperature (Aldredge *et al.*, 2012), or pollutants (Richard *et al.*, 2021), hence re-pairing with a new mate alone may not improve hatching success. Further, there is evidence that birds that retain their mates over multiple seasons have greater reproductive success than those that change mate (Coulson, 1966; Erickson & Morris, 1972; Mills, 1973; Yamamoto *et al.*, 1989) and several studies of captive birds have shown that reproductive success, particularly fertilisation success, improves the longer pairs are kept together (Brosset, 1981; Olsen & Olsen, 1981; Wiemeyer, 1981; Fournier *et al.*, 2007). Therefore, an erroneous assumption of fertilisation failure, leading to the splitting of established pairs, might set back reproductive success even further. As many of the species held at ZSL are part of endangered species breeding programmes and are maintained in studbooks, the potential misclassification of a potentially viable male as infertile could have an impact not only within its current breeding pair, but also across the entire managed population when making pairing recommendations.

Even if all eggs with undetermined fertility status based on fluorescence microscopy were unfertilised, less than a third of the eggs laid by the captive ZSL populations that appeared undeveloped or addled were unfertilised, with similar values for both threatened and non-threatened species. However, as it is likely that at least some of these undetermined eggs were in fact fertilised, the true proportion of unfertilised eggs may be much lower. The maximum estimate of fertilisation failure across the captive ZSL eggs (30.2%) is similar to the proportion of undeveloped eggs found to be unfertilised in wild house sparrow (*Passer domesticus*) (Birkhead et al., 1995), wild managed kākāpō (*Strigops habroptilus*) (Savage et al., 2021), and captive helmeted honeyeater (*Lichenostomus melanops cassidix*) (Hemmings *et al.*, 2012b), and lower than that found for captive orange-bellied parrot (*Neophema chrysogaster*), and captive Spix's macaw (*Cyanopsitta spixii*) (Hemmings *et al.*, 2012b) (**Table B1**, **Appendix B**). For kakī in 2019/20, fluorescence microscopy results indicated that all unhatched captive-laid eggs were fertilised, and 0-2.9% of unhatched wild-laid eggs were unfertilised, similar to the proportion of unhatched eggs found to be unfertilised in wild managed tree sparrow (*Passer montanus*) (Birkhead *et al.*, 2008), wild managed great tit (*Parus major*) and blue tit (*Cyanistes caeruleus*) (Hemmings & Evans, 2020), and wild Northern wheatear (*Oenanthe oenanthe*) (van Oosten *et al.*, 2019), while the overall population fertilisation failure rate for wild-laid kakī eggs of 0-0.82% was similar to that of captive zebra finch (Hurley *et al.*, 2020) (**Table B1**, **Appendix B**). Taken together, my interspecific results and the findings from other studies do not appear to support captive populations being more likely to have experienced fertilisation failure than wild populations (Hemmings *et al.*, 2012b), suggesting that there may be more species- or population-specific factors at play. However, the most appropriate method to investigate this is through intraspecific comparisons comparing the fertilisation rate of captive- and wild-laid eggs from the same species, which have so far been limited to only this and one other study (Hemmings *et al.*, 2012b). While Hemmings *et al.* (2012b) did find a much higher proportion of undeveloped captive-laid orange-bellied parrot eggs to be unfertilised compared to wild-laid eggs, the sample sizes were very small, while in my study I found inconsistent support for captive-laid kakī eggs having elevated fertilisation failure compared to wild-laid eggs. This demonstrates the need for more intraspecific comparisons of wild and captive populations,

particularly as a difference in fertilisation success could represent a mechanism of adaptation to captivity that could potentially impact reintroduction success.

One potentially key factor when considering rates of fertilisation failure in a population is a species' threat status, with populations of threatened species shown to have elevated rates of hatching failure compared to non-threatened species (**Chapter 2**; Marshall *et al.*, 2023). Several studies of wild or wild managed populations of species classified as Least Concern have found overall population fertilisation failure rates close to 0% (Birkhead *et al.*, 1995; Kempenaers *et al.*, 1999; Hemmings & Evans, 2020), while higher rates have been reported for threatened species such as 13.7% for the Critically Endangered wild managed kākāpō (Savage et al., 2021), indicating that fertilisation failure may only become an issue once a species has already started to decline due to other threats. However, it is difficult to discern whether this is a general pattern across threatened and non-threatened species due to the paucity of studies differentiating between fertilisation failure and embryo mortality in general, and particularly in studies of threatened species (**Table B1**, **Appendix B**). There is a clear need for more studies of both threatened and non-threatened species to identify the causes of hatching failure as part of their investigations to enable us to better understand any overall patterns and ultimately identify the drivers of these failures.

Intraspecific patterns

In kakī, for breeding seasons where fluorescence microscopy methods were not used, it is clear that the treatment of addled eggs (excluded, assumed to be fertilised, or assumed to be unfertilised) in estimations of the proportion of hatching failure attributed to embryo mortality *versus* fertilisation failure often resulted in substantial differences (**Figure 3-3**). By using fluorescence microscopy to accurately determine the fertility status of addled eggs, we can greatly improve our understanding of egg failure in a population. As all addled kakī eggs from the 2019/20 breeding season examined using fluorescence microscopy were fertilised it is likely that at least some, and potentially many, of the addled eggs in previous seasons were also fertilised, hence excluding addled eggs or assuming them to be infertile in the past would have led to overestimations of fertilisation failure. Under the assumption that all addled

eggs were fertilised, which is likely given the results of fluorescence microscopy in 2019/20, I did find that a greater proportion of hatching failure was attributed to fertilisation failure than embryo mortality in captive-laid eggs compared to wild-laid eggs based on a simple two-proportion z-test, which could potentially fit the hypothesis that rates of fertilisation failure are higher in captive populations than wild populations (Hemmings *et al.*, 2012b). However, this was not supported by a GLMM comparing fertilisation failure and embryo mortality, as whether an egg was wild- or captive-laid was not found to be a significant predictor, regardless of how addled eggs were treated in the model. Also, as there were only three addled kakī eggs in the 2019/20 season, care must be taken when extrapolating this finding more generally, although the finding that two thirds of the addled ZSL eggs examined using fluorescence microscopy were also fertilised does add support to this conclusion. Additionally, the analyses do not account for the finding that fluorescence microscopy methods also indicated that almost all kakī eggs lacking signs of embryo development in 2019/20, which may have been classified as 'infertile' based on necropsy alone, were fertilised. One of the potential explanations for lower fertilisation success in captive populations is the lack of opportunity for extra-pair copulations, but as kakī are socially monogamous (Marchant & Higgins, 1993) with limited evidence of alternative mating strategies (Overbeek *et al.*, 2020), this may not be an issue for this species in captivity. We may expect more problems with fertilisation failure in species which normally have more complex mating systems which can't be easily replicated in captive settings, although there is a need for more studies of species with different breeding and mating systems to enable comparisons and test this theory.

The mean hatching failure rate for kakī across breeding seasons was similar to that previously reported by Heber & Briskie (2010), suggesting that hatching failure is fairly consistent within this population. I found no difference in hatching failure rate between captive- and wild-laid eggs in this time period, differing from an earlier study on the same population by van Heezik *et al.* (2005) which found that hatchability of fertile eggs (i.e. those that showed any sign of embryonic development) was higher for wild-laid eggs than captive-laid eggs. The same study also found that eggs laid by captive birds were more likely to be 'infertile', although this was likely overestimated due to the use of candling and necropsy

methods only; it is not stated how addled eggs were treated in calculations of fertility. The lower hatchability and fertilisation of captive-laid eggs at this time was suspected to be due to diet deficiencies, senescence effects of aging captive males, and incompatibility between breeding pairs (van Heezik *et al.*, 2005). While these suspected drivers were not empirically tested, they were used to inform management decisions, which appear to have worked to eliminate this difference between captive- and wild-laid eggs during this study period.

The finding of no difference in hatching failure between captive- and wild-laid eggs may also appear to contrast the findings presented in **Chapter 2** (Marshall *et al.*, 2023), showing that captive populations generally experience higher rates of hatching failure than wild populations. However, while made up of both captive and free-living pairs, kakī can be considered overall as one wild managed population as all captive-bred individuals are released into the wild as juveniles, and almost every egg laid by both free-living and captive pairs is artificially incubated and reared in captivity. This may suggest that differences in parental incubation or other aspects of parental care between wild and captive breeding pairs could be one underappreciated driver of higher hatching failure rates in captive populations rather than any inherent differences in egg quality, but this requires further investigation to elucidate.

Eggs from larger clutches, eggs that were heavier, and those containing older embryos at the time of collection for artificial incubation had higher rates of hatching success, all of which was consistent with the findings of Bourke *et al.* (2023) who used the same initial kakī dataset but different methods to investigate the hatchability of fertile eggs only (i.e. those that showed any sign of embryonic development during initial candling after collection for artificial incubation). The typical size of kakī clutches is four eggs, with only around 2% of eggs in my analysis belonging to clutches of five or six, and around 15% belonging to clutches of one to three eggs. As smaller clutches may result from nests being partially depredated or otherwise disturbed, the higher hatching failure of eggs from smaller clutches may be due to incubation being disrupted prior to collection, an example of indirect predator effects (Travers *et al.*, 2010; Fernández & Carro, 2021). Alternatively, a higher rate of hatching failure in eggs from smaller clutches could be the result of females in poor condition or

with limited resources laying fewer eggs (Lack, 1967; Xu *et al.*, 2023), with an increased likelihood that these eggs may also be of reduced quality due to decreased maternal investment (Blount *et al.*, 2002; Parker, 2002; Rutkowska & Cichoń, 2002; Assersohn *et al.*, 2021a).

Heavier eggs being more likely to hatch is consistent with findings that egg size is positively correlated to hatching success (Krist, 2011), and may reflect higher levels of maternal investment. Eggs that undergo a period of parental incubation prior to artificial incubation have been shown to experience lower rates of hatching failure than eggs removed immediately after laying (Burnham, 1983; Elliott *et al.*, 2001; Robertson *et al.*, 2006; Smith *et al.*, 2011; Hoeck *et al.*, 2015), which is consistent with the observation that eggs containing older embryos at the time of collection were more likely to hatch. Interestingly, these factors are likely to work against each other, as eggs lose weight during incubation due to water loss, hence eggs which are collected when they contain older embryos will be lighter than their initial weight at laying. Supporting this, I found a significant, although weak, negative correlation between egg weight and estimated embryo age at time of collection for artificial incubation. Therefore, while these results suggest that hatching success may be improved by only collecting heavier eggs and those containing older embryos, in order to make the most informed decision about which eggs to collect practitioners would need to measure both attributes in the field, which is impractical and likely to result in more disturbance to nests and breeding adults (Bourke *et al.*, 2023). Furthermore, current management guidelines recommend that eggs are collected from wild nests as soon as possible upon discovery due to the high level of threat to eggs from predators and flooding, which currently exceeds any potential improvement in hatching success attained by waiting to collect eggs until partially through the incubation period. The potential trade-off between egg predation in the wild and increased hatching failure of early-collected eggs should be considered in future, particularly if the predation risk is reduced.

Failed eggs that were lighter at time of collection were more likely to have failed due to fertilisation failure than embryo mortality. Poor diet and/or nutritional deficiencies have been shown to influence egg fertility (Assersohn *et al.*, 2021a), and this could also result in females being smaller and laying smaller eggs, resulting in a correlated effect. However, this relationship requires further

investigation to elucidate. Similarly, the findings that failed eggs from larger clutches were more likely to have experienced fertilisation failure, but that those that did experience embryo mortality were more likely to fail later in incubation, do not have a clear biological explanation, and require further investigation beyond the scope of this study.

Embryo mortality appears to occur more often early in incubation in kakī rather than mid-incubation or later, partially reflecting studies from domesticated birds (Lodge *et al.*, 1971; Christensen, 2001; Sellier *et al.*, 2005). Due to the application of assisted hatching in this population, some eggs that potentially would have died hatching were able to successfully hatch which may have slightly suppressed the number of later incubation deaths. Additionally, any addled or 'infertile' eggs from seasons without fluorescence microscopy analyses were excluded from this analysis, and as my results indicate that many of these may have actually been fertilised (and hence experienced pre- or early incubation mortality), the true proportion of eggs failing early in incubation may have been higher than currently shown in **Figure 3-3**. Early and pre-incubation deaths may reflect problems with inbreeding depression within the population, as major genes are typically expressed early in development and inbreeding causes the exposure of recessive lethal alleles in the homozygous state (Koenig, 1982; Keller & Waller, 2002). The kakī population experienced a severe bottleneck in 1981, reaching an estimated minimum of 23 adults (Maloney & Murray, 2001), hence it is possible that they may be experiencing some effects of inbreeding. However, previous studies on this have shown somewhat inconsistent findings. Steeves *et al.* (2010) found no evidence of inbreeding depression in free-living pairs, while Hagen *et al.* (2011) found a significant negative relationship between inbreeding and fitness within captive pairs. More recently, Bourke *et al.* (2023) found inbreeding coefficient to be a significant predictor of kakī survivability, but not of hatchability. Recent developments facilitating the genetic analysis of early embryos (e.g. Kato *et al.*, 2017) could potentially be used to identify the level of inbreeding in embryos which have died early in development relative to those that die later or go on to hatch, helping to uncover whether inbreeding could be an explanation for the relatively high rates of early reproductive losses in kakī, or whether other factors

such as egg quality or female traits should be investigated as potential drivers (Assersohn *et al.*, 2021b).

Conclusions and future directions

The results reported show that key information about hatching failure such as timing of embryo mortality and rates of unfertilised eggs may be missed if only macroscopic investigations are applied to unhatched eggs, as is the case in most studies. These findings are not only applicable to birds, but are also of relevance to other oviparous taxa, as demonstrated by the application of fluorescence microscopy methods to the eggs of freshwater turtles (Croyle *et al.*, 2016) and crocodiles (Augustine, 2017). By improving our understanding of the causes of hatching failure in threatened populations, more effective mitigation strategies can be developed, improving hatching success and increasing population numbers.

Chapter 4: Threatened bird eggshell microbiomes and the effect of management

Abstract

Animal microbiomes are increasingly being shown to play important roles in host health, physiology, development, and behaviour, and to be influenced by both internal and external factors. There is also a growing appreciation for the effects of microbiomes in the context of reproduction. While there is a large and increasing body of work investigating gut, feather, and faecal microbiomes in birds, the eggshell microbiome remains poorly understood, and has been particularly understudied in non-commercial and threatened species. The effects of captivity on eggshell microbiomes have also not been explored, despite evidence of differences in gut, cloacal, oral, and uropygial gland microbiomes between wild and captive bird populations. Here, I characterise the eggshell microbiomes of two threatened species with elevated rates of hatching failure, the kakī (black stilt; *Himantopus novaezelandiae*) and the hihi (stitchbird; *Notiomystis cincta*), for the first time, and draw comparisons between them. I explore the influence of management-related factors on the kakī eggshell microbiome, as well as the influence of parental and environmental factors on the eggshell microbiomes of both species, and discuss the potential implications for their conservation. Results show that the eggshell microbiomes of kakī and hihi differ significantly from each other, but broadly resemble other non-commercial avian microbiomes in their overall composition. Captive-laid kakī eggs had a greater microbial richness and evenness in their eggshell microbiomes compared to wild-laid eggs, with data suggesting that the effects of captivity on the eggshell microbiome may persist trans-generationally. The eggshell microbiomes of eggs from the same clutch were more similar to each other than to eggs from different clutches, and there was little indication that different clutches produced by the same parents were more similar to each other than to other non-sibling clutches. Several parental variables, such as breeding experience and social sire age, and environmental variables such as rainfall, were also found to contribute towards shaping the eggshell microbiome composition. These findings highlight the importance of considering ecological and behavioural traits when studying microbiome variation in wild or free-living animal populations, as well as considering the potential effects of captivity on microbiomes in managed populations.

Introduction

Microbial infection has long been of major concern within the commercial poultry industry due to both the negative effects on egg viability and the risk posed to human health during egg consumption (Haines, 1939; Board, 1966;

Mayes & Takeballi, 1983). This has led to substantial research into bacterial contamination of commercial eggs destined for human consumption and confirmation of the presence of potentially pathogenic bacteria in their eggshell microbiomes (i.e. the communities of microorganisms on their shell surface) (Fardows & Shamsuzzaman, 2015; Olsen et al., 2017; Shi et al., 2020; Chousalkar et al., 2021; Rychlik et al., 2023). Similar studies in non-commercial species remain relatively limited in comparison, although there is growing evidence that potentially pathogenic bacteria frequently occur as part of the eggshell microbiome of non-threatened wild birds (Cook *et al.*, 2005a; Shawkey *et al.*, 2009; Ruiz-de-Castañeda *et al.*, 2011b; Soler *et al.*, 2012a; Brandl *et al.*, 2014; Lee *et al.*, 2014). Few studies have considered the potential importance of the eggshell microbiome in bird populations managed for conservation, despite them being subject to many of the same management techniques used in commercial breeding. Changes in the eggshell microbiome caused by management interventions, and associated increases in risk of trans-shell infection, may provide one explanation for the high levels of hatching failure seen in captive and otherwise managed populations (Marshall *et al.*, 2023).

The eggshell microbiome has been shown to be present on avian eggs from soon after laying (Baggott & Graeme-Cook, 2002; Cook *et al.*, 2003, 2005b), with evidence that microbial communities can vary both between species (Kuehler, 1993; Baggott & Graeme-Cook, 2002) and within and between clutches of the same species (Martín-Platero *et al.*, 2010). Eggshell microbiomes have generally received less attention than other avian microbiomes (Rowe *et al.*, 2020; Campos-Cerda *et al.*, 2022), despite potentially being an important driver of egg viability (Berrang *et al.*, 1999; Cook *et al.*, 2003) and their putative role in establishing the initial chick gut microbiome (Pedroso, 2009; Roto *et al.*, 2016; Trevelline *et al.*, 2018; Maki *et al.*, 2020; Těšický *et al.*, 2023) which could impact individual development, fitness, and survival long-term. Characterising the eggshell microbiome and uncovering the factors shaping its composition could therefore not only improve our understanding of its potential impact on avian hatching success, but may also have implications for broader aspects of threatened species management.

There is a lack of agreement regarding the source of the eggshell microbiome, with some research supporting the hypothesis that it is vertically transmitted,

originating from the mother's cloacal microflora during egg-laying (Ruiz-de-Castañeda *et al.*, 2011a, 2011b; Grizard *et al.*, 2014), while other studies promote the 'direct-contact inoculation hypothesis', whereby horizontal uptake from nest material and/or the incubating parent plays the dominant role (Godard *et al.*, 2007; Martínez-García *et al.*, 2016; van Veelen *et al.*, 2018; Song *et al.*, 2022). Regardless of the initial source, there is evidence that eggshell bacterial loads and/or microbiome composition can be influenced by a wide variety of external or environmental conditions such as temperature and humidity (Ruiz-de-Castañeda *et al.*, 2011c, 2011a; Darolová *et al.*, 2018), nest structure (Godard *et al.*, 2007; Peralta-Sánchez *et al.*, 2012), nest reuse (Walls *et al.*, 2012; Bakermans *et al.*, 2019), nesting environment/habitat (Lee *et al.*, 2017; Basso *et al.*, 2022), nest-lining materials (Peralta-Sánchez *et al.*, 2010; Martínez-García *et al.*, 2016; Ruiz-Castellano *et al.*, 2016), and ectoparasite presence (Tomás *et al.*, 2018).

The influences of parental factors on the eggshell microbiome have generally received less attention than environmental factors, with the exception of incubation. Parental incubation has been shown to have a bacteriostatic effect on eggshells, preventing the growth of bacteria, although the precise mechanism behind this remains poorly understood (e.g. Cook *et al.*, 2005b; Shawkey *et al.*, 2009; Soler *et al.*, 2015; Bollinger *et al.*, 2018; Song *et al.*, 2022). Incubation conditions and efficiency may be linked to clutch size (Monaghan & Nager, 1997; Reid *et al.*, 2000), hence, given the putative links between eggshell microbiome and incubation, clutch size may be expected to be associated with eggshell microbial load or community structure. Few studies have investigated this, although one multi-species study found a negative relationship between eggshell bacterial load and clutch size (Darolová *et al.*, 2018), while a study of great tits (*Parus major*) and blue tits (*Cyanistes caeruleus*) found no association (Boonyarittichaikij *et al.*, 2018). An under-investigated area in non-commercial birds is the effect of parental age and/or breeding experience on the eggshell microbiome. Female age has been shown to be correlated with cloacal microbiomes (Hernandez *et al.*, 2021) and there is evidence that cloacal contact and/or ejaculate transfer during copulation can lead to the transmission of bacteria between birds (Sheldon, 1993; Lombardo *et*

al., 1996; Westneat & Rambo, 2000; Hupton *et al.*, 2003; Kulkarni & Heeb, 2007; White *et al.*, 2010), which could impact the eggshell microbiome.

In addition to environmental and parental factors, common management methods related to captivity such as supplementary feeding, handling, or housing, are likely to influence various aspects of an animal's microbiome (Dallas & Warne, 2022). There is growing evidence that captivity affects the microbiome of the avian gut (Wienemann *et al.*, 2011; Ushida *et al.*, 2016; Xie *et al.*, 2016; Salgado-Flores *et al.*, 2019; Becker *et al.*, 2020; Oliveira *et al.*, 2020; San Juan *et al.*, 2020; West *et al.*, 2022), cloaca (Xenoulis *et al.*, 2010; Kelly *et al.*, 2022), oral cavity (Alba *et al.*, 2022), and uropygial gland (Grieves *et al.*, 2022), and there have been calls for microbiomes to be considered as part of threatened species management (Xie *et al.*, 2016; Trevelline *et al.*, 2019; Becker *et al.*, 2020; Oliveira *et al.*, 2020; Comizzoli *et al.*, 2021; Matheen *et al.*, 2022; West *et al.*, 2022). However, there is currently no evidence on the impact of conservation management on the eggshell microbiome.

In this study I analyse the eggshell microbiomes of two species endemic to Aotearoa New Zealand, a Vulnerable passerine, the hihi (stitchbird; *Notiomystis cincta*) (BirdLife International, 2022b), and a Critically Endangered non-migratory wader, the kakī (black stilt; *Himantopus novaezelandiae*) (BirdLife International, 2018b). Both experience relatively high rates of hatching failure (35% for hihi (Brekke *et al.*, 2010), 28.4% for kakī (Heber & Briskie, 2010)) and are subject to ongoing conservation management, but differ in many aspects of their evolutionary and life histories as well as in the management they experience, enabling the consideration of different ecological and behavioural traits in regards to eggshell microbiome.

The hihi population studied here were translocated to Tiritiri Matangi Island beginning in 1995 (Castro, 2013), with the current population reliant on supplementary feeding and almost exclusively nesting in artificial nestboxes (Ewen *et al.*, 2018; BirdLife International, 2022b). Although socially monogamous, they have a complex breeding system including polygynandry and forced copulation by males (Castro *et al.*, 1996), and eggs are incubated by females only over a 14-15 day incubation period (Ewen *et al.*, 2018). The kakī population consists of free-living individuals in Te Manahuna (the Mackenzie

Basin) and captive pairs held at two captive-breeding facilities. Kakī are monogamous solitary nesters with strong pair bonds and mate fidelity (Reed *et al.*, 1993; van Heezik *et al.*, 2009; Pierce, 2013). They breed in braided rivers and wetlands and lay their eggs in ground nests made up of plant material and small stones (Pierce, 2013), with natural incubation being biparental over a 24-27 day incubation period (Pierce, 1982, 2013). The population is subject to headstarting, with almost all eggs from both wild and captive nests removed for artificial incubation and hand-rearing in captivity before individuals are released as juveniles (BirdLife International, 2018b). Both species are subject to regular nest checks throughout the breeding season, enabling the collection of eggshell swabs and observations about clutch characteristics, and have long-term datasets including detailed information on parentage, survival, and reproductive success of almost every individual in the population, making them suitable for investigation into the drivers of eggshell microbiome composition.

To better understand the factors influencing the eggshell microbiomes of these two very different species I apply culture-independent molecular techniques to characterise and compare their eggshell microbiomes and assess the influence of environmental factors such as temperature and rainfall and parental factors such as parental age and breeding experience. Using kakī alone (since there are no captive-breeding populations of hihi), I also assess the extent to which the eggshell microbiome differs between captive and wild-laid eggs. I discuss the results in the context of our current understanding of eggshell microbiomes and the potential consequences for the conservation and management of these and other threatened species.

Materials and Methods

Study Species and Sample Collection

A summary of life history traits, threat status, and management of hihi and kakī is included in **Table C1**, **Appendix C.1**. All samples from both species were collected during the 2019/20 breeding season (October 2019 – January 2020) in Aotearoa New Zealand. Hihi samples were collected from eggs laid in the translocated population on Tiritiri Matangi Island. Of a total of 364 eggs laid in the 2019/20 breeding season, 288 eggs (79.1%) were swabbed. All nesting

occurred within artificial nestboxes which were treated for ectoparasites and cleaned between breeding seasons (Ewen *et al.*, 2009, 2018). Hihi in this population generally lay up to two successful clutches of two to five eggs per season, and will typically lay a replacement clutch in the event of an unsuccessful breeding attempt (Castro *et al.*, 1996; Taylor *et al.*, 2005; Ewen *et al.*, 2018). Hihi eggshell swabbing was covered under Research Permit 44300-FAU (2015-2025). Kakī samples were collected from eggs in wild nests in Te Manahuna, and from eggs laid by captive pairs at the Department of Conservation (DOC) Kakī Recovery Centre in Twizel and the Isaac Conservation and Wildlife Trust (ICWT) in Christchurch. Of a total of 166 eggs laid in the 2019/20 breeding season, 93 eggs (56.0%) were swabbed. Free-living kakī nests were found in braided riverbeds, wetlands, swamps, tarns (small mountain and high-country lakes), and farmland. Captive pairs were maintained in open-air flight aviaries with ponds supplied by freshwater springs (Reed *et al.*, 1993; Jakob-Hoff, 2001). The practice of egg pulling for artificial incubation encourages pairs to re-clutch, with both free-living and captive pairs laying up to four clutches of three to six (usually four) eggs per season (Reed *et al.*, 1993; van Heezik *et al.*, 2005; Pierce, 2013).

Due to the species' threat status and the limitations of the field sites, a bespoke swabbing method was developed for each species, in collaboration with conservation teams and based on existing literature, to minimise potential risk to the eggs. To avoid excessive touching and moving of the eggs only the exposed surface was sampled, and non-moistened swabs were used to avoid introducing liquid to the eggshell surface which could potentially facilitate trans-shell infection. All eggs were swabbed using Tubed Sterile fine tip Dryswab™ (MW100; MWE) and each egg was sampled individually with a separate swab. Due to the very small size of hihi eggs the entire exposed surface of each egg was swabbed for approximately 5 seconds to maximise bacteria collection. For the much larger kakī eggs the swab was gently swiped along the entire exposed surface of the eggs 4 times without overlapping strokes, gently rotating the swab at the end of each stroke. Hihi eggs were swabbed on approximately day 13 after commencement of incubation, and where possible, each egg's developmental status (developed or not) was also recorded based on visual inspection through the eggshell at this stage. Kakī eggs were swabbed

opportunistically during collection for artificial incubation, which varied from 0-21 days after laying. All swabs were individually sealed and placed into a freezer (-18°C) after collection.

Any samples with incomplete information or where swabs were suspected to have been contaminated during or after collection through accidental contact with nest materials or lab surfaces were excluded from sequencing. Samples from any nests where females were suspected to have abandoned the nest, or where eggs disappeared during incubation, were also excluded. Due to time and cost constraints, and the paucity of samples from multiple clutches from the same hihi pairs, only first clutches from the hihi population were included in sequencing. Since there were fewer kakī clutches in total all samples were sequenced for this species, which included multiple clutches from some pairs although these were generally not sequential. One kakī sample did not successfully amplify and was excluded from the final analysis. This left 92 kakī samples and 157 hihi samples in the final dataset.

The rate of hatching success (excluding depredated, abandoned, or damaged eggs) across the entire managed hihi population on Tiritiri Matangi in 2019/20 was 75.21% (267/355). In my final dataset, 79.25% (84/106) hihi eggs of known fate hatched, and the mean hatching success per clutch was 75.16%. For kakī in 2019/20, hatching success of all eggs in the population was 75.93% (123/162) for all eggs, 83.72% (36/43) for captive-laid eggs and 73.11% (87/119) for wild-laid eggs. In my final dataset, 79.35% (73/92) eggs had hatched, with mean hatching success per clutch of 77.17%, while 91.30% (21/23) of captive-laid eggs and 75.36% (52/69) of wild-laid eggs hatched successfully.

Environmental Data

Environmental data from the closest weather stations to the breeding sites was downloaded from New Zealand's National Climate Database via CliFro (*https://cliflo.niwa.co.nz/*). The only environmental data that was consistently available for all sites during the study period was the daily maximum temperature (°C), daily minimum temperature (°C), and the total daily rainfall (mm). The daily mean temperature (°C) was calculated as the mean of the

minimum and maximum temperature each day. Additional weather station details are available in **Table C2**, **Appendix C.1**.

DNA extraction and 16S rRNA gene sequencing

DNA was extracted from swabs using a Qiagen DNeasy PowerSoil HTP 96 Kit at the University of Canterbury (UC), Aotearoa New Zealand. Successful DNA extraction was confirmed using a NanoDrop™ Spectrophotometer. DNA samples were dried using a speed-vac (vacuum pressure = 0.1, ramp speed = 3, time = 2 hours). Dried samples were imported to the UK under a General Import Licence (IMP/GEN/2008/03).

The dried samples were rehydrated, DNA quantities were checked, and DNA was amplified using an Illumina MiSeq platform. To identify bacterial communities, the DNA was amplified for the 16S rRNA V4 region using dual indexed forward and reverse primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) (Caporaso *et al.*, 2011), with the library construction and sequencing protocol adapted from Kozich et al. (2013).

Along with the final dataset of 92 kakī sample and 157 hihi samples, three mock community samples and three no-template controls were also sequenced.

Sequence data processing

Microbiome bioinformatics were performed with a combination of QIIME 2 2022.8 (Quantitative Insights In Microbial Ecology; Bolyen *et al.*, 2019) and packages in RStudio version 2022.7.0.548 (RStudio Team, 2022) running R version 4.2.1 (R Core Team, 2022). The raw sequence data was in the format of Casava 1.8 paired-end demultiplexed fastq files. An alternative methodology following the DADA2 Pipeline Tutorial (1.16) (Callahan *et al.*, 2016) was also trialled to provide a robustness check for the processing (see **Appendix C.1**). As results of the two methodologies were overwhelmingly similar only the processed data from the QIIME2 pipeline was used in downstream analysis.

The data was quality filtered via the *q2-demux* plugin followed by denoising with DADA2 (Callahan *et al.*, 2016) via the *q2-dada2* plugin to exclude singletons (variants supported by a single read), remove chimeras, and assemble error-corrected amplicon sequence variants (ASVs) representing unique bacterial

taxa. ASVs accurately illuminate cryptic diversity with fewer false-positive taxon inferences than traditional operational taxonomic unit (OTU) approaches (Callahan *et al.*, 2016; van Veelen *et al.*, 2022). The truncation lengths of forward and reverse reads were set to 240 and 160 bp respectively, based on the quality score criteria (≤30). An average of 87.64% of reads remained after processing. Non-target-length sequences outside of the range 250-256 nucleotides were removed, reducing the total number of ASVs seen across the samples from 7648 to 6969.

Feature classifiers were trained on the sequenced region of the target sequences via the *q2-feature-classifier* (Bokulich et al., 2018) *fit-classifier-naive-bayes* (Pedregosa et al., 2011) as this has been shown to improve taxonomic classification accuracy (Werner et al., 2012). Taxonomy was assigned to ASVs via the *q2-feature-classifier* (Bokulich et al., 2018) *classify-sklearn* (Pedregosa et al., 2011), naïve Bayes taxonomy classifier against the Greengenes v13.8 99% OTUs reference sequences (McDonald et al., 2012) and the Silva v138.1 99% OTUs reference sequences formatted for use with QIIME2 (Quast et al., 2013; Robeson et al., 2021; Kaehler, 2022). As there are pros and cons to both the Greengenes and Silva databases (Balvočiute & Huson, 2017), both were trialled for taxonomic classification of the data and assessed for any major differences. There appeared to be minimal differences between the classifications (**Table C3**, **Appendix C.1**), and as the Silva database is larger, has been more recently updated, and was used to assign taxonomy in two recent studies of eggshell microbiome (Basso et al., 2022; Song et al., 2022), it was selected for use in further analyses. The taxonomic names seen in the Silva v138.1 database (Quast et al., 2013; Yilmaz et al., 2014) are used throughout this study for consistency and ease of comparison with previous research, although it is acknowledged that these may not align with the updated phyla names published following the decision of the International Committee on Systematics of Prokaryotes to include the rank of phylum in the rules of the International Code of Nomenclature of Prokaryotes (Oren & Garrity, 2021). The ASVs were filtered to retain only features annotated to at least phylum level, and any non-target sequences assigned to Archaea, Eukaryota, chloroplasts, or mitochondria were removed, reducing the total number of ASVs to 6571.

The *decontam* package version 1.16.0 (Davis *et al.*, 2018) in R was used to detect any contaminants, applying the “prevalence” method which compares sequences in negative controls to those in samples. Contaminant detection was performed on all eggshell samples together and also on hihi- and kakī-only subsets independently to account for any potential differences in contaminant profile (Davis *et al.*, 2018). Samples were not subset by sequencing run, as is sometimes recommended, as only one negative control had been included on each plate, which is insufficient for the prevalence method (Davis *et al.*, 2018). A probability threshold of 0.4 for all samples identified 15 potential contaminants, all of which were also identified as potential contaminants for the hihi and kakī subsets at thresholds up to 0.5 (whereby all sequences which are more prevalent in negative controls than in true samples are identified as contaminants) (**Table C4**, **Appendix C.1**). These ASVs appeared likely to be true contaminants based on their unexpected presence given the nature of the samples and the fact that they included several genera that have previously been reported as contaminants in other studies (Salter *et al.*, 2014) (**Table C4**, **Appendix C.1**), so they were removed. Removal of a further 28 ASVs only present in mock communities and negative control samples reduced the final dataset to 6528 ASVs in total. No further abundance filtering was performed, except where specifically mentioned for certain statistical analyses, as it is generally not considered necessary following denoising with DADA2.

All ASVs were aligned with MAFFT (Katoh *et al.*, 2002; Katoh & Standley, 2013) via *q2-alignment* and used to generate a phylogenetic tree with fasttree2 (Price *et al.*, 2010) via *q2-phylogeny*. To determine whether sufficient surveying effort had been achieved, the sampling depth of the samples was evaluated by generating alpha rarefaction curves via *q2-diversity* using the median frequency per sample (Estaki *et al.*, 2020). These indicated that collecting additional sequences was unlikely to result in any significant changes to the estimated diversity of the samples, validating the eggshell swabbing method used. ASVs were grouped at various taxonomic levels using the ‘tax_glom’ function within the *phyloseq* package version 1.40.0 (McMurdie & Holmes, 2013) in R and *collapse* via the *q2-taxa* plugin in QIIME2.

Statistical Analyses

Alpha diversity was used to analyse the diversity of samples, while beta diversity was used to study the dissimilarities in the community composition of the samples. Alpha diversity can be split into measures of richness (total number of taxa in a sample) and evenness (incorporating taxa abundances), and both alpha and beta diversity metrics can either be qualitative (based on presence or absence of sequences), or quantitative (incorporating sequence abundances) (Lozupone *et al.*, 2007). The quantitative alpha diversity metrics used in this study were Shannon's diversity index (Shannon & Weaver, 1949), which equally weights species richness and evenness, and Pielou's evenness (Pielou, 1966), which measures community evenness, while the qualitative alpha diversity metrics were Faith's Phylogenetic Diversity (PD) (Faith, 1992) and observed features, which both measure community richness. For beta diversity, quantitative measures were weighted UniFrac distance (Lozupone *et al.*, 2007) and Bray-Curtis distance (Sørensen, 1948; Bray & Curtis, 1957), while the qualitative measures used were unweighted UniFrac distance (Lozupone & Knight, 2005) and Jaccard distance (Jaccard, 1908). Faith's PD and both UniFrac distances incorporate phylogenetic relationship between features into their calculations. Multiple alpha and beta diversity metrics were used as differences between diversity measures can result in misleading results if only one metric is interpreted to draw conclusions (Johnson & Burnet, 2016; Kers & Saccenti, 2022). All diversity metrics were computed using *core-metrics-phylogenetic* via *q2-diversity* after samples were rarefied (randomly subsampled without replacement) to 4896 sequences per sample for the full dataset and for the hihi samples only, and to 14,396 sequences per sample for the kakī samples only. Sampling depths were chosen to capture the overall signature of the alpha diversity metrics based on examination of the alpha rarefaction curves while still retaining all samples (Estaki *et al.*, 2020). The EMPeror (Vázquez-Baeza *et al.*, 2013, 2017) plots included in the outputs of *core-metrics-phylogenetic* were used to visualise the Principal Coordinate Analysis (PCoA) matrices computed from each of the beta diversity metrics.

Characterising the eggshell microbiome

To estimate relative abundances of various taxa within the eggshell microbiomes, *relative-frequency* via *q2-feature-table* was used to convert frequencies to relative frequencies. To assess and visualise the number of unique and shared taxa between hihi and kakī eggshells, lists of all ASVs, genera, and phyla were created using *core-features* via *q2-feature-table* with p-min-fraction 0.001 and p-max-fraction 1.0. These were imported into the online tool InteractiVenn (Heberle *et al.*, 2015) to generate Venn diagrams, which were then manually adjusted to be area-proportional.

How do the eggshell microbiomes of hihi and kakī compare?

Differential abundance testing was applied to detect differences in microbial mean taxa abundance at the ASV, genus and phylum level between the hihi and kakī eggshell microbiomes. As recommended by Wallen (2021) and Nearing et al. (2022), the data was analysed with multiple methods in a consensus approach. The methods applied were ANCOM within QIIME2 via the *q2-composition* (Mandal *et al.*, 2015) plugin, and DESeq2 within R using the *DESeq2* package version 1.36.0 (Love *et al.*, 2014). As recommended for ANCOM, features with very low abundance and prevalence were filtered out (Estaki *et al.*, 2020), removing all ASVs appearing in less than 20 samples (<10% of all samples) and with a total frequency of less than 20 counts across all samples. The *add-pseudocount* action was used to both apply a relative abundance transformation and add a pseudocount of 1 to all remaining counts to fulfil the requirements of ANCOM. Benjamini-Hochberg (Benjamini & Hochberg, 1995) adjusted p-values (p-adj) were reported for results derived from DESeq2 and the W statistic was reported for ANCOM results. A normalised W value was also calculated based on the number of features tested at each level and used with a fixed threshold of 0.7 to assess significance of taxa identified as differentially abundant by ANCOM.

To compare the alpha diversity (within-sample richness and evenness) of hihi and kakī eggshells, *alpha-group-significance* via *q2-diversity* was used to apply Kruskal-Wallis tests (Kruskal & Wallis, 1952). The effect size (η^2) was calculated as $\eta^2 = (H – k + 1)/(n – k)$, where H was the H statistic obtained in the Kruskal-Wallis test, k was the number of groups, and n was the total number of

observations (Tomczak & Tomczak, 2014). To compare the beta diversity (between-sample dissimilarity), *adonis* (Anderson, 2001; Oksanen *et al.*, 2018) via *q2-diversity* was used to apply PERMANOVA tests based on 999 permutations, with permutational multivariate analysis of dispersion (PERMDISP) (Anderson, 2006) tests also performed using *beta-group-significance* via *q2-diversity* to test whether any significant PERMANOVA results were due to a high degree of dispersion (i.e. within-group variance) in either hihi or kakī. The same tests were applied to a dataset excluding samples from captive-laid kakī eggs to compare the eggshell microbiomes of just free-living kakī with the free-living hihi.

What factors drive differences in the eggshell microbiome?

To investigate the factors driving differences in the eggshell microbiome within each study species, associations between parental, environmental, and management-related variables and alpha and beta diversity metrics were analysed. For categorical variables, Kruskal-Wallis tests with calculated η^2 effect size were applied as above, while *alpha-correlation* via *q2-diversity* was used to apply Spearman correlation tests (Spearman, 1904) to continuous variables, with the Spearman ρ used as a measure of effect size. As with the comparison between species, differences in beta diversity metrics across variables were tested using PERMANOVA tests via *q2-diversity adonis*, with PERMDISP also applied to any categorical variables with significant PERMANOVA results. Additionally, *beta-group-significance* via *q2-diversity* was used to apply pairwise PERMANOVA tests based on 999 permutations to check for differences in beta diversity between individual clutches and dams (kakī only). To further explore differences in microbiome composition between groups, ANCOM and DESeq2 differential abundance testing was applied as described above to any variables with two sample groups which significantly differed in at least one alpha or beta diversity metric.

The parental, environmental, and management-related factors tested for each species were based on the characteristics of each population and previous findings from the literature of variables which are likely to impact the eggshell microbiome. Due to the relatively small sample sizes and the likelihood of correlations between the variables, each factor was tested independently,

although it is acknowledged that such multiple testing could increase the chances of false-positive results.

As both species are socially monogamous, dam and social sire ID overlapped completely and were combined in reporting results. For both species the parental variables tested were: dam/social sire age; dam/social sire age^2; dam/social sire breeding experience (experienced *versus* naïve); dam/social sire total social partners to date; and clutch size. Kakī are socially monogamous (Marchant & Higgins, 1993), with limited evidence of alternative mating patterns such as extra-pair copulations (Overbeek *et al.*, 2020) hence only the numbers of social partners were included, but due to the high rates of extra-pair copulations in this hihi population (Ewen *et al.*, 2004; Low, 2005), the dam/social sire total genetic partners to date was also tested. This data was calculated based on totalling the number of genetic sires of every egg laid by the female in the 2019/20 and previous breeding seasons. The effect of the estimated number of days of parental incubation at swab was tested for kakī but not for hihi as all eggs were swabbed on approximately day 13 of incubation. Clutch number was also tested for kakī to test for any impact of re-clutching on the eggshell microbiome. As multiple clutches were only available for a subset of the females, a multivariate Adonis test was used to check the effect of clutch number after accounting for both dam ID and whether the eggs were captive- or wild-laid.

For both species the environmental variables tested were: mean daily temperature (°C), mean daily rainfall (mm), and overall minimum and maximum temperature (°C) between estimated clutch initiation and swab date. Hihi nest latitude/longitude and kakī nest location were also tested. As hihi almost exclusively breed in artificial nestboxes provided by conservation managers the latitude and longitude of each nest was readily available as part of the long-term breeding program records. Nest locations of the free-living kakī are recorded by location name.

Although the hihi population undergoes several management interventions (supplementary feeding, artificial nestbox provision, etc.), this management is applied in the same way across the entire population hence no comparisons could be made in terms of potential management-related drivers of eggshell

microbiome. However, for kakī, some sampled eggs were laid in captivity while others were laid by free-living pairs, and the breeding individuals themselves had either been captive- or wild-laid. All known adults in this dataset had been artificially incubated and hatched in captivity except for one female and two males which were naturally incubated in captivity, and every known adult had been reared in captivity, hence there was no opportunity for useful comparisons of parental hatching or rearing environment. The potential effect of captivity on eggshell microbiomes was therefore investigated in kakī by including the following variables: egg captive- or wild-laid; dam/sire captive- or wild-laid.

Finally, two variables were included as controls for methods: sequencing plate ID (plate number of sample for sequencing) and freezer delay (mins) (time between swab collection and placement in freezer).

Throughout the Results section, any association characterised as 'significant' will refer to a statistically significant association at the alpha level of 0.05.

Results

Characterising the eggshell microbiome

In both hihi and kakī eggshell samples, the most abundant phylum was Proteobacteria, followed by Actinobacteriota, Firmicutes, Bacteroidota

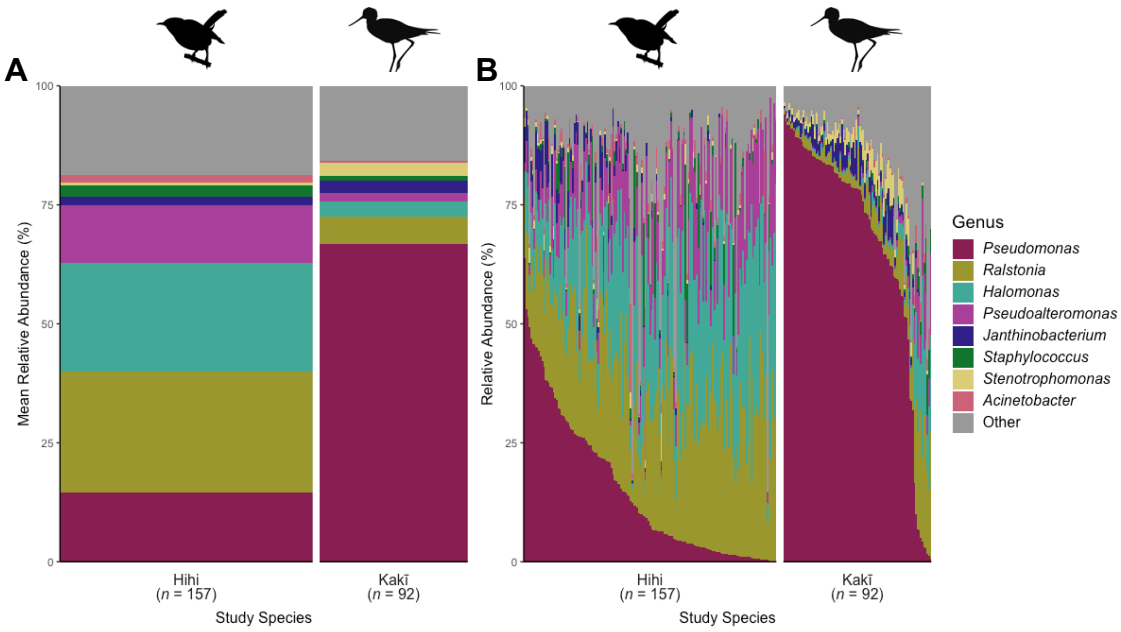

Figure 4-1. Relative abundances of genera across eggshell samples from hihi and kakī eggs shown as A) the mean relative abundance across all samples, and B) the relative abundance in each sample. The eight most abundant genera are named, with the remaining taxa represented by 'Other'. The width of each panel is representative of the sample size of the groups.

(Bacteroidetes), and Cyanobacteria (**Figure 4-1**; **Table C5, Appendix C.2**). Of the top six most abundant genera in both study species, five (*Pseudomonas*, *Ralstonia*, *Halomonas*, *Pseudoalteromonas*, *Janthinobacterium*) were the same (**Figure 4-1**; **Table C5**, **Appendix C.2**). The most abundant families were also similar within both hihi and kakī samples, with Pseudomonadaceae, Burkholderiaceae, Halomonadaceae, Pseudoalteromonadaceae, and Oxalobacteraceae within the top six most abundant families for both bird species (**Table C5**, **Appendix C.2**).

At the ASV level, 631 taxa were found to be shared between both hihi and kakī eggshell microbiomes, with 2476 ASVs unique to hihi and 3421 unique to kakī (**Figure 4-2**). At the genera level, 464 taxa were shared, with 162 unique to hihi and 387 unique to kakī, while at the phyla level 26 phyla were shared, with only 2 unique to hihi and 7 unique to kakī (**Figure 4-2**).

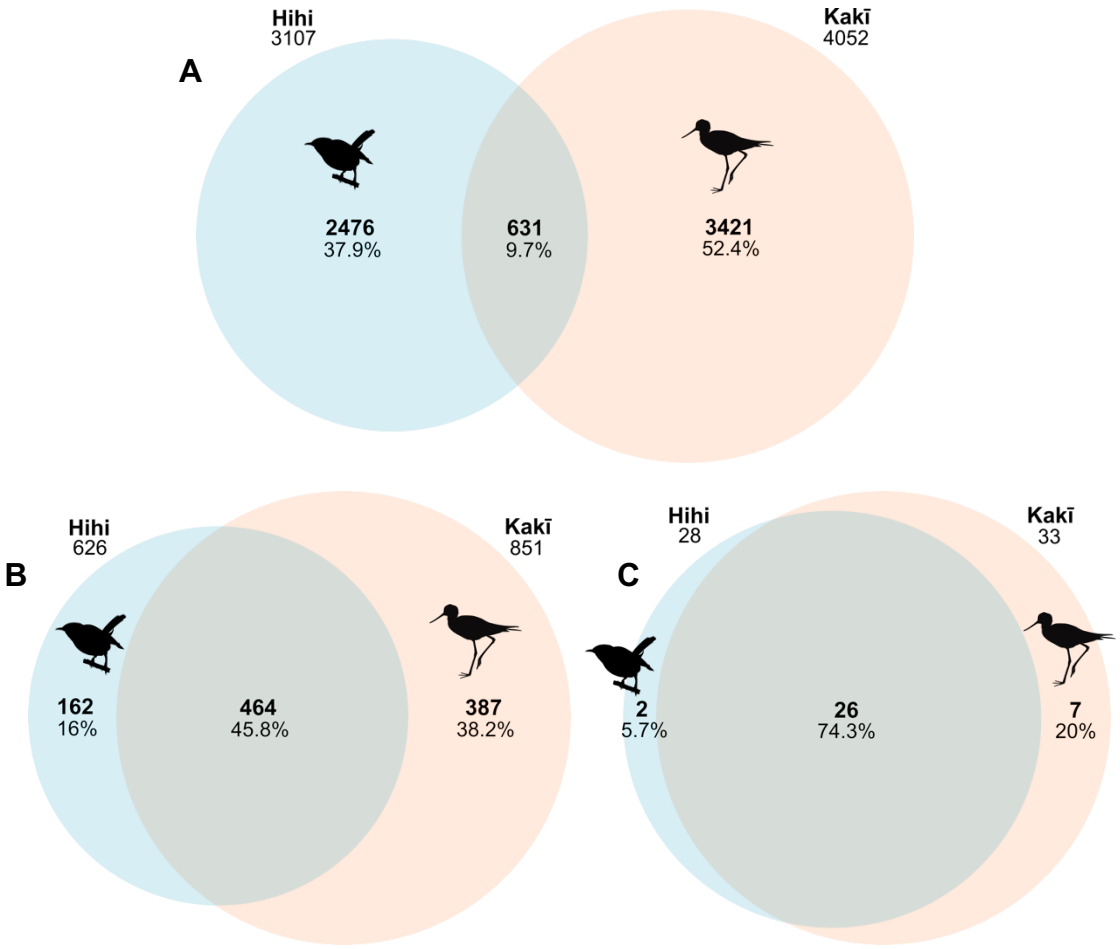

Figure 4-2. Venn diagrams visualising the shared and unique A) ASVs, B) genera, and C) phyla between hihi and kakī eggshell samples. The area of the circles is proportional to the total number of taxa at each level found across hihi and kakī eggshell samples.

How do the eggshell microbiomes of hihi and kakī compare?

Differential abundance

The results of differential abundance testing using ANCOM and DESeq2 revealed 32 ASVs and 35 ASVs, respectively, to be significantly differentially abundant between hihi and kakī eggshell samples, with a greater number found to be more abundant in kakī samples compared to hihi samples than vice versa (**Figure 4-3A; Figure C1**, **Table C6**, **Appendix C.1**). At the genera level, ANCOM indicated 18 genera and DESeq2 identified 112 to be differentially abundant between the study species, and again more taxa were of greater abundance in kakī samples than in hihi samples (**Figure 4-3B; Figure C2**, **Table C7**, **Appendix C.1**). Only *Pseudoalteromonas* and *Halomonas* were found to be of greater abundance in hihi by both methods, while the ANCOM method also identified *Ralstonia* as being more abundant in hihi and DESeq2 identified it as being more abundant in kakī samples. There were similar

findings at the family level, with the DESeq2 and ANCOM methods identifying 92 and 14 significantly differentially abundant families respectively (**Figure 4-3C; Figure C3**, **Table C8**, **Appendix C.2**). In keeping with the findings at the genera level, Pseudoalteromonadaceae (which contains *Pseudoalteromonas*) and Halomonadaceae (which contains *Halomonas*) were found to more be abundant in hihi simples by both differential abundance testing methods, and Burkholderiaceae (which contains *Ralstonia*) was identified as more abundant in hihi samples by ANCOM and more abundant in kakī samples by DESeq2. At the phyla level, Firmicutes was found to be significantly more abundant in hihi compared to kakī (**Figure 4-3D; Figure C4**, **Table C9**, **Appendix C.2**).

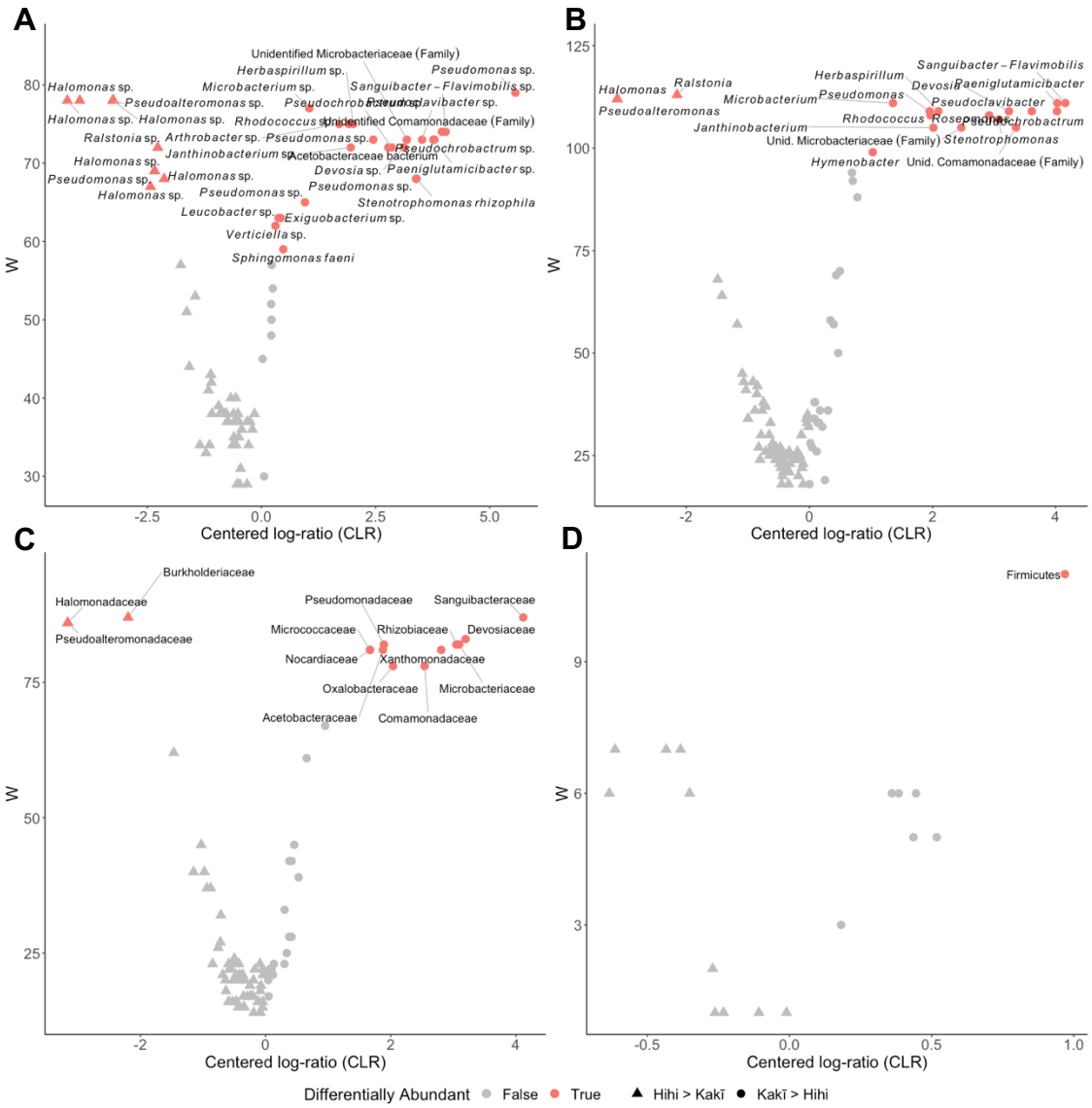

Figure 4-3. Volcano plots showing the A) ASVs, B) genera, C) families, and D) phylum identified by ANCOM as being differentially abundant between hihi and kakī eggshells. Red points represent statistically significantly differentially abundant taxa. Triangles represent taxa that are more abundant in hihi eggshell samples, while circles represent taxa that are more abundant in kakī eggshell samples. Full results are included in **Table C6–Table C9**, **Appendix C.2**.

Table 4-1. Kruskal-Wallis (categorical variables) and Spearman correlation (numeric variables) test results for alpha diversity metrics for all samples, hihi only samples, and kakī only samples. Only variables with a statistically significant difference in at least one alpha diversity metric are reported. Statistically significant p-values are highlighted in bold. The effect size (η^2) for the Kruskal-Wallis test was calculated as $\eta^2 = (H - k + 1)/(n - k)$, where H was the H statistic obtained in the Kruskal-Wallis test, k was the number of groups, and n was the total number of observations.

			Shannon's diversity index			**Observed features**			**Faith's PD**			**Pielou's evenness**		
			H	η^2	*p*-value	*H*	η^2	*p*-value	*H*	η^2	*p*-value	*H*	η^2	*p*-value
All	Hihi (*n* = 157)	Kakī (*n* = 92)	56.190	0.223	**<0.001**	70.111	0.280	**<0.001**	93.242	0.373	**<0.001**	95.723	0.383	**<0.001**
Wild only	Hihi (*n* = 157)	Kakī (*n* = 69)	63.577	0.279	**<0.001**	55.589	0.244	**<0.001**	71.489	0.315	**<0.001**	100.737	0.445	**<0.001**
Hihi only	Clutch/dam/sire ID (41 groups)		55.542	0.134	0.052	74.476	0.297	**<0.001**	72.421	0.279	**0.001**	51.415	0.098	0.107
(n = 157)	Dam experienced (*n* = 79) vs. naïve (*n* = 78)		0.503	-0.003	0.478	4.719	0.024	**0.030**	4.161	0.020	**0.041**	0.019	-0.006	0.891
Kakī only	Clutch ID (27 groups)		50.841	0.382	**0.002**	47.697	0.334	**0.006**	48.231	0.342	**0.005**	49.739	0.365	**0.003**
(n = 92)	Dam/sire ID (20 groups)		28.860	0.137	0.068	31.567	0.175	**0.035**	36.984	0.250	**0.008**	28.795	0.136	0.069
	Egg captive- (*n* = 23) vs. wild-laid (*n* = 69)		4.940	0.044	**0.026**	0.024	-0.011	0.878	0.020	-0.011	0.889	6.814	0.065	**0.009**
			ρ	–	*p*-value	*ρ*	–	*p*-value	*ρ*	–	*p*-value	*ρ*	–	*p*-value
Hihi only	Sire age (linear and quadratic)		0.061	–	0.449	0.208	–	**0.009**	0.239	–	**0.003**	-0.082	–	0.310
(n = 157)	Dam total genetic partners		-0.075	–	0.352	-0.199	–	**0.013**	-0.164	–	**0.041**	-0.001	–	0.996
	Mean daily rainfall (mm)		-0.135	–	0.091	-0.259	–	**0.001**	-0.276	–	**0.001**	0.059	–	0.460
Kakī only	Clutch size		0.246	–	**0.018**	0.312	–	**0.003**	0.307	–	**0.003**	0.216	–	**0.039**
(n = 92)	Est. days parental incubation at swab		0.260	–	**0.012**	0.013	–	0.904	-0.002	–	0.988	0.289	–	**0.005**
	Mean daily temp. (°C)		-0.138	–	0.191	-0.291	–	**0.005**	-0.164	–	0.117	-0.087	–	0.408

Table 4-2. Results of univariate Adonis testing (PERMANOVA) based on 999 permutations for beta diversity metrics generated using *adonis* in QIIME2. Only variables with a statistically significant difference in at least one beta diversity metric are reported. Statistically significant *p*-values are highlighted in bold. Shading indicates variables that accounted for at least 5% of the variation in at least one beta diversity metric.

			Weighted UniFrac			**Unweighted UniFrac**			**Jaccard**			**Bray-Curtis**		
			pseudo-*F*	R^2	*p*-value	pseudo-*F*	R^2	*p*-value	pseudo-*F*	R^2	*p*-value	pseudo-*F*	R^2	*p*-value
All	Hihi (*n* = 157)	Kakī (*n* = 92)	73.216	0.229	**0.001**	22.827	0.085	**0.001**	17.341	0.066	**0.001**	204.491	0.453	**0.001**
Wild only	Hihi (*n* = 157)	Kakī (*n* = 69)	108.880	0.327	**0.001**	17.117	0.071	**0.001**	15.620	0.065	**0.001**	208.451	0.482	**0.001**
Hihi only	Clutch/dam/sire ID (41 groups)		1.570	0.351	**0.001**	1.183	0.290	**0.001**	1.098	0.275	**0.001**	1.717	0.372	**0.001**
(*n* = 157)	Dam experienced (*n* = 79) vs. naïve (*n* = 78)		3.128	0.020	**0.007**	1.570	0.010	**0.016**	1.227	0.008	**0.013**	2.800	0.018	**0.004**
	Sire age		2.031	0.013	0.054	1.965	0.013	**0.006**	1.471	0.009	**0.001**	2.955	0.019	**0.003**
	Sire age^2		2.696	0.017	**0.021**	2.035	0.013	**0.003**	1.506	0.010	**0.001**	3.438	0.022	**0.003**
	Sire total social partners		2.533	0.016	**0.022**	1.313	0.008	0.072	1.184	0.008	**0.026**	2.403	0.015	**0.019**
	Sire total genetic partners		1.621	0.010	0.128	1.482	0.009	**0.046**	1.157	0.007	**0.047**	2.118	0.013	**0.028**
	Mean daily temp. (°C)		1.250	0.008	0.264	1.152	0.007	**0.048**	1.152	0.007	**0.048**	2.091	0.013	**0.026**
	Mean daily rainfall (mm)		2.351	0.015	**0.027**	2.004	0.013	**0.003**	1.518	0.010	**0.001**	2.979	0.019	**0.004**
	Overall min temp. (°C)		1.234	0.008	0.253	1.223	0.008	0.135	1.133	0.007	0.069	1.867	0.012	**0.038**
	Overall max temp. (°C)		1.564	0.010	0.134	1.594	0.010	**0.011**	1.296	0.008	**0.006**	3.777	0.024	**0.001**
	Sequencing plate ID		1.800	0.011	0.096	1.610	0.010	**0.021**	1.404	0.009	**0.002**	3.181	0.020	**0.007**
	Freezer delay		2.305	0.015	**0.033**	1.036	0.007	0.354	1.107	0.007	0.098	1.808	0.012	**0.047**
Kakī only	Clutch ID (27 groups)		5.538	0.689	**0.001**	1.436	0.365	**0.001**	1.314	0.344	**0.001**	6.547	0.724	**0.001**
(*n* = 92)	Dam/sire ID (20 groups)		2.515	0.399	**0.002**	1.425	0.273	**0.001**	1.224	0.244	**0.001**	3.027	0.444	**0.001**
	Sire experienced (*n* = 44) vs. naïve (*n* = 48)		3.581	0.038	**0.030**	1.771	0.019	**0.005**	1.333	0.015	**0.011**	3.416	0.037	**0.030**
	Sire age		4.227	0.045	**0.017**	1.678	0.018	**0.008**	1.325	0.015	**0.011**	3.572	0.038	**0.027**
	Sire age^2		5.300	0.056	**0.015**	1.609	0.018	**0.013**	1.333	0.015	**0.009**	4.828	0.051	**0.013**
	Sire total social partners		1.266	0.014	0.273	1.749	0.019	**0.006**	1.274	0.014	**0.021**	1.131	0.012	0.280
	Clutch size		2.098	0.023	0.097	1.909	0.021	**0.002**	1.475	0.016	**0.004**	1.596	0.017	0.162
	Clutch number		15.456	0.147	**0.001**	1.841	0.020	**0.006**	1.848	0.020	**0.001**	13.335	0.129	**0.001**
	Est. days parental incubation at swab		20.865	0.188	**0.001**	1.378	0.015	**0.043**	1.790	0.020	**0.002**	17.818	0.165	**0.001**
	Mean daily temp. (°C)		0.882	0.010	0.388	1.440	0.016	**0.036**	1.235	0.014	**0.037**	0.777	0.009	0.434
	Mean daily rainfall (mm)		3.263	0.035	**0.031**	1.049	0.012	0.313	1.308	0.014	**0.024**	3.217	0.035	**0.039**
	Overall min temp. (°C)		0.490	0.005	0.635	1.295	0.014	0.081	1.226	0.013	**0.042**	0.706	0.008	0.487
	Overall max temp. (°C)		7.423	0.076	**0.001**	1.772	0.019	**0.005**	1.425	0.016	**0.005**	5.554	0.058	**0.014**
	Nest location (14 locations)		2.517	0.295	**0.002**	1.338	0.182	**0.001**	1.178	0.164	**0.001**	2.681	0.309	**0.001**
	Egg captive- (*n* = 23) vs. wild-laid (*n* = 69)		8.774	0.089	**0.002**	1.865	0.020	**0.005**	1.451	0.016	**0.005**	6.645	0.069	**0.004**
	Sire captive- (n = 31) or wild-laid (n = 61)		2.531	0.027	0.058	1.711	0.019	**0.004**	1.328	0.015	**0.015**	1.894	0.021	0.128
	Freezer delay		11.221	0.115	**0.001**	1.320	0.015	0.064	1.438	0.016	**0.005**	9.764	0.102	**0.001**

Table 4-3. PERMDISP analysis results for beta diversity metrics generated using *beta-group-significance* via *q2-diversity* in QIIME2. Variables reported are categorical variables with a statistically significant difference in at least one beta diversity metric according to univariate Adonis testing (PERMANOVA) analyses (see **Table 4-2**). Statistically significant *p*-values are highlighted in bold.

			Weighted UniFrac		Unweighted UniFrac		Jaccard		Bray-Curtis	
			F	*p*-value	*F*	*p*-value	*F*	*p*-value	*F*	*p*-value
All	Hihi (*n* = 157)	Kakī (*n* = 92)	15.473	**0.001**	0.141	0.724	24.104	**0.001**	15.749	**0.001**
Wild only	Hihi (*n* = 157)	Kakī (*n* = 69)	24.601	**0.001**	0.255	0.617	31.752	**0.001**	38.161	**0.001**
Hihi only (*n* = 157)	Dam experienced vs. naïve		10.953	**0.001**	0.717	0.377	6.217	**0.021**	14.127	**0.002**
	Sequencing plate ID		0.668	0.414	0.000	0.992	0.233	0.657	0.105	0.735
Kakī only (*n* = 92)	Sire experienced vs. naïve		3.788	0.060	0.958	0.332	0.082	0.771	3.739	**0.046**
	Nest location (14 locations)		5.042	**0.001**	0.895	0.950	2.790	0.135	7.600	**0.001**
	Egg captive- or wild-laid		10.104	**0.002**	7.386	**0.026**	0.004	0.954	8.201	**0.005**
	Sire captive- or wild-laid		3.149	0.080	1.007	0.338	0.248	0.658	2.579	0.113

Alpha diversity

All alpha diversity metrics significantly differed between study species ($p < 0.001$), with hihi samples ($n = 157$) having higher Shannon's diversity and Pielou's evenness than kakī samples ($n = 92$), and lower observed features and Faith's PD (**Table 4-1**; **Figure C5**, **Appendix C.2**). The same results were seen when comparing only wild samples (**Table 4-1**).

Beta diversity

Similarly, all beta diversity metrics significantly differed between study species based on PERMANOVA analyses, with the R^2 effect size indicating that study species accounted for between 6.56% and 45.3% of the variation in beta diversity across all samples depending on the metric used (**Table 4-2**). PCoA plots visualising the dissimilarities in microbial communities among the study species based on each of the beta diversity metrics and showing the variation explained by the first two axes exhibited clustering of communities according to study species (**Figure C5**, **Appendix C.2**). The results of PERMDISP tests of weighted UniFrac, Jaccard, and Bray-Curtis distances were all significant, indicating that the significant PERMANOVA results for these metrics may have been due to a high degree of within-group variance rather than between-group variance (**Table 4-3**). The same results were seen for the wild only dataset (**Table 4-2 & Table 4-3**).

What factors drive differences in the eggshell microbiome?

Parental drivers

Hihi

Differential abundance

Differential abundance testing using DESeq2 identified two ASVs (*Pseudomonas* sp. and *Sanguibacter-Flavimobilis* sp.) and a genus (*Sanguibacter-Flavimobilis*) to be significantly more abundant in eggs laid by naïve females than experienced females (**Table 4-4**).

Table 4-4. Summary of differential abundance testing within hihi and kakī subsets of variables with two groups using ANCOM in QIIME2 and DESeq2 in R. Only statistically significantly differentially abundant taxa are shown. For DESeq2, significance is based on the Benjamini-Hochberg adjusted *p*-values (*p-adj* < 0.05). For ANCOM, significance is based on the internal ANCOM threshold calculation verified using a calculated normalised W value based on the number of features being tested at each taxonomic level, with a fixed significance threshold of 0.7.

	ANCOM	DESeq2
Hihi		
Dam experienced vs. naïve		
ASV	–	*Pseudomonas sp.*, *Sanguibacter-Flavimobilis* sp. **Naïve > experienced**
Genus	–	*Sanguibacter-Flavimobilis* **Naïve > experienced**
Kakī		
Egg captive- vs. wild-laid		
Phylum	Deinococcota **Captive-laid > wild-laid**	Deinococcota, Firmicutes **Captive-laid > wild-laid**
Sire experienced vs. naïve		
Phylum	Deinococcota **Experienced > naïve**	Deinococcota, Firmicutes **Experienced > naïve**
Sire captive- or wild-laid		
ASV	–	*Chryseobacterium* sp., *Ureaplasma gut_metagenome* **Captive-laid > wild-laid**
Genus	–	*Ureaplasma* **Captive-laid > wild-laid**
Phylum	–	Deinococcota **Wild-laid > captive-laid**

Alpha diversity

For hihi, clutch ID (also dam/sire ID) was associated with significant differences in both observed features and Faith's PD (**Table 4-1**), with no significant pairwise comparisons according to the adjusted *p*-value ($q > 0.05$).

Within hihi samples, eggs laid by naïve dams ($n = 78$) had significantly higher observed features and Faith's PD than eggs laid by experienced dams ($n = 79$). There was a significant positive relationship between social sire age (and social

sire age^2) and both observed features and Faith's PD, and a significant negative relationship between these alpha diversity metrics and dam total genetic partners (**Table 4-1**).

Beta diversity

There were significant differences in eggshell microbial communities between different clutches across all beta diversity metrics (**Table 4-2**), although pairwise comparisons between clutches were not significant ($q > 0.05$). Clutch ID (also dam/sire ID) had by far the largest R^2 effect size of all examined variables, accounting for 27.5-37.2% of the variation in beta diversity depending on the metric used (**Figure 4-4**,**Table 4-2**). No other variable accounted for >5% of the variation in any beta diversity metric. PCoA plots visualising the dissimilarities in microbial communities among the eggs laid by different females showed some clustering of communities, although the number of females made this difficult to see clearly (**Figure C6**, **Appendix C.2**). There did appear to be some variation in how closely clustered clutches were, indicating variation in similarity of eggs laid by the same female.

There was significant clustering of eggs laid by experienced dams *versus* those laid by naïve dams based on all beta diversity metrics (**Table 4-2**), although significant PERMDISP results for weighted UniFrac, Jaccard, and Bray-Curtis distances indicate these differences may have been due to within- rather than between-group variance (**Table 4-3**). Social sire age was associated with significant differences in unweighted UniFrac, Jaccard, and Bray-Curtis distances, and sire age^2 was associated with significant differences in all beta diversity metrics (**Table 4-2**). There were significant differences in community structure between eggs sired by males with different numbers of social partners based on weighted UniFrac, Jaccard, and Bray-Curtis distances, and between eggs socially sired by males with different numbers of genetic partners based on unweighted UniFrac, Jaccard, and Bray-Curtis distances (**Table 4-2**).

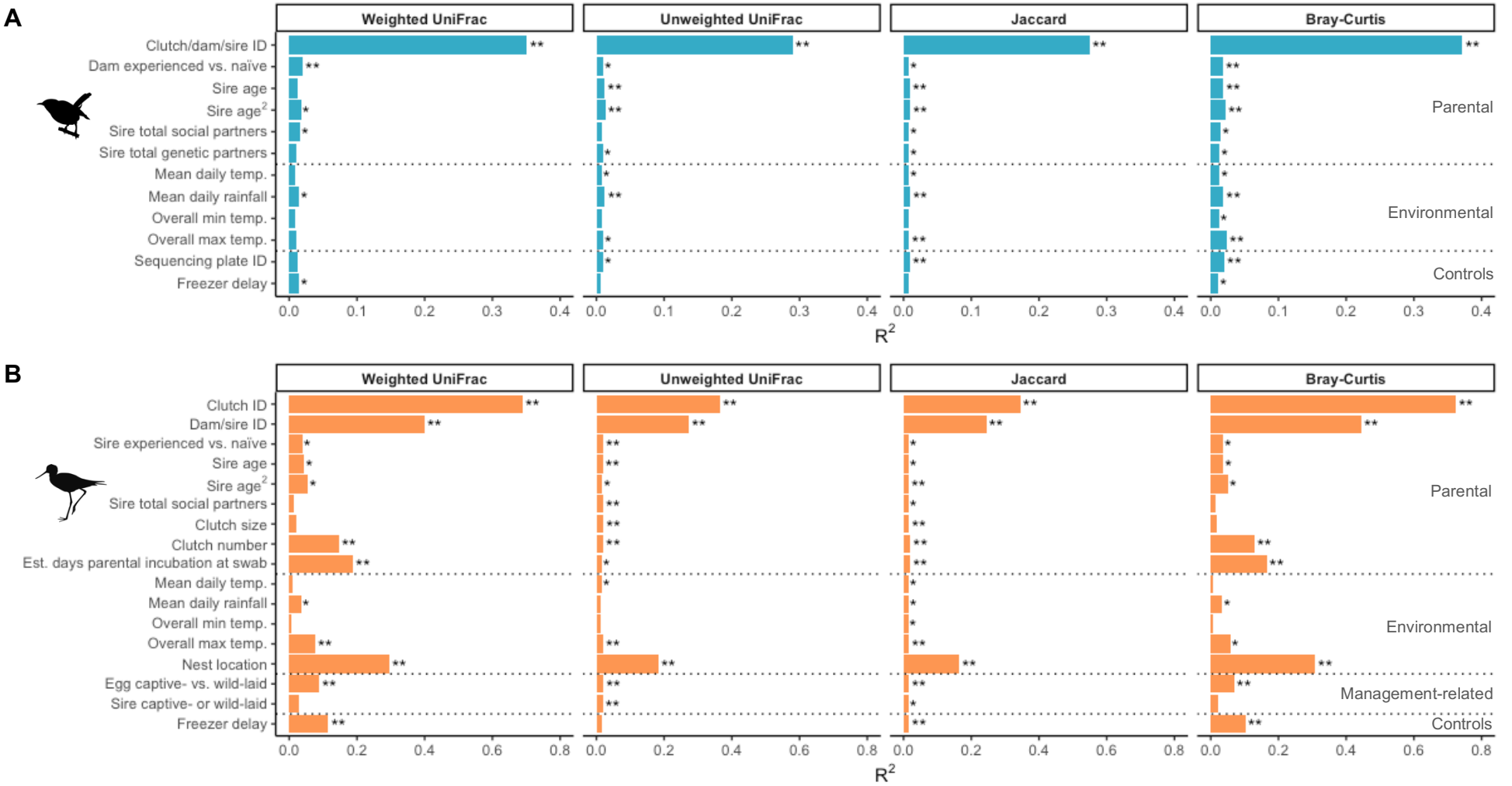

Figure 4-4. Results of univariate Adonis testing (PERMANOVA) based on 999 permutations for beta diversity metrics showing the R^2 effect size (explanatory power) of each variable for A) hihi eggshell microbiomes, B) kakī eggshell microbiomes. Only variables with a statistically significant difference in at least one beta diversity metric are shown. Levels of significance are: **, $p < 0.01$, *, $p < 0.05$.

Kakī

Differential abundance

The results of differential testing with both ANCOM and DESeq2 identified the phylum Deinococcota to be significantly more abundant in kakī eggs sired by males with previous breeding experience than in those sired by naïve males, with DESeq2 also showing this relationship for Firmicutes (**Table 4-4**).

Alpha

For kakī, clutch ID was associated with significant differences across all alpha diversity metrics, while dam/sire ID was significant for both observed features and Faith's PD (**Table 4-1**). Neither of these variables showed any significant pairwise comparisons ($q > 0.05$).

There was a significant positive relationship between kakī clutch size and all alpha diversity metrics, and a significant positive relationship between the estimated days of parental incubation at swab and both Shannon's diversity index and Pielou's evenness (**Table 4-1**).

Beta

There were significant differences in eggshell microbiome communities according to clutch ID and dam/sire ID across all beta diversity metrics, and clutch ID had the largest R^2 effect sizes of all examined variables for all metrics, accounting for particularly high proportions of the variation in weighted UniFrac and Bray-Curtis distance (**Figure 4-4**, **Table 4-2**). Dam/sire ID had the second highest R^2 effect size. Neither clutch ID nor dam/sire ID showed any significant pairwise comparisons ($q > 0.05$). PCoA plots visualising the dissimilarities in microbial communities among the eggs showed some clustering of communities according to both dam ID and female ID (**Figure C7**, **Appendix C.2**). Comparisons between clusters based on clutch ID and those based on dam ID indicate that eggs within individual clutches are more similar than eggs from multiple clutches laid by the same female, with some repeated clutches appearing to be quite distant from each other along the first two principal coordinate axes (**Figure C7**, **Appendix C.2**).

Eggs sired by experienced *versus* naïve males significantly differed in all beta diversity metrics, accounting for <5% of the variation in any metric (**Figure 4-4**, **Table 4-2**). A significant PERMDISP result for Bray-Curtis distance indicated that the significant PERMANOVA result for this metric may have been due to within- rather than between-group variance (**Table 4-3**). Both sire age and sire age^2 were associated with significant differences in all beta diversity metrics, with relatively small effect sizes (**Figure 4-4**, **Table 4-2**). Kakī clutch size was associated with significant differences in eggshell microbiome based on unweighted UniFrac and Jaccard distances, accounting for <5% of the variation of both metrics (**Figure 4-4**, **Table 4-2**). Both clutch number and estimated number of days of parental incubation at swab were significantly associated with differences in all beta diversity metrics, with effect sizes indicating these variables both accounted for >10% of the variation in weighted UniFrac and Bray-Curtis distances (**Figure 4-4**, **Table 4-2**). The results of a multivariate Adonis test showed that clutch number was still a significant driver of differences in beta diversity metrics after accounting for dam ID and whether eggs were captive- or wild-laid (**Table C10**, **Appendix C.2**). A sire's total number of social partners was associated with significant differences in unweighted UniFrac and Jaccard distances, with relatively small effect sizes (**Figure 4-4**, **Table 4-2**).

Environmental drivers

Hihi

Alpha diversity

There was a significant negative relationship between both observed features and Faith's PD of hihi eggshell samples and mean daily rainfall between estimated clutch initiation and swab date (**Table 4-1**).

Beta diversity

Mean daily temperature between estimated clutch initiation and swab date was associated with significant differences in eggshell microbial communities based on unweighted UniFrac, Jaccard, and Bray-Curtis distances, while overall minimum temperature between estimated clutch initiation and swab date was

associated with significant differences in Bray-Curtis distance (**Table 4-2**). Overall maximum temperature between estimated clutch initiation and swab date was significantly associated with differences in unweighted UniFrac, Jaccard, and Bray-Curtis distances, while daily mean rainfall between estimated clutch initiation and swab date was associated with significant differences in all beta diversity metrics (**Table 4-2**).

Kakī

Alpha diversity

In kakī eggshell samples there was a significant negative relationship between the mean daily temperature between estimated clutch initiation and swab date and observed features (**Table 4-1**).

Beta diversity

Mean daily temperature between estimated clutch initiation and swab date was associated with significant microbiome differences based on unweighted UniFrac and Jaccard distances, with relatively small effect sizes (**Figure 4-4**, **Table 4-2**). Mean daily rainfall, overall minimum temperature, and overall maximum temperature between estimated clutch initiation and swab date all significantly differed in at least one beta diversity metric, but only overall maximum temperature accounted for >5% of the variation in any metric (**Figure 4-4**, **Table 4-2**). Kakī nest location was significantly associated with differences in all beta diversity metrics, accounting for the third highest amount of variation after clutch and dam/sire ID (**Figure 4-4**, **Table 4-2**), although significant PERMDISP results for both weighted UniFrac and Bray-Curtis distances may indicate these differences were driven by within-group rather than between-group variance (**Table 4-3**).

Management-related drivers

Kakī

Overall, the relative abundances of taxa were similar between captive- and wild-laid eggs, although there did appear to be a greater abundance of the genus *Pseudomonas* and its family Pseudomonadaceae in eggshell microbiomes of

wild-laid eggs compared to those of captive-laid eggs (**Table C11**, **Figure C9**, **Appendix C.2**).

Differential abundance

Both ANCOM and DESeq2 results showed the phylum Deinococcota to be significantly more abundant in eggs laid in captivity than eggs laid by free-living kakī, with DESeq2 also showing the same relationship for the phyla Firmicutes (**Table 4-4**). The DESeq2 method also indicated that two ASVs (*Chryseobacterium* sp. and *Ureaplasma gut_metagenome*) and one genus (*Ureaplasma*) were significantly more abundant in eggs sired by captive-laid males, while the phylum Deinococcota appeared to be significantly more abundant in eggs sired by wild-laid males (**Table 4-4**).

Alpha diversity

Captive-laid eggs (n = 23) had significantly higher Shannon's diversity and Pielou's evenness than wild-laid eggs (n = 69), but they did not significantly differ in observed features or Faith's PD (**Table 4-1; Figure C8**, **Appendix C.2**).

Beta diversity

The microbiome communities of captive- and wild-laid eggs significantly differed according to all beta diversity metrics, accounting for 1.59-8.88% of the variation (**Figure 4-4**, **Table 4-2**). The significant differences for weighted UniFrac, unweighted UniFrac, and Bray-Curtis distance may have been driven by within-group variance based on significant PERMDISP results (**Table 4-3**). PCoA plots visualising the dissimilarities in microbial communities between captive-laid and wild-laid eggs based on each of the beta diversity metrics showed clustering of communities according to laying condition, but with substantial overlaps (**Figure C8**, **Appendix C.2**). Eggs sired by captive-laid *versus* wild-laid kakī males significantly differed in unweighted UniFrac and Jaccard distances, although with relatively small effect sizes (**Figure 4-4**, **Table 4-2**).

Controls

Alpha diversity

There were no significant differences in any of the alpha diversity metrics for either of the two variables included as technical controls for either hihi or kakī.

Beta diversity

Freezer delay was associated with significant differences in weighted UniFrac and Bray-Curtis distances in hihi eggshell microbiomes, and with significant differences in weighted UniFrac, Jaccard, and Bray-Curtis distances in kakī samples, all with relatively small effect sizes (**Figure 4-4**, **Table 4-2**). Sequencing plate ID was not associated with any significant differences in beta diversity within kakī samples, and within hihi samples it was associated with significant differences in unweighted UniFrac, Jaccard, and Bray-Curtis distances, accounting for ~2% or less of the variation in these metrics (**Figure 4-4**, **Table 4-2**). The results of PERMDISP indicated that these results were likely due to between- rather than within-group variance (**Table 4-3**). While differences in beta diversity related to controls raises some concerns about potential differences in the sequencing process which could be influencing the relationships seen in the samples, the effect sizes are relatively small, and as clutches were distributed randomly across the plates, this should help to avoid reinforcing any possible sequencing biases.

Discussion

In this study I characterised the eggshell microbiomes of two threatened bird species and assessed the drivers of alpha (within-sample richness and/or evenness) and beta (between-sample dissimilarity) diversity. While the eggshell microbiomes of hihi and kakī significantly differ from each other, the main microbial taxa found in each remain broadly similar to those previously reported for non-commercial birds, indicating a level of consistency in the bacterial taxa that colonise egg surfaces. In both species, the microbiomes of eggs within clutches were found to be distinct from other clutches in the population, likely due to varying parental, environmental, and management-related factors. In hihi, parental drivers appeared to contribute the most towards eggshell

microbiome composition, with eggshell microbiomes having greater microbiome richness and phylogenetic diversity when eggs were laid by first-time breeding females and socially sired by middle-aged males. In kakī, while nest location accounted for a large amount of variation in beta diversity between samples, parental drivers also played a major role in the microbiome composition, with greater eggshell microbiome diversity associated with eggs laid in larger clutches and greater richness and evenness in the microbiome of eggs experiencing longer periods of parental incubation. Captivity also appeared to play a role in kakī eggshell microbiome composition, with greater quantitative richness and evenness in the microbiome associated with captive-laid eggs relative to wild-laid eggs.

Characterising the eggshell microbiome

As in many other non-commercial bird species, the most abundant phyla making up the eggshell microbiome community structure of both kakī and hihi were Proteobacteria, Actinobacteria, Firmicutes, Bacteroidota (Bacteroidetes), and Cyanobacteria (**Table C12**, **Appendix C.1**). Pseudomonadaceae was one of the most abundant families across the samples and is a common family in passerine nests (Singleton & Harper, 1998; Goodenough & Stallwood, 2010), and on avian eggshells (Lee *et al.*, 2014; Peralta-Sánchez *et al.*, 2018). The driver of the family's abundance was *Pseudomonas*, a common and species-rich genus including a number of saprophytic and opportunistic species which have been linked to various infections across birds (Bailey *et al.*, 2000; Rich, 2003; Benskin *et al.*, 2009), including trans-shell infection and resulting embryo mortality (Walker *et al.*, 2002). *Ralstonia* was the most abundant genus in hihi samples and the second most abundant in kakī, driving the high relative abundance of the family Burkholderiales across samples. Several *Ralstonia* spp. are considered to be emerging global opportunistic pathogens based on their frequency in causing hospital-acquired infections in humans (Ryan & Adley, 2014), but there does not appear to be any recorded incidences of *Ralstonia* acting as an avian pathogen, although it has previously been identified on avian eggshells (Grizard *et al.*, 2014; van Veelen *et al.*, 2018) and is a significantly representative microorganism in egg yolk of laying hens (Jin *et al.*, 2022). Ambrosini et al. (2019) identified *Ralstonia* as one of the most abundant genera in the cloacal microbiome of barn swallows (*Hirundo rustica*),

with a significant association to nestlings, and it has also been shown to be an important genus in the gut microbiome of yellow-legged gull (*Larus michahellis*) hatchlings (Velando *et al.*, 2021). Grieves et al. (2022) found *Ralstonia* to be at elevated frequency in the uropygial gland microbiota of captive song sparrows (*Melospiza melodia*) compared to free-living individuals, but *Ralstonia* was not found to be differentially abundant between captive- and wild-laid kakī eggs.

How do the eggshell microbiomes of hihi and kakī compare?

The study species differ greatly in their evolutionary and life history, habitat, and management, so it is not unexpected that a large proportion of detected taxa were unique to either hihi or kakī and that many taxa were differentially abundant between them. Two halophilic or marine genera (*Halomonas* and *Pseudoalteromonas*), were among the small number of taxa found to be more abundant in hihi eggshells compared to kakī eggshells. Hihi are terrestrial and the presence of these bacteria may initially appear unexpected, but as Tiritiri Matangi is a small island with hihi nests often located near to the coasts it is possible that these bacteria reached eggshells via sea spray, either directly or carried on nest material or parent feathers/skin.

The kakī eggshell microbiome has lower evenness and greater richness and phylogenetic diversity than the hihi eggshell microbiome, with a higher abundance of many taxa. The studied hihi population are limited to a relatively small island (<2.2km^2), with all individuals nesting in artificial nestboxes and supplementary fed within a restored, and therefore relatively homogenous, habitat. Consequently, both eggs and breeding pairs likely experience very similar external conditions. In contrast, kakī samples were taken from both captive and free-living individuals, with free-living nests spread across a large (~1000km^2) and varied area, so it is likely that both nest materials and diet, as well as other external conditions, differed more strongly between individuals.

Even when captive-laid eggs were excluded from comparisons, the average kakī eggshell microbiome was still found to have greater richness and phylogenetic diversity than the average hihi eggshell microbiome. Nesting environment/habitat (Lee *et al.*, 2017; Basso *et al.*, 2022) and nest materials (e.g. Martínez-García *et al.*, 2016) have been shown to influence the eggshell microbiome, and diet has been reported to be the most influential factor shaping

the gut microbiome of wild birds (Matheen *et al.*, 2022), which could impact eggshell microbiomes. Taken together, results could indicate that the eggshell microbiome reflects the diversity of its surrounding habitat, with populations in less diverse environments having lower eggshell bacterial richness. As many threatened species live in small, fragmented, or otherwise non-optimal habitats, this could be a common feature of their eggshell microbiomes and should be taken into consideration when undertaking egg management.

Another aspect of threatened species which could be reflected in the eggshell microbiome is their genetic diversity. It has been proposed that inbreeding depression (i.e. the loss of fitness resulting from inbreeding) could affect the microbiome either directly or through interaction with the host's genotype (Bahrndorff *et al.*, 2016), with some evidence that inbreeding shapes the gut microbiome of gopher tortoises (*Gopherus polyphemus*) (Yuan *et al.*, 2015) and population bottlenecks shown experimentally to constrain microbiome diversity in *Drosophila melanogaster* (Ørsted *et al.*, 2022). Both hihi and kakī have experienced recent population bottlenecks (Maloney & Murray, 2001; Brekke *et al.*, 2011; Duntsch *et al.*, 2023), but while there is strong evidence of this leading to reduced genetic diversity and inbreeding depression in hihi (Brekke *et al.*, 2010; Duntsch *et al.*, 2023), the findings for kakī have been less conclusive (Steeves *et al.*, 2010; Hagen *et al.*, 2011; Bourke *et al.*, 2023). Low genetic diversity in hihi could therefore be another explanation for their lower eggshell microbiome diversity compared to kakī, although caution must be taken when comparing genetic diversity between species, and further investigation is needed in order to adequately interpret any potential relationship between inbreeding and the eggshell microbiome.

What factors drive differences in the eggshell microbiome?

The eggshell microbiome of eggs was more similar within than between clutches from the same species, but there was little indication that sibling kakī clutches (produced by the same parents at different times) were more similar to each other than to other clutches in the population. While the latter may be taken as support for the hypothesis that the eggshell microbiome is acquired horizontally, rather than resulting from the vertical transmission of cloacal microflora during egg-laying, there are several complexities that make this

interpretation less clearcut. Firstly, kakī clutches were sampled after different lengths of parental incubation, with length of parental incubation itself found to be associated with differences in microbiome. Therefore, even if sibling clutches were more similar to each other than to non-sibling clutches immediately after laying, this similarity may have been reduced by differing amounts of incubation prior to sampling. Eggs within a clutch share both a mother (source of cloacal microbiota) and a nesting environment and incubating parent or parents (source of external microbiota from nest materials, feathers, and skin), so similarities could result from either, or both, vertical and horizontal transmission. As a female's cloacal microbiome may change through time, the eggshell microbiomes of sibling clutches could show substantial variation even if they resulted purely from vertical transmission, while if both vertical and horizontal transmission occur the different external conditions of repeated clutches could mask the similarities due to vertical transmission. Ultimately, sampling of nest materials, dam cloaca/faeces, and brood patch of the incubating parent(s) alongside eggshell sampling, as well as experimental manipulations such as swapping eggs between clutches, is needed to truly elucidate the process of eggshell microbiome acquisition. The parental, environmental, and management-related factors found in this study to drive differences in the eggshell microbiome are discussed in turn.

Parental drivers

The strongest parental drivers of eggshell microbiome differences in hihi were dam breeding experience and social sire age, with sire age also appearing to drive differences in kakī microbiomes alongside clutch size, clutch number, and length of parental incubation. Eggs laid by experienced hihi females (i.e. those that had bred previously) had significantly lower eggshell microbiome richness and phylogenetic diversity than eggs laid by first-time breeders, with a *Pseudomonas* sp. and a *Sanguibacter-Flavimobilis* sp. found to be less abundant on eggs laid by experienced dams. *Pseudomonas* is a common avian pathogen (Benskin *et al.*, 2009), hence its higher abundance on the eggs of naïve dams could potentially be related to the lower hatching success reported in first-time hihi breeders (Low *et al.*, 2007). *Sanguibacter* has been found to be specific to early incubation in eggshell microbiomes (Potter *et al.*, 2013) and

related to diet in avian cloacal (Silva *et al.*, 2022) and gut (Mohr *et al.*, 2022) microbiomes.

Experienced females may have a less diverse gut and/or cloacal microbiome than first-time breeders, leading to lower diversity on their eggshells, perhaps due to being able to compete for better food resources or due to selection on bacterial communities as females reach sexual maturity (Grond *et al.*, 2018; Rowe *et al.*, 2020). Experienced females may also incubate eggs more efficiently or keep their nests cleaner, reducing eggshell microbiome diversity. Alternatively, this result could reflect selective disappearance of individuals with a greater bacterial load who may be less likely to survive to become experienced breeders, leaving females of higher fitness which are better able to control bacterial communities and reduce transmission to eggs. It has been shown that eggs laid by females with better immune system functioning have lower eggshell bacterial loads (Soler *et al.*, 2011a; Darolová *et al.*, 2021), potentially providing support to this explanation.

As bacteria can be transmitted between birds during copulation through cloacal contact and/or ejaculate transfer (e.g. Sheldon, 1993; White *et al.*, 2010), mating with a greater number of partners could increase and diversify an individual's bacterial load (Thrall *et al.*, 2000; but see Hernandez *et al.*, 2021), leading to the prediction that eggs of females with more previous partners have a more diverse microbiome. However, eggshell microbiome richness and phylogenetic diversity both decreased as a hihi dam's total number of genetic mates increased, and it was also seen that the eggshell microbiome of kakī eggs had greater richness and phylogenetic diversity than the hihi eggshell microbiome despite kakī consistently only have one mate per season. As hihi eggs were sampled near to the end of incubation it is possible that females with more previous mates did initially lay eggs with a more diverse microbiome, but were able to compensate for any effects of this on their eggs during the incubation period.

Although breeding experience is intrinsically connected with age in hihi, with all naïve females being first year females, no significant relationships between dam age and eggshell microbiome diversity were found. This is interesting given evidence of age-dependent hatching success in hihi (e.g. Low *et al.*, 2007),

which could be related to eggshell microbiome, and contrasts with the results of Hernandez *et al.* (2021) who found that female tree swallow (*Tachycineta bicolor*) age was positively correlated with cloacal microbiome richness. The potentially contrasting results seen here, which suggest a change in eggshell microbiome mainly with the initial transition of a breeder from naïve to experienced, could indicate the occurrence of selection in female microbiomes in line with the onset of reproduction or sexual maturity in hihi (Grond *et al.*, 2018; Rowe *et al.*, 2020). Alternatively, female cloacal microbiome diversity could change with age in hihi, but as-yet-unknown mechanisms may be at play which prevent these changes being translated to the eggshell microbiome.

Social sire age (and age^2) was positively correlated with microbiome richness and phylogenetic diversity in hihi and associated with variation in beta diversity in both species. Male hihi are not believed to be involved in incubation and appear to rarely enter the nest after egg-laying is complete (Castro *et al.*, 1996), hence their impact on eggshell microbiomes might be expected to be minimal, particularly as hihi eggs were sampled long after social males are assumed to have last entered the nest. However, there is still some uncertainty surrounding non-social male behaviour and it is possible that these males enter nests throughout incubation seeking extra-pair copulations. Middle-aged hihi males have been shown to sire a greater number of extra-pair offspring than younger or older males (Brekke *et al.*, 2013), which may mean they carry a greater microbial load or diversity of microbes either in their cloaca or on their body feathers due to entering more nests and copulating with more females. This diversity could potentially then be passed directly on to the eggs in their own nest (horizontal transfer) or to the cloaca of their social mate during copulation and then to eggs during vertical transmission. Also, as middle-aged males have better territories (Brekke *et al.*, 2015) it is possible that their nests are entered more frequently by intruders seeking extra-pair mating opportunities, potentially increasing the microbial diversity of the nest materials or incubating female and hence driving the increased richness and phylogenetic diversity of eggshell microbiomes as a result of horizontal uptake. In kakī, incubation is biparental (Pierce, 1982, 2013), hence it is perhaps anticipated that males would have an effect on eggshell microbiomes through incubation effects and/or horizontal transmission of microbes, although it is also possible that microbes transferred

from the male to the female's cloaca during copulation are then vertically transmitted to the egg. Overall, the characteristics of males may have more of an impact on eggshell microbiome than is often assumed, suggesting that studies of parental factors should not be limited to females to avoid potentially missing these complexities.

Kakī eggs which experienced a longer period of parental incubation prior to swabbing had a more diverse and even eggshell microbiome, indicating that incubation may have prevented any taxa, such as pathogenic bacteria, from becoming dominant, increasing egg viability. Kakī eggs containing older embryos at the time of collection have been shown to have higher hatching success than those collected with younger embryos (Bourke *et al.*, 2023), and in general eggs that undergo a period of parental incubation prior to artificial incubation experience lower rates of hatching failure than eggs removed immediately after laying (Burnham, 1983; Elliott *et al.*, 2001; Robertson *et al.*, 2006; Smith *et al.*, 2011; Hoeck *et al.*, 2015), potentially due to the acquisition of a beneficial microbiome from natural incubation. A better understanding of this process could support conservation decision-making about the best time to perform egg-pulling to optimise hatching success.

Microbiomes of kakī eggs from larger clutches had greater richness, phylogenetic diversity, and evenness than eggs from smaller clutches. It may be easier for parents to incubate smaller clutches, meaning that eggs from smaller clutches experience an amplified bacteriostatic effect of incubation. Another possible explanation is that due to the high vulnerability of wild nests, any incomplete clutches found with at least two eggs were removed for artificial incubation (and swabbed) and replaced with ceramic 'dummy' eggs to prevent nest desertion and encourage clutch completion, after which the final eggs were also collected and swabbed. Dummy eggs could potentially introduce bacteria to the nest, impacting the microbiome of the final eggs compared to those collected earlier. Given the small number of such occurrences in this dataset, further investigation, including experimental clutch manipulation, would be required to verify this hypothesis. However, as the use of dummy eggs for conservation and monitoring purposes is relatively widespread, this could be of key interest to conservation practitioners.

Finally, clutch number accounted for a relatively large amount of variation in beta diversity between kakī eggs. Previous work on kakī has indicated that multi-clutching has no negative impact on hatchability (Bourke *et al.*, 2023), in contrast to evidence of reduced reproductive success in replacement clutches for other species (Cade & Jones, 1993; Jones *et al.*, 1994). As replacement clutches are laid by pairs in response to simulated nest loss, it is possible that eggshell microbiomes of later clutches benefit from increased investment by parents attempting to compensate for previous losses, but there does not appear to have been any previous consideration of, or investigation into, this hypothesis in the literature. Alternatively, as clutch number reflects when eggs are laid in the breeding season, differences in microbiomes could reflect environmental differences such as resource availability and changes in climatic conditions.

Environmental drivers

The only environmental driver associated with microbiome diversity in hihi was the daily mean rainfall between estimated clutch initiation and swab date. For kakī, daily mean temperature and overall maximum temperature between estimated clutch initiation and swab date had some influence, and nest location also accounted for a large amount of variation between samples. This hihi population exclusively use artificial nestboxes, which may be expected to offer eggs protection from climatic conditions. However, cavity nests have been shown to have a higher relative humidity than open nests (Kern & Cowie, 1995), and greater rainfall levels may exacerbate this. While water on the egg surface has been shown to increase microbial growth on eggshells, parental incubation appears to nullify these effects (D'Alba *et al.*, 2010). The finding of a negative relationship between eggshell microbiome richness and phylogenetic diversity with mean rainfall could result from increased growth of opportunistic bacteria in humid conditions, reducing richness and diversity as these outcompete other taxa. Alternatively, it could be a consequence of incubation effects on eggshell microbiome composition compensating for increased humidity by reducing overall eggshell microbiome richness. As rainfall may not accurately depict nestbox humidity, direct measures of the nest microclimate and monitoring of incubation may be required for a better understanding of this relationship (Ruiz-de-Castañeda *et al.*, 2011c). Compared to hihi, the eggs of ground-nesting kakī

are more directly exposed to the elements, with even captive birds maintained in outdoor aviaries with minimal protection from the environment. As high ambient temperatures during laying have been shown to promote bacterial growth on eggshells during incubation (Ruiz-de-Castañeda *et al.*, 2011c), the lower richness of kakī eggshell microbiomes with greater mean daily temperature could be related to the proliferation of certain bacteria in higher temperatures at the expense of other taxa, reducing the overall richness.

The large proportion of kakī eggshell microbiome variation accounted for by nest location indicates the presence of location-specific factors influencing either the eggs themselves or the breeders. Kakī breed in varied habitats, including braided riverbeds, swamps, tarns, farmland, and open-air aviaries, which may be expected to be subject to multiple different abiotic and biotic conditions. Nest location has previously been related to differences in hatchability of kakī eggs (Bourke *et al.*, 2023), and it is possible that eggshell microbiome variation plays a role in this, although more work is needed to confirm if the eggshell microbiome significantly differs between hatched and unhatched kakī eggs (see **Chapter 5**). Location-specific differences in eggshell microbiome could be taken into consideration during management, for example in future scenarios where only a subset of wild nests can be managed. At this time there may be a trade-off between collecting eggs from varied environments to ensure the diversity of eggshell microbiomes is maintained, or limiting collection of eggs to locations selected due to parameters such as easier accessibility or higher mean hatching success.

Management-related drivers

This is the first study to compare eggshell microbiomes between wild and captive populations of a non-commercial species. I found that the eggshell microbiomes of captive-laid eggs had greater richness and evenness than those of wild-laid eggs. Animals in captivity generally experience a more standardised diet and housing conditions than wild individuals, which may be expected to lead to lower microbiome diversity, as has been seen in previous comparisons of gut (Wienemann *et al.*, 2011; San Juan *et al.*, 2020) and cloacal (Kelly *et al.*, 2022) microbiota of captive compared to wild samples. However, Oliveira *et al.* (2020) found no effect of captivity on raptor faecal diversity, and Grieves *et al.*

(2022) found higher diversity in uropygial gland microbiota of captive *versus* free-living song sparrows, indicating that there may be considerable complexity in the relationship between captivity and microbiomes. Captive birds are likely to be exposed to both cleaning products and human microbiomes more than wild individuals, which could influence their microbiomes.

The relative stability of captivity may also create less of a trade-off between microbial load and condition than might be expected in the wild, with captive birds able to harbour greater bacterial diversity with fewer negative consequences. The phyla Firmicutes, which was found to be more abundant in eggshell microbiomes of captive- than wild-laid kakī eggs, has previously been found to be more abundant in gut microbiota of artificially-reared *versus* wild rock ptarmigans (Ushida *et al.*, 2016), but other studies have found Firmicutes to be more abundant in the microbiomes of wild individuals compared to captive individuals (Xenoulis *et al.*, 2010; San Juan *et al.*, 2020; Alba *et al.*, 2022), hence any relationship with captivity is unclear. Both *Chryseobacterium* and *Ureaplasma,* species of which were found to be more abundant in eggs sired by captive-laid males, have previously been found in avian microbiomes (Wang *et al.*, 2011; Drobniak *et al.*, 2022; Song *et al.*, 2022) and are potentially pathogenic (Ran *et al.*, 2021; Kubovčiak *et al.*, 2022). Differences in eggshell microbiome between eggs sired by captive- *versus* wild-laid males may indicate that differences present at the egg-laying stage could persist for many years, which should be taken into consideration in long-term conservation programmes, particularly as a potential aspect of adaptation to captivity.

Conclusions and future directions

This study has characterised, for the first time, the eggshell microbiomes of two very different threatened bird species, and has explored some possible reasons for their similarities and differences in relation to their habitats, life history, and management. Key findings include differences in the eggshell microbiome related to captivity, parental breeding experience, and social sire age, as well as rainfall, temperature, and nest location. However, microbiomes are complex, and the variables tested here only accounted for a small proportion of the variation in diversity between samples, indicating that other factors may be at play that are influencing the composition of the eggshell microbiome. Further

work exploring other factors such as the inclusion of different nest materials in the nest, the presence or absence of ectoparasites, and the nest microclimate could help to shed light on other aspects shaping the eggshell microbiome. Future work could also expand upon the findings of this study by sampling eggs at multiple points throughout incubation to explore how eggshell microbiomes change over time and identify which, if any, specific taxa are being promoted or suppressed. Finally, studies incorporating swabs of nest material, female cloaca, and incubating parent(s) feather and brood patch alongside eggshell swabs, with the possible addition of experimental egg manipulations with real or dummy eggs, could help to determine the source of the eggshell microbiome and conclusively test the hypotheses of vertical transmission *versus* horizontal uptake.

Chapter 5: Does eggshell microbiome predict hatching success?

Abstract

Hatching failure is a ubiquitous and persistent, yet generally poorly understood, issue for many bird species. In particular, the high rates of hatching failure experienced by many populations of threatened species can impact conservation efforts and limit potential for species recovery. Trans-shell infection and subsequent embryo mortality is thought to be a major driver of reproductive failure in both the wild and in captivity, however there are still limitations in our understanding of how widely this is the case and the mechanisms behind this process. A growing body of research into the microbial community on the surface of eggshells, i.e. the eggshell microbiome, has indicated that it can play an important role in both probability of infection and egg viability. Despite this, the relationships between the eggshell microbiome and hatching failure remain largely unexplored, particularly in threatened bird species for which an improved understanding of any interactions could potentially be used to inform strategies to mitigate high rates of hatching failure. Here, I investigate whether the eggshell microbiome predicts hatching failure in two phylogenetically distinct threatened species, the kakī (black stilt; *Himantopus novaezelandiae*) and the hihi (stitchbird; *Notiomystis cincta*), both of which experience relatively high levels of hatching failure and are subject to ongoing conservation management. I examine the relationship between eggshell microbiomes and hatching failure in these species by comparing the eggshell microbiome of hatched and unhatched eggs and exploring the influence of parental, environmental, and management-related factors on the relationship between the eggshell microbiome and hatching failure. Results indicate that the eggshell microbiomes of eggs that go on to hatch and those that go on to fail do not differ in either their within-sample richness and evenness (alpha diversity), or their between-sample community composition dissimilarities (beta diversity). However, a small number of taxa, including both potential pathogens and beneficial bacteria, were found to differ in abundance between hatched and unhatched hihi eggs. Results also showed that maternal age plays a role in hihi hatching success, but with no indication of any interactive effects between eggshell microbiome and female age on hatching. Overall, this study indicates that the eggshell microbiome does not predict hatching success of hihi and kakī eggs, suggesting that trans-shell infection may not be a major threat to the reproductive success of these two threatened species. Efforts to reduce hatching failure should therefore be directed towards other factors which have previously been shown to impact egg viability in these populations, while it would also be advisable to conduct further research into the causes of failure to better support informed conservation decision-making.

Introduction

Disease plays an important role in limiting population growth across taxa, and birds are no exception (Newton, 1998). Pathogenic bacteria are associated with infection and mortality at all stages of avian development and have been subject to a large amount of research effort (Benskin *et al.*, 2009), but there are still many gaps in our understanding of how infection and disease influence the earliest stages of reproduction. In birds, the impact of disease is often manifested via poor parental condition, which can have knock-on consequences for hatching success due to factors such as reduced egg quality and inefficient incubation (e.g. Blount *et al.*, 2002; Parker, 2002; Rutkowska & Cichoń, 2002). However, pathogens may also cause hatching failure directly, by invading the egg through pores in the eggshell, causing infection and subsequent embryo mortality (e.g. Godard *et al.*, 2007; Soler *et al.*, 2010; Hansen *et al.*, 2015).

It was previously commonly believed that avian eggs and their embryos were sterile prior to hatching, however, more recently, research has indicated that eggs can contain an internal microbiome and that *in ovo* colonisation by these microbiota may be key in establishing the gut microbiota of the chick (Pedroso, 2009; Roto *et al.*, 2016; Trevelline *et al.*, 2018; Těšický *et al.*, 2023). The source of this internal microbiome is thought to be via transfer of microbiota from the mother's reproductive tract to the developing egg (Pedroso *et al.*, 2016; Ding *et al.*, 2017; Trevelline *et al.*, 2018; Dietz *et al.*, 2019; Lee *et al.*, 2019), via trans-shell migration of eggshell and environmental microbiota after laying (Maki *et al.*, 2020), or both (Pedroso, 2009; Roto *et al.*, 2016). However, along with these commensal bacteria, there is evidence that pathogenic bacteria can also be introduced to embryos either from the mother's reproductive tract prior to laying (De Buck *et al.*, 2004; Wigley *et al.*, 2005; Cobb, 2011; Cox *et al.*, 2012) or via trans-shell infection from the eggshell surface (Berrang *et al.*, 1999; Cook *et al.*, 2003), potentially leading to embryo mortality.

The egg surface has been found to host a community of bacteria and fungi from shortly after laying, known as the eggshell microbiome, and thought to established either through vertical transmission of cloacal microflora from the mother during egg-laying (Ruiz-de-Castañeda et al., 2011a, 2011b; Grizard et al., 2014), through horizontal uptake from the egg's surroundings (e.g. nest

material and incubating parent) (Godard et al., 2007; Martínez-García et al., 2016; van Veelen et al., 2018; Song et al., 2022), or from a mixture of both. While there is a known risk to eggs from trans-shell infection of pathogenic bacteria, the role of the overall eggshell microbiome in hatching failure is unclear, with evidence that eggshell microbiomes can have positive effects (Soler *et al.*, 2008; e.g. Martín-Vivaldi *et al.*, 2014), negative effects (e.g. Cook *et al.*, 2003; Hansen *et al.*, 2015), and no effects (e.g. Peralta-Sánchez *et al.*, 2010; Schmitt *et al.*, 2017a) on avian hatching failure. These differences may be species or situation dependent, with previous studies suggesting that differing ambient conditions in the tropics and temperate regions may explain inconsistencies in microbial effects on egg viability (Wang *et al.*, 2011; Giraudeau *et al.*, 2014). A number of factors have also been shown to influence the antimicrobial defence mechanisms of eggs (D'Alba & Shawkey, 2015), including chemical and physical egg attributes (Board & Fuller, 1974; Board, 1980, 1982), nest composition (Gwinner & Berger, 2005; Mennerat *et al.*, 2009b, 2009a; Peralta-Sánchez *et al.*, 2010; Ruiz-Castellano *et al.*, 2016), and parental incubation behaviour (D'Alba *et al.*, 2010; Ruiz-de-Castañeda *et al.*, 2011a; Martín-Vivaldi *et al.*, 2014). Egg antimicrobial defences can also be linked to aspects such as maternal condition, with females with lower immunity potentially being more likely to transmit pathogens to their eggs (Soler *et al.*, 2011a; Schmitt *et al.*, 2017a; Darolová *et al.*, 2021), and also likely to provide lower levels of maternal investment into the eggs, reducing the eggs' natural defences and making the embryos more vulnerable to infection (Hargitai *et al.*, 2006; Schoepf *et al.*, 2022).

One limitation in our understanding of the eggshell microbiome's role in hatching failure is that few studies have directly compared the eggshell microbiomes of hatched *versus* unhatched eggs. Eggshell bacterial load is often considered to be a good predictor for the probability of trans-shell infection and subsequent hatching failure based on experimental studies (Cook *et al.*, 2003, 2005b; Godard *et al.*, 2007), and has been used as a proxy for egg viability in studies which have not explicitly included analyses of actual hatching success (Cook *et al.*, 2005a; Soler *et al.*, 2011a, 2015; Peralta-Sánchez *et al.*, 2012). However, studies finding no apparent relationship between bacterial loads and hatching failure have called this into question (Peralta-Sánchez *et al.*, 2010;

Ruiz-de-Castañeda *et al.*, 2011c; Walls *et al.*, 2012). As many of the bacterial species making up the eggshell microbiome may be commensal, or even beneficial, bacterial species composition and the presence or dominance of particular pathogenic strains may in fact be the driving factor in embryo mortality (but see Peralta-Sánchez *et al.*, 2018). Two recent studies comparing the eggshells of hatched and unhatched sea turtle eggs found differences in their microbiomes, with both studies pointing towards the potentially pathogenic genus *Pseudomonas* as a likely cause of hatching failure (McMaken, 2022; Capri *et al.*, 2023).

Several studies which have investigated the role of parental incubation in controlling microbial infection of eggs have suggested that rather than reducing overall eggshell bacterial loads, incubation protects against trans-shell infection by facilitating changes to the microbiome composition (Shawkey *et al.*, 2009; Giraudeau *et al.*, 2014; Grizard *et al.*, 2014; Lee *et al.*, 2017), specifically promoting the relative abundance of harmless or antibiotic-producing bacteria and suppressing the growth of pathogenic bacteria (Brandl *et al.*, 2014; Lee *et al.*, 2014; Song *et al.*, 2022). The acquisition of a beneficial eggshell microbial community via parental incubation could be one explanation for the frequent finding that eggs that undergo a period of natural incubation prior to artificial incubation experience higher rates of hatching success than those removed from nests immediately post-laying (Burnham, 1983; Elliott *et al.*, 2001; Robertson *et al.*, 2006; Smith *et al.*, 2011; Hoeck *et al.*, 2015), a phenomenon which is particularly relevant to conservation as populations in which eggs are artificially incubated generally experience some of the highest rates of hatching failure across all wild and managed birds (Marshall *et al.*, 2023).

Microbial infection of eggs has long been recognised as an issue within the poultry industry given the potential risks to human health and negative economic consequences of egg losses (Haines, 1939; Board, 1966; Mayes & Takeballi, 1983), and thus numerous measures such as biosecurity and hygiene practices, vaccination, the use of probiotics and antibiotics, and continuous monitoring of the health of the poultry flock are taken to prevent and control infection (Meunier *et al.*, 2016; Stegniy *et al.*, 2020; Gast *et al.*, 2022). Microbial infection is also often suggested as a driver of avian hatching failure in non-commercial species, but there are gaps in our understanding of how

widespread this may be and the underlying mechanisms. In fact, hatching failure overall is generally poorly understood despite its ubiquity (Assersohn *et al.*, 2021b), and the particularly high rates experienced by threatened and managed populations (Marshall *et al.*, 2023) highlight how important an improved understanding of hatching failure could be in our efforts to conserve species at risk from extinction.

Given that some of the techniques used in commercial breeding, such as artificial incubation, are also applied to threatened species under conservation, there is potential to use the knowledge gained from the poultry-dominated avian microbiome literature along with the relative smaller body of research into the eggshell microbiomes of wild bird species to better understand how conservation interventions may impact the eggshell microbiome and the risk of trans-shell infection in managed, threatened species. Ultimately, this improved understanding could be used to support informed conservation decision-making towards action to reduce hatching failure in threatened species, aligning with the conclusions of several studies which have called for the consideration of animal microbiomes in conservation management practices (Trevelline *et al.*, 2019; Comizzoli *et al.*, 2021).

In this study, the eggshell microbiomes of hihi (stitchbird; *Notiomystis cincta*), a Vulnerable passerine (BirdLife International, 2022b), and kakī (black stilt; *Himantopus novaezelandiae*), a Critically Endangered non-migratory wader (BirdLife International, 2018b), are investigated to discern whether or not the eggshell microbiome plays a role in hatching failure. These species are both endemic to Aotearoa New Zealand and although they differ substantially in their evolutionary history, life history, habitat, nesting habits, and management interventions (**Table C1, Appendix C.1**), they both experience relatively high hatching failure rates of 28.4% (Heber & Briskie, 2010) for kakī and 35% (Brekke *et al.*, 2010) for hihi. They are subject to regular nest checks, and undergo a variety of management interventions including artificial incubation, headstarting, and captive-breeding for kakī (BirdLife International, 2018b), and translocation, artificial nestbox provision, and supplementary feeding for hihi (BirdLife International, 2022b).

Both hihi and kakī have suffered from issues related to disease in recent history (Ewen *et al.*, 2007; Rippon *et al.*, 2010; Brown *et al.*, 2020, 2021, 2022; Alley & Hunter, 2023), but there has not been any direct investigation into the incidence of trans-shell infection in relation to hatching failure in these populations. There is evidence that a major driver of embryo mortality in hihi is inbreeding depression (Brekke *et al.*, 2010), and maternal age has also been linked to hatching success in multiple studies (Low *et al.*, 2007; Low & Pärt, 2009; de Villemereuil *et al.*, 2019b, 2019a; Mather *et al.*, 2020). For kakī, previous studies have found that hatchability is higher for wild-laid eggs (van Heezik *et al.*, 2005), and have also identified maternal age, clutch number, clutch size, and egg weight and estimated embryo development stage at the time of collection for artificial incubation as factors influencing the likelihood of hatching (Bourke *et al.*, 2023). It is noted that these previous findings for kakī applied only to eggs assessed as being 'fertile' based on candling and apparent signs of embryo development, hence excluded eggs with no evidence of embryo development which may have experienced either fertilisation failure or early embryo mortality. They also excluded addled eggs, which could have been the result of embryo mortality due to trans-shell infection based on high levels of microbial infection and presence of pathogenic bacteria in addled eggs of other species (Pinowski *et al.*, 1994; Houston *et al.*, 1997; Hansen *et al.*, 2015), although it is possible that these microbes contaminated the eggs post-failure rather than causing the failure.

The potential relationship between eggshell microbiome and hatching failure in these two threatened species is tested in two ways. First, building on previous work in which the eggshell microbiome of these two species was characterised for the first time (**Chapter 4**), hatched and unhatched eggs from each species were compared to test the hypothesis that the eggshell microbiome of failed eggs will have a stronger bias towards pathogenic bacteria. Then, the influence of eggshell microbiome diversity and the relative abundance of several potentially pathogenic taxa on hatching failure is tested for, as well as any interaction between these measures and other variables previously shown to impact hatching failure in these, and other, species. The results are discussed in the context of our current understanding of eggshell microbiomes and the

potential consequences for the conservation and management of these, and other, threatened species.

Materials and Methods

Study populations

The hihi population included in this study is the translocated population on Tiritiri Matangi Island where almost all nesting occurs within artificial nestboxes and supplementary food is provided (Ewen *et al.*, 2018; BirdLife International, 2022b). Hihi pairs lay up to two successful clutches of two to five eggs in a season, and will typically lay a replacement clutch if a breeding attempt fails (Castro *et al.*, 1996; Taylor *et al.*, 2005; Ewen *et al.*, 2018). Kakī samples were collected from the only remaining free-living population located in Te Manahuna (the Mackenzie Basin) along with a captive pair held at the Department of Conservation (DOC) Kakī Recovery Centre, Twizel, and two captive pairs held at The Isaac Conservation and Wildlife Trust (ICWT) in Christchurch. Almost every egg from both free-living and captive pairs is removed for artificial incubation as part of headstarting, with this egg-pulling encouraging pairs to re-clutch and lay up to four clutches of three to six (usually four) eggs in each breeding season (Reed *et al.*, 1993; van Heezik *et al.*, 2005; Pierce, 2013). Both the hihi population and free-living and captive kakī are included in conservation management programmes with long-term datasets of parentage, survival, and reproductive success. A summary of hihi and kakī life history traits, threat status, and management is included in **Table C1**, **Appendix C.1**.

Sample collection

All samples were collected during the 2019/20 breeding season (October 2019 – January 2020) in Aotearoa New Zealand. Out of 364 hihi eggs laid, a total of 288 eggs (79.1%) were sampled, and of 166 kakī eggs laid, a total of 93 eggs (56.0%) were sampled. All eggs were swabbed individually using a separate Tubed Sterile fine tip Dryswab™ (MW100; MWE). Please see **Chapter 4** for full details of the sampled populations and the swabbing procedure.

Environmental data

Daily maximum temperature (°C), daily minimum temperature (°C), and the total daily rainfall (mm) were downloaded from New Zealand's National Climate Database via CliFro (*https://cliflo.niwa.co.nz/*) from the closest weather stations to the breeding sites. The daily mean temperature (°C) was calculated as the mean of the minimum and maximum temperature each day. Additional details are available in **Appendix C.1**.

DNA extraction and 16S rRNA gene sequencing

DNA extraction from swabs was conducted using a Qiagen DNeasy PowerSoil HTP 96 Kit. DNA was amplified using an Illumina MiSeq platform for the 16S rRNA V4 region using dual indexed forward and reverse primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) (Caporaso *et al.*, 2011), with the library construction and sequencing protocol adapted from Kozich et al. (2013). Due to experimental constraints, repeated hihi clutches and any samples with incomplete information were excluded from sequencing. As there was a smaller number of kakī clutches in total all samples were sequenced, which included multiple (although often non-sequential) clutches from some pairs. As one kakī sample did not successfully amplify, the final dataset consisted of 92 kakī samples and 157 hihi samples. Three mock community samples and three negative (no-template) controls were also sequenced. Please see **Chapter 4** for additional details.

Hatching success information

Hatching success information for each egg was calculated both as a binary (individual egg went on to hatch or fail) and as a numeric (overall hatching success of the clutch). To ensure failed eggs included only those that did not hatch due to fertilisation failure or embryo mortality (rather than, for example, predation, damage, or desertion), samples from any nests where eggs disappeared or females were suspected to have abandoned the nest during incubation were excluded. For kakī, the hatching fate of each individual egg was known, while for hihi this information was only available for 106/157 (67.5%) of eggs included in the study, although clutch success rate was known for all clutches for both species. Across the final samples, 79.25% (84/106) hihi eggs

of known fate hatched, and the mean hatching success per clutch was 75.16%, consistent with the overall hatching success rate of 75.21% (267/355) for the entire managed hihi population on Tiritiri Matangi in 2019/20. Across kakī samples, 79.35% (73/92) of sampled eggs hatched, with mean hatching success per clutch of 77.17%. Of captive-laid eggs, 91.30% (21/23) hatched, and of wild-laid eggs 75.36% (52/69) hatched. The overall hatching success of the entire managed kakī population for the 2019/20 breeding season was 75.46% (123/163) for all eggs, 83.72% (36/43) for captive-laid eggs and 72.50% (87/120) for wild-laid eggs.

Sequence data processing

Microbiome bioinformatics were performed with a combination of QIIME 2 2022.8 (Quantitative Insights In Microbial Ecology; Bolyen *et al.*, 2019) and appropriate packages in RStudio version 2022.7.0.548 (RStudio Team, 2022) running R version 4.2.1 (R Core Team, 2022).

The data was quality filtered via the *q2-demux* plugin followed by denoising with DADA2 (Callahan *et al.*, 2016) via the *q2-dada2* plugin. The truncation lengths of forward and reverse reads were set to 240 and 160 bp respectively, based on the quality score criteria (≤30), and non-target-length sequences outside the range of 250-256 nucleotides were removed. Feature classifiers were trained on the sequenced region of the target sequences via the *q2-feature-classifier* (Bokulich *et al.*, 2018) *fit-classifier-naive-bayes* (Pedregosa *et al.*, 2011). Taxonomy was assigned to Amplicon Sequence Variants (ASVs) via the *q2-feature-classifier* (Bokulich *et al.*, 2018) *classify-sklearn* (Pedregosa *et al.*, 2011) naïve Bayes taxonomy classifier against the Silva v138.1 99% OTUs reference sequences formatted for use with QIIME2 (Quast *et al.*, 2013; Robeson *et al.*, 2021; Kaehler, 2022).

The ASVs were filtered to retain only features annotated to at least phylum level, and non-target sequences assigned to Archaea, Eukaryota, chloroplasts, or mitochondria were removed. The *decontam* package version 1.16.0 (Davis *et al.*, 2018) in R was used to detect contaminants by applying the “prevalence” method. A probability threshold of 0.4 identified 15 ASVs as potential contaminants, and these were removed from all samples. The final dataset

consisted of 6528 ASVs in total. All ASVs were aligned with mafft (Katoh *et al.*, 2002; Katoh & Standley, 2013) via *q2-alignment* and used to generate a phylogenetic tree with fasttree2 (Price *et al.*, 2010) via *q2-phylogeny*. Additional details of sequence data processing can be found in **Chapter 4** and **Appendix C.1.**

Alpha diversity metrics were used to analyse the diversity of the samples, while beta diversity metrics were used to study the dissimilarities in the community composition of the samples. Both alpha and beta diversity metrics can either be qualitative (based on presence or absence of sequences), or quantitative (incorporating sequence abundances) (Lozupone *et al.*, 2007), and alpha diversity metrics can summarise either the richness (number of taxonomic groups) and/or evenness (distribution of abundances of the taxonomic groups) of samples. The quantitative alpha diversity metrics used in this study were Shannon's diversity index (Shannon & Weaver, 1949), which measures richness and evenness, and Pielou's evenness (Pielou, 1966), which measures evenness, while the qualitative alpha diversity metrics were observed features and Faith's Phylogenetic Diversity (PD) (Faith, 1992), which both measure richness. For beta diversity, quantitative measures were weighted UniFrac distance (Lozupone *et al.*, 2007) and Bray-Curtis distance (Sørensen, 1948; Bray & Curtis, 1957), while the qualitative measures used were unweighted UniFrac distance (Lozupone & Knight, 2005) and Jaccard distance (Jaccard, 1908). Faith's PD and both UniFrac distances incorporate phylogenetic relationship between features into their calculations.

Alpha and beta diversity metrics were estimated using *core-metrics-phylogenetic* via *q2-diversity* after samples were rarefied (subsampled without replacement) to 4896 sequences per sample for the full dataset and for the hihi samples only, and to 14,396 sequences per samples for the kakī samples only. Multiple alpha and beta diversity metrics were estimated as it has been shown that using only one metric in analyses and for conclusions can lead to misleading results due to differences in their sensitivities to rare taxa and to different components of diversity (e.g. richness *versus* evenness) (Johnson & Burnet, 2016; Kers & Saccenti, 2022). The alpha diversity metrics estimated were the Shannon's diversity index (Shannon & Weaver, 1949), observed features, Pielou's evenness (Pielou, 1966), and Faith's PD (Faith, 1992), while

the beta diversity metrics estimated were the weighted UniFrac distance (Lozupone *et al.*, 2007), unweighted UniFrac distance (Lozupone & Knight, 2005), Jaccard distance (Jaccard, 1908), and Bray-Curtis distance (Sørensen, 1948).

Statistical analyses

As both species are socially monogamous, dam and sire ID overlapped completely. Further, as only first clutches were included for hihi, dam/sire ID also overlapped completely with clutch ID.

How does the eggshell microbiome differ between hatched and unhatched eggs?

To compare the alpha diversity of eggshells from hatched and unhatched eggs, *alpha-group-significance* was used to apply Kruskal-Wallis tests (Kruskal & Wallis, 1952) and *alpha-correlation* (both via *q2-diversity*) was used to apply Spearman correlation tests (Spearman, 1904) to binary (hatched *versus* unhatched) and numeric (percentage hatching success per clutch) hatching success respectively. To test for differences in beta diversity metrics, *adonis* (Anderson, 2001; Oksanen *et al.*, 2018) via *q2-diversity* was used to apply PERMANOVA tests based on 999 permutations. Permutational multivariate analysis of dispersion (PERMDISP) (Anderson, 2006) tests were also performed using *beta-group-significance* via *q2-diversity* to test for significant differences between the within-group variance of hatched eggs and unhatched eggs.

Differential abundance testing was applied to detect differences in microbial mean taxa abundance between the eggshell microbiomes of hatched and unhatched eggs from each study species. As the results of different differential abundance testing methods have been shown to vary due to characteristics of both the data and the differential abundance tool itself, the data was analysed with both ANCOM and DESeq2 in a consensus approach to ensure robust biological interpretations as previously recommended (Wallen, 2021; Nearing et al., 2022). ANCOM was applied within QIIME2 via the *q2-composition* (Mandal et al., 2015) plugin and DESeq2 was applied in R using the *DESeq2* package version 1.36.0 (Love et al., 2014). For the ANCOM method, all ASVs appearing

in less than 20 samples (<10% of all samples) and with a total frequency of less than 20 counts across all samples were filtered out and the add-pseudocount action was used to both apply a relative abundance transformation and add a pseudocount of 1 to all remaining counts. Benjamini-Hochberg (Benjamini & Hochberg, 1995) adjusted p-values (p-adj) were reported for results derived from DESeq2 and the W statistic was reported for ANCOM results, with a normalised W value based on the number of features being tested at each level calculated and used with a fixed threshold of 0.7 to assess significance of taxa identified as differentially abundant by ANCOM. Differentially abundant taxa were identified at the ASV, genus, family, and phylum level. ASVs were grouped at the various taxonomic levels using the 'tax_glom' function within the *phyloseq* package version 1.40.0 (McMurdie & Holmes, 2013) in R and *collapse* via the *q2-taxa* plugin in QIIME2.

Does eggshell microbiome diversity or composition predict hatching success?

To test whether the eggshell microbiome composition predicts hatching success the 'glmer' function from the *lme4* package version 1.1.31 (Bates et al., 2015) was used to fit Generalised Linear Mixed Models (GLMMs) with hatching success as the dependent (response) variable. Separate sets of GLMMs were constructed with either a binary hatched *versus* unhatched status for each egg or a clutch-level hatching success measure (as a proportion) as the response variable. All GLMMs were binomial with a logit link function, and for models of clutch-level hatching success, the 'weights' argument was set to equal clutch size. Dam ID was included as a random effect in all models which controlled for dam and sire individual differences, and partially controlled for kakī nest location differences as dam ID and nest location were strongly correlated. When considering the available data and the factors suspected to influence hatching success in the study populations, potential multicollinearity was identified using correlation measures. Correlations between categorical variables were assessed via pairwise chi-squared tests of independence and computations of the Cramér's V (Cramér, 1946) for each relationship, while correlations between continuous variables were assessed via Pearson correlation coefficient values and correlations between continuous variables and dichotomous categorical variables were assessed via point biserial correlation coefficient values (Tate,

1954, 1955) (**Table D1–Table D3**, **Appendix D)**. Due to high degrees of correlation between dam and sire age and breeding experience in both species, only age was included in the models. Similarly, dam age and sire age were found to be strongly correlated for kakī hence only dam age was included in the models, but for hihi there was a negligible correlation, and both were included. Different measures of alpha diversity (Shannon's diversity index, Pielou's evenness, Faith's PD, and observed features) were used to test for potential effects of the eggshell microbiome on hatching success, as were the relative abundance of the genera *Staphylococcus* and *Enterococcus* and the family Enterobacteriaceae, which are all known avian pathogens which have previously been considered in studies of eggshell microbiomes and hatching success (Soler *et al.*, 2011a, 2012a, 2012b; Peralta-Sánchez *et al.*, 2012; Martín-Vivaldi *et al.*, 2014; Ruiz-Castellano *et al.*, 2016; Tomás *et al.*, 2018). The relative abundance of the *Pseudomonas* genus was also tested as this is another recognised avian pathogen (Benskin *et al.*, 2009) which was found to be a major component of the eggshell microbiomes of the study species in **Chapter 4,** and has been identified as a major driver of differences between the eggshell microbiomes of hatched and unhatched sea turtle eggs (McMaken, 2022).

The additional parental and environmental factors with putative or suspected links to hatching success in these or similar species, and for which consistent data was available, which were included as potential explanatory variables in the models were: clutch size, dam age (linear and quadratic), mean daily temperature (°C) between estimated clutch initiation and swab date, and mean daily rainfall (mm) between estimated clutch initiation and swab date. For the hihi models, sire age (linear and quadratic) was also included, while for kakī models, clutch number, egg captive- or wild-laid, egg weight at collection, and estimated days of parental incubation at swab (i.e. the estimated age of embryo at time of collection for artificial incubation) were included. All continuous variables were scaled and centred and age was modelled as both a linear and quadratic term as females have been found to show age-dependent reproductive success in both hihi (Low *et al.*, 2007; Brekke *et al.*, 2013, 2015; de Villemereuil *et al.*, 2019a; Mather *et al.*, 2020) and kakī (Bourke *et al.*, 2023). To test the significance of variables for the model, each variable was evaluated

individually against a null model, with the Akaike's information criterion (AIC) (Akaike, 1981; Burnham & Anderson, 2002) used to compare between them. Variables from univariate models with a significantly lower AIC value than the null model were included in an additive full model which was compared to models with each variable removed one by one. Interactions between any of these significant variables were also checked, and where these significant variables included alpha diversity metrics, any correlations identified in **Chapter 4** were also tested in the form of interactions.

The full models and models with interactions were checked for overdispersion using the 'check_overdispersion' function from the *performance* package version 0.10.1 (Lüdecke et al., 2021), with no overdispersion being detected ($p > 0.05$) for any model. The 'aictab' function from the package *AICcmodavg* version 2.3.1 (Mazerolle, 2020) was then used to compare all models (null, univariate, full additive, and interactive) by the AIC corrected for small sample size (AIC_c) (Akaike, 1973; Hurvich & Tsai, 1989). All models within $\Delta AIC_c \leq 2$ of the model with the lowest AIC_c (the best model) were averaged using the 'model.avg' function from the *MuMIn* package version 1.47.1 (Bartón, 2022) to obtain weighted parameter estimates and the relative importance of each parameter. Full-model averaging was used to calculate the weighted parameter estimates (Symonds & Moussalli, 2011). The results of both the best model and the results of model averaging were reported.

Throughout the Results section, any association characterised as 'significant' will refer to a statistically significant association at the alpha level of 0.05.

Results

How does the eggshell microbiome differ between hatched and unhatched eggs?

Neither individual egg hatching success nor proportional hatching success per clutch were associated with significant differences in any of the alpha or beta eggshell microbiome diversity metrics in either hihi or kakī, although both observed features and Faith's PD were close to significance in kakī (**Figure 5-1**, **Table 5-1** & **Table 5-2**). PCoA plots visualising the dissimilarities in microbial communities among hatched and unhatched eggs for hihi and kakī based on

each of the beta diversity metrics, and showing the variation explained by the first two axes, displayed substantial overlaps between the groups (**Figure 5-1**). The results of PERMDISP also did not indicate any significant differences between the within-group variances of hatched eggs compared to unhatched eggs for either species (**Table 5-3**).

Table 5-1. Kruskal-Wallis and Spearman correlation test results for alpha diversity metrics for hatching success measured as both a binary (hatched *versus* unhatched) and numeric (percentage hatching success per clutch (%)) for hihi and kakī eggshell samples. The effect size (η^2) for the Kruskal-Wallis test was calculated as $\eta^2 = (H - k + 1)/(n - k)$, where H was the H statistic obtained in the Kruskal-Wallis test, k was the number of groups, and n was the total number of observations.

	Shannon's diversity index			**Observed features**			**Faith's PD**			**Pielou's evenness**		
	H	η^2	*p*-value	*H*	η^2	*p*-value	*H*	η^2	*p*-value	*H*	η^2	*p*-value
Hatched vs. unhatched [hihi: *n* = 106; kakī: *n* = 92]												
Hihi	1.106	0.001	0.293	0.261	-0.007	0.610	0.152	-0.008	0.697	1.593	0.006	0.207
Kakī	1.442	0.005	0.230	3.835	0.032	0.050*[2]*	3.412	0.027	0.065	1.115	0.001	0.291
	ρ	–	*p*-value	*ρ*	–	*p*-value	*ρ*	–	*p*-value	*ρ*	–	*p*-value
Hatching success per clutch % [hihi: *n* = 157; kakī: *n* = 92]												
Hihi	-0.084	–	0.294	-0.082	–	0.305	-0.036	–	0.655	-0.046	–	0.565
Kakī	0.181	–	0.085	0.133	–	0.207	0.135	–	0.201	0.182	–	0.082

Table 5-2. Univariate Adonis testing (PERMANOVA) results for beta diversity metrics for hatching success measured as both a binary (hatched *verus* unhatched) and numeric (percentage hatching success per clutch (%)) for hihi and kakī eggshell samples generated using *adonis* in QIIME2.

	Weighted UniFrac			**Unweighted UniFrac**			**Jaccard**			**Bray-Curtis**		
	pseudo-*F*	R^2	*p*-value	pseudo-*F*	R^2	*p*-value	pseudo-*F*	R^2	*p*-value	pseudo-*F*	R^2	*p*-value
Hatched vs. unhatched [hihi: *n* = 106; kakī: *n* = 92]												
Hihi	0.723	0.007	0.647	0.809	0.008	0.841	0.896	0.009	0.910	0.731	0.007	0.689
Kakī	0.746	0.008	0.458	1.148	0.013	0.187	0.941	0.010	0.709	0.346	0.004	0.881
Hatching success per clutch % [hihi: *n* = 157; kakī: *n* = 92]												
Hihi	1.540	0.010	0.136	1.013	0.006	0.410	0.970	0.006	0.609	1.520	0.010	0.104
Kakī	1.143	0.013	0.258	1.065	0.012	0.293	0.945	0.010	0.666	0.823	0.009	0.389

Table 5-3. PERMDISP analysis results for beta diversity metrics for hatching success measured as a binary (hatched *versus* unhatched) for hihi and kakī eggshell samples generated using *beta-group-significance* via *q2-diversity* in QIIME2.

	Weighted UniFrac		**Unweighted UniFrac**		**Jaccard**		**Bray-Curtis**	
	F	*p*-value	*F*	*p*-value	*F*	*p*-value	*F*	*p*-value
Hihi hatched vs. unhatched (*n* = 106)	0.521	0.460	2.339	0.188	2.649	0.389	0.075	0.761
Kakī hatched vs. unhatched (*n* = 92)	0.600	0.438	2.589	0.194	1.833	0.284	0.355	0.562

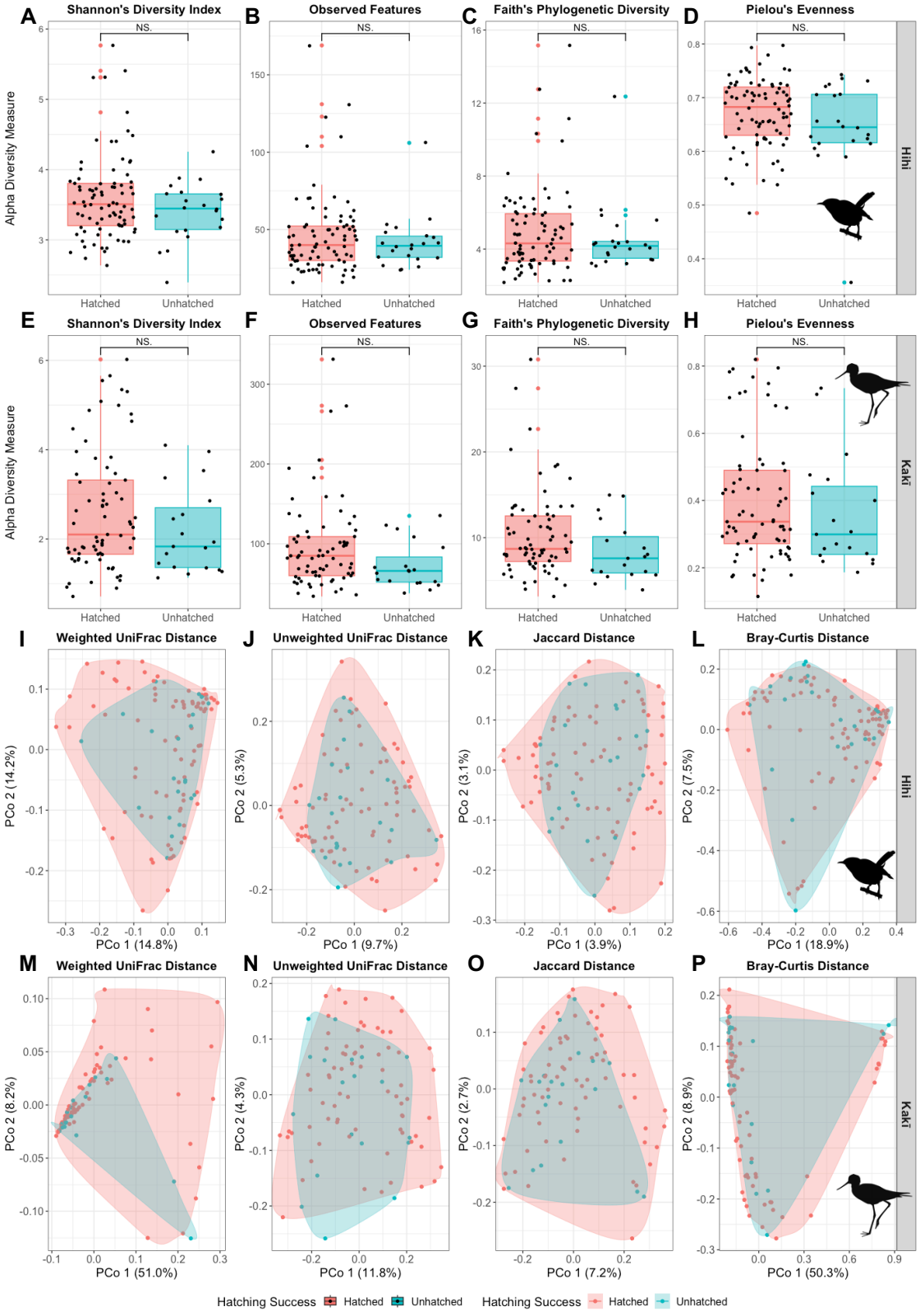

Figure 5-1. Visualisations of alpha and beta diversity metrics for eggshells of hatched and unhatched hihi and kakī eggs. Box-and-whisker plots of Shannon's diversity index, observed features, Faith's phylogenetic diversity, and Pielou's Evenness for A–D) hihi samples, E–H) kakī samples. Boxes show the interquartile range (IQR) of alpha diversity values, with lower and upper whiskers extending to the smallest value at most 1.5 × IQR from the first quartile and the largest value no further than 1.5 × IQR from the third quartile. Individual coloured points represent 'outlying' data beyond this range, and black points represent the underlying distribution of the data. Principal coordinates analysis (PCoA) based on weighted UniFrac, weighed UniFrac, Jaccard, and Bray-Curtis distances for I–L) hihi samples, M–P) kakī samples.

The most abundant phyla in the eggshells of both hatched and unhatched eggs were Proteobacteria, Firmicutes, Actinobacteria, Bacteroidota, and Cyanobacteria (**Table D4**, **Appendix D**). The relative abundances of different phyla, families, and genera were generally very similar between hatched and unhatched eggs in both study species (**Figure 5-2**; **Table D4**, **Appendix D**). The genus *Orbus* and its family Orbaceae were seen only in unhatched hihi eggshell samples, but this was due to the presence of *Orbus* at a relatively high level in just one sample, hence is unlikely to be biologically meaningful.

The results of differential abundance testing with ANCOM did not identify any significantly differentially abundant taxa between hatched and unhatched eggs in either hihi or kakī. Similarly, the results of DESeq2 showed no taxa to be

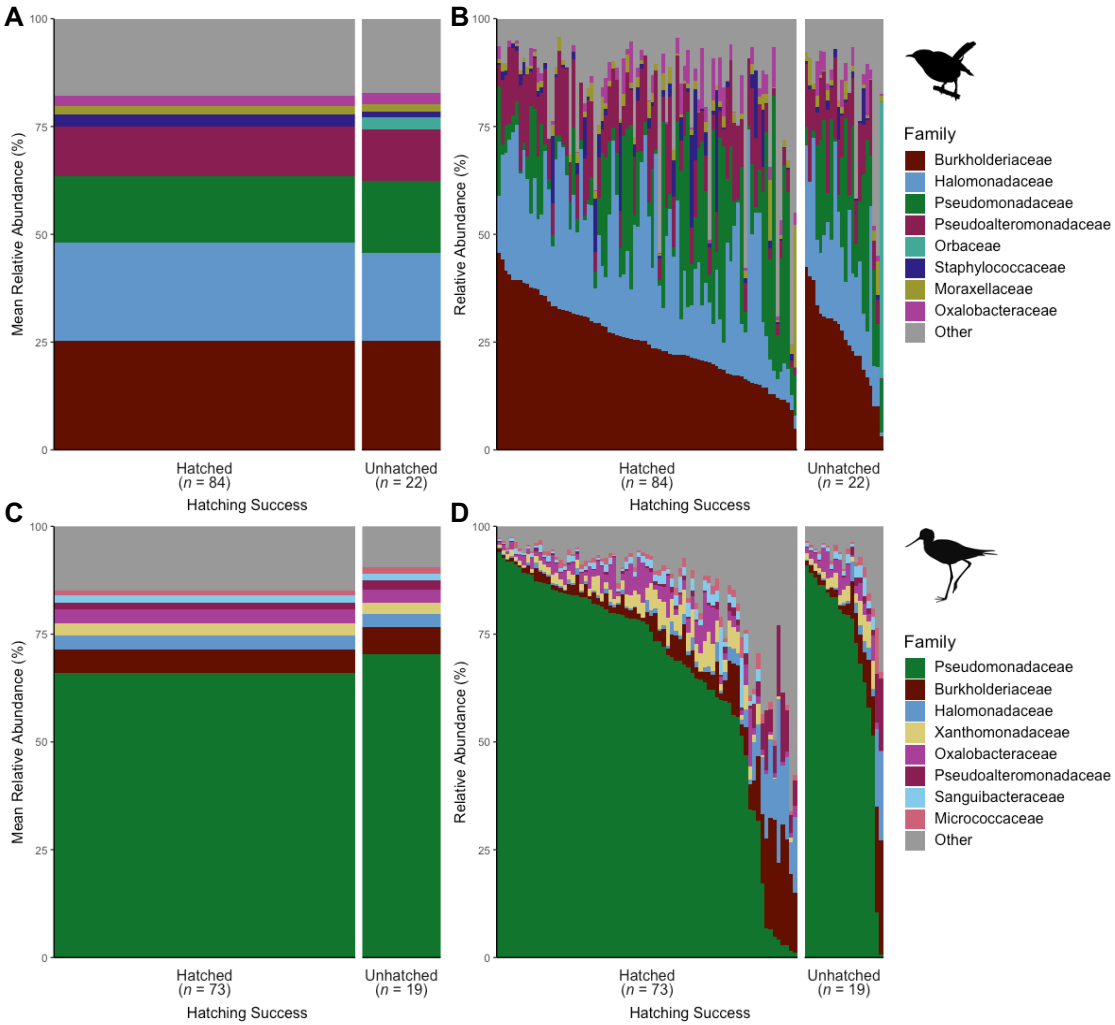

Figure 5-2. Relative abundances of families across eggshell samples from hatched and unhatched A–B) hihi eggs, C–D) kakī eggs. The left of each pair of figures shows the mean relative abundances across all samples, while the right shows the relative abundance in each sample. The top five most abundant phyla and top eight most abundant families and genera are named, with the remaining taxa represented by 'Other'. The width of each panel is representative of the sample size of the groups.

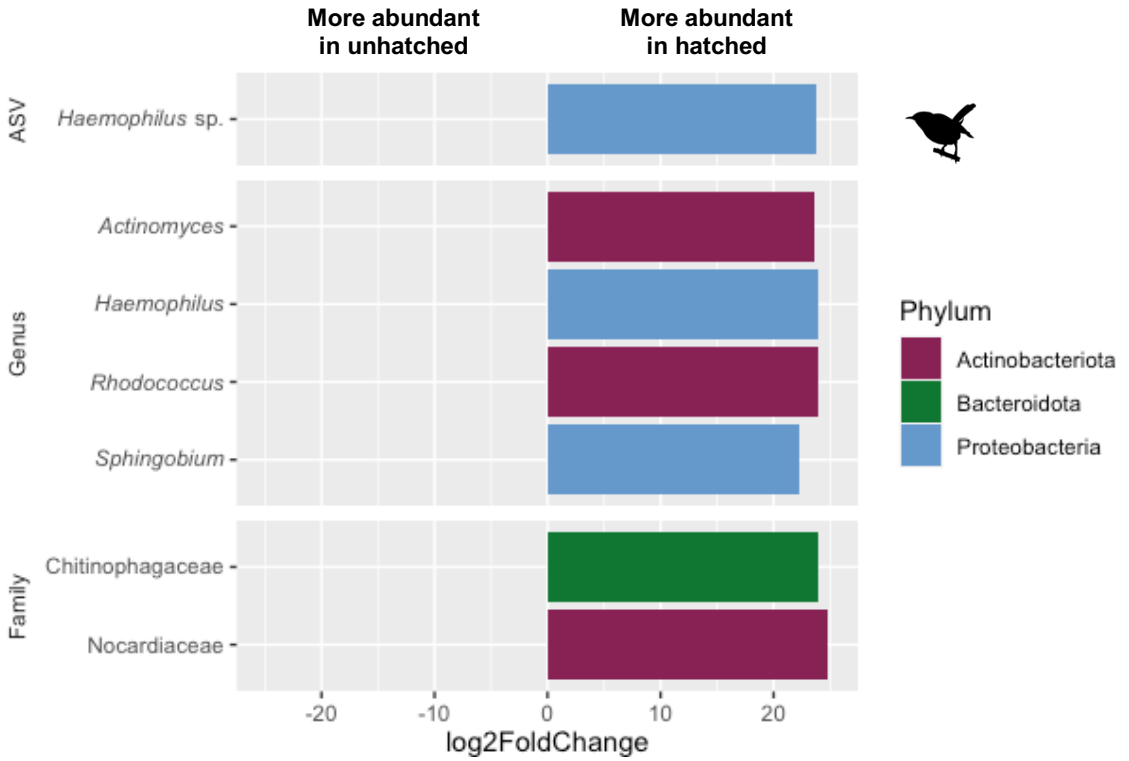

Figure 5-3. Symmetrical barplot showing the taxa identified by DESeq2 as being significantly differentially abundant between the eggshells of hatched and unhatched hihi eggs. Significance is based on the Benjamini-Hochberg adjusted p-values (p-adj < 0.05). Negative log2FoldChange values indicate taxa were more abundant in eggshell samples from unhatched eggs, while positive log2FoldChange values indicate taxa were more abundant in eggshell samples from hatched eggs.

significantly differentially abundant between the eggshell microbiomes of hatched and unhatched kakī eggs. The results of DESeq2 for hihi revealed one ASV (an unclassified species of *Haemophilus*), four genera (*Rhodococcus*, *Haemophilus*, *Actinomyces*, and *Sphingobium*), and two families (Nocardiaceae and Chitinophagaceae) to be significantly more abundant in the eggshell microbiomes of hatched hihi eggs compared to unhatched hihi eggs (**Figure 5-3**).

Does eggshell microbiome diversity or composition predict hatching success?

Hihi

The best model to predict binary hatching success in hihi was an additive model with the parameters Shannon's diversity index and dam age^2 (**Table D5**, **Appendix D**). Dam age^2 had a significant effect on binary hatching success, with eggs laid by older females being significantly less likely to hatch (effect ±

SE = -0.820 ± 0.399, *z* = -2.056, *p* = 0.0398). Eggs with greater Shannon's diversity in their eggshells appeared to be more likely to hatch, although this relationship was only close to being statistically significant (effect ± SE = 0.943 ± 0.482, *z* = 1.957, *p* = 0.0503) (**Table 5-4**). The models within $\Delta AIC_c \leq 2$ of the best model included the parameters Shannon's diversity index, dam age^2, dam age, and the interactions between Shannon's diversity index and both linear and quadratic dam age, however model averaging showed none of these parameters to be significant predictors of binary hatching success. For proportional hatching success per clutch, the best model was a univariate model of dam age indicating a negative, but non-significant relationship (effect ± SE = -0.551 ± 0. 289, z = -1.909, p = 0.0563). Similarly, model-averaging of the top models showed negative correlations between proportional hatching success and both dam age and dam age^2, but these were non-significant (**Table 5-4**).

Kakī

For kakī, the best model to predict binary hatching success was a univariate model of estimated days of parental incubation at swab, indicating a positive but non-significant correlation (effect ± SE = 1.794 ± 0.946, z = 1.896, p = 0.0579) (**Table 5-4**). Alongside the estimated days of parental incubation at swab, the top models also included the parameter of egg captive- or wild-laid and the interaction between these, with model-averaging again showing no significant relationships (**Table 5-4**). Finally, the best model to predict proportional hatching success per clutch for kakī was an additive model with clutch number and mean daily temperature between estimated clutch initiation and swab date, with neither of these parameters having a significant relationship with hatching success. The top models included these parameters with the addition of the estimated days of parental incubation at swab, but model-averaging indicated no significant relationships (**Table 5-4**).

Table 5-4. Summary of results from the best model and from model averaging of all models within $\Delta AIC_c \leq 2$ of the best model. Parameter estimates were calculated using the full-model averaging method. Dam ID was included as a random term in all models. All continuous variables were scaled and centred. SE = adjusted standard error; CI = confidence intervals; RI = relative importance. The relative importance is calculated as the sum of the Akaike weights of all models within $\Delta AIC_c \leq 2$ of the model with the lowest AIC_c containing this parameter. Variables in bold indicate significant p-values ($p < 0.05$) and where the 95% confidence interval did not overlap zero.

Explanatory variables	Estimate	95% CI		SE	RI	*z*	*p*
Hihi binary hatching success (*n* = 106)							
Best model: Shannon's diversity index + dam age²							
(Intercept)	**1.827**	**1.071**	**3.042**	**0.461**	**–**	**3.964**	**<0.001**
Shannon's diversity index	0.943	0.146	2.082	0.482	–	1.957	0.050*[3]*
Dam age²	**-0.820**	**-1.874**	**-0.123**	**0.399**	**–**	**-2.056**	**0.040**
Model averaging							
(Intercept)	**1.847**	**0.930**	**2.764**	**0.468**	**–**	**3.948**	**<0.001**
Shannon's diversity index	0.921	-0.056	1.898	0.498	1.00	1.849	0.065
Dam age²	-0.400	-1.358	0.558	0.489	0.51	0.819	0.413
Dam age	-0.397	-1.354	0.561	0.489	0.49	0.812	0.417
Dam age² : Shannon's diversity index	-0.084	-0.576	0.408	0.251	0.19	0.334	0.738
Dam age : Shannon's diversity index	-0.082	-0.557	0.394	0.243	0.19	0.336	0.737
Hihi proportional hatching success per clutch (*n* = 157)							
Best model: dam age							
(Intercept)	**1.595**	**1.050**	**2.288**	**0.300**	**–**	**5.324**	**<0.001**
Dam age	-0.551	-1.193	0.005	0.289	–	-1.909	0.056
Model averaging							
(Intercept)	**1.597**	**1.001**	**2.192**	**0.304**	**–**	**5.254**	**<0.001**
Dam age	-0.239	-0.892	0.415	0.333	0.43	0.717	0.474
Dam age²	-0.203	-0.819	0.414	0.314	0.38	0.644	0.519
Kakī binary hatching success (*n* = 85)							
Best model: est. days of incubation							
(Intercept)	**1.886**	**1.084**	**3.399**	**0.526**	**–**	**3.587**	**<0.001**
Est. days of incubation	1.794	0.449	4.394	0.946	–	1.896	0.058
Model averaging							
(Intercept)	**2.448**	**0.464**	**4.432**	**1.012**	**–**	**2.418**	**0.016**
Est. days of incubation	1.418	-0.715	3.550	1.088	1.00	1.303	0.193
Egg captive- or wild-laid	-0.693	-2.801	1.416	1.076	0.56	0.644	0.520
Est. days of incubation : egg captive- or wild-laid	0.275	-1.534	2.084	0.923	0.18	0.298	0.766
Kakī proportional hatching success per clutch (*n* = 85)							
Best model: clutch number + mean daily temp.							
(Intercept)	**1.590**	**0.525**	**2.971**	**0.550**	**–**	**2.891**	**0.004**
Clutch number	-0.385	-0.875	0.089	0.243	–	-1.582	0.114
Mean daily temp.	-0.543	-1.294	0.039	0.327	–	-1.661	0.097
Model averaging							
(Intercept)	**1.569**	**0.594**	**2.545**	**0.498**	**–**	**3.153**	**0.002**
Clutch number	-0.225	-0.766	0.315	0.276	0.54	0.817	0.414
Mean daily temp.	-0.368	-1.115	0.380	0.382	0.64	0.963	0.335
Est. days of incubation	0.084	-0.348	0.516	0.220	0.17	0.381	0.703

Discussion

The relationship between eggshell microbiome and hatching failure in the eggs of free-living and captive threatened species has so far lacked detailed investigation, and is explored here in hihi and kakī for the first time. The results of this study showed no significant differences in alpha or beta diversity of the

eggshell microbiomes of eggs that went on to hatch and eggs that went on to experience hatching failure for either species. However, a small number of taxa were found to be more abundant in the eggshell microbiomes of hihi eggs that went on to hatch in comparison to eggs which failed to hatch. Results also indicated that hihi eggs laid by older females were significantly less likely to hatch, and while no factors related to the eggshell microbiome were found to significantly interact with hatching success, a positive relationship between Shannon's diversity index and hatching success of hihi eggs was close to significance. Overall, although disease has been suggested to be one of the main drivers of hatching failure in non-commercial avian species, my results show that either this is not the case in these species, or that the eggshell microbial species composition does not reflect the risk of trans-shell infection.

How does the eggshell microbiome differ between hatched and unhatched eggs?

The eggshell microbiome of hatched and unhatched eggs from both hihi and kakī did not differ in either alpha (within-sample richness and evenness) or beta (between-sample dissimilarity) diversity. The relative abundances of taxa at genus, family, and phylum level were also similar, with only a small number of taxa being identified as being more abundant in the eggshell microbiomes of hatched *versus* unhatched hihi eggs. While two studies of sea turtles found differences between the eggshell microbiomes of hatched and unhatched eggs, with *Pseudomonas* being a key genus associated with unhatched eggs (McMaken, 2022; Capri *et al.*, 2023), there does not appear to be any studies which have directly compared the eggshell microbiome composition of hatched and unhatched avian eggs. One reason for the lack of comparisons of microbiome composition between hatched and unhatched eggs may be that the presence of certain bacteria inside or on the eggshell of an unhatched egg cannot necessarily be interpreted as being the cause of hatching failure, as trans-shell infection by opportunistic bacteria can also occur after embryo mortality (Soler *et al.*, 2011b). To avoid this, during this study eggs were sampled during incubation rather than after they had hatched or failed to hatch, with the samples then related to the egg's fate at the end of incubation.

Given the findings from sea turtle studies of an association between unhatched eggs and *Pseudomonas*, it is interesting that this was one of the most abundant genera on both hihi and kakī eggshells, with a particularly high abundance on kakī. *Pseudomonas* is a common and species-rich genus, including a number of saprophytic species, and is recognised as a common avian pathogen (Benskin *et al.*, 2009). It is part of the family Pseudomonadaceae, which has been found on the eggshells of a number of bird species (Ruiz-de-Castañeda *et al.*, 2011b; Lee *et al.*, 2014; Peralta-Sánchez *et al.*, 2018), and may be influential in hatching success as members of the family have the potential to degrade the cuticle of eggshells and increase the number of unplugged pores (Ruiz-de-Castañeda *et al.*, 2011b), facilitating trans-shell infection. Consequently, the family has been described as the most common egg-spoilage microorganism in poultry (Dockstater, 1952; Florian & Trussell, 1957). However, comparisons between hatched and unhatched eggs did not show any differences in the abundance of either Pseudomonadaceae or *Pseudomonas* in either hihi or kakī. This appears to be consistent with the findings of Ruiz-de-Castañeda *et al.* (2011b), who identified Pseudomonadaceae and *Pseudomonas* as the most common family and genus on eggshells and in cloacae of female pied flycatchers (*Ficedula hypoleuca*), but with no effect of Pseudomonadaceae (or *Pseudomonas luteola*) on hatching success detected.

It is possible that Pseudomonadaceae may not be as detrimental to the eggs of free-living bird species as they have been shown to be in poultry, with conditions related to the commercial hatchery process potentially playing a role in the activity of pathogenic bacteria. However, another explanation is that eggs examined here were only sampled at one timepoint, and therefore it was not possible to capture changes in microbiome composition related to hatching success which arose after the point of sampling. Ideally, eggs would have been subject to repeated sampling throughout incubation and at the point of hatching or failure to hatch, but in threatened bird populations interventions and disturbance are typically minimised, hence this was not possible for these study species. However, hihi eggs were sampled near to the end of incubation (day 13 in a 14-15 day incubation period (Ewen *et al.*, 2018)) so it might be expected that the microbiome of eggshells at this point was not dissimilar to that at the point of either hatching or failure. The relative abundance of *Pseudomonas* in

hihi eggshell microbiomes was much lower than in kakī samples, which were sampled at different timepoints, including when no parental incubation had taken place. This may align with a study by Lee *et al.* (2014) showing that the relative abundance of *Pseudomonas* on Eurasian Magpie (*Pica pica*) eggshells significantly decreased after incubation. This is further supported by the finding of a significant negative correlation between the estimated days of parental incubation at swab and the relative abundance of *Pseudomonas* in kakī eggshell microbiome samples (**Table D1**, **Appendix D)**. Given its potential as a key pathogen causing embryo mortality, this putative relationship between relative abundance of *Pseudomonas,* the bacteriostatic effects of incubation, and the underlying mechanisms warrant a more direct investigation.

Several genera from the Neisseriaceae family and at least 2 *Neisseria* genera were identified in the hihi and kakī eggshell samples, but there was no evidence that any of these were differentially abundant between eggs that went on to fail or hatch successfully. A study on greater white-fronted goose (*Anser albifrons*) identified a *Neisseria* species as the predominant bacterium present in addled eggs, and found that this bacterium was widespread within the nest environment and on eggshell samples (Hansen *et al.*, 2015). However, not all eggs associated with *Neisseria*-contaminated nests were addled, indicating trans-shell infection by *Neisseria* when it is present is not ubiquitous. A number of other genera which have been identified as bacterial pathogens in birds (Benskin *et al.*, 2009) were either not found in any of the samples (e.g. *Salmonella*, *Klebsiella*, *Enterobacter*), or were found but did not appear to be differentially abundant between hatched and unhatched eggs (e.g. *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Campylobacter*, *Clostridium*, *Mycoplasma, Escherichia*). As *Salmonella* in particular has previously had negative effects on the adult hihi population on Tiritiri Matangi (Ewen *et al.*, 2007; Thorogood *et al.*, 2013; van Andel *et al.*, 2015), its absence on eggshells could be seen as a successful outcome of disease risk management protocols (Ewen *et al.*, 2018).

Differential abundance testing using DESeq2 identified a *Haemophilus* sp., the genera *Rhodococcus, Haemophilus, Actinomyces*, and *Sphingobium*, and the families Nocardiaceae and Chitinophagaceae as being significantly more abundant in the eggshell microbiomes of hihi eggs that went on to hatch than

those that went on to experience hatching failure. *Rhodococcus* has previously been identified on avian eggshells, and has been found to be specific to the late incubation stage (Potter *et al.*, 2013, 2014). All of the genera found to be differentially abundant are known to include pathogens (Rimler, 1979; Bell *et al.*, 1998; Ryan & Adley, 2010; Gajdács & Urbán, 2020), while *Haemophilus* and *Actinomyces* are also known to include some commensal organisms (Bell *et al.*, 1998; Steininger & Willinger, 2016) which could potentially be involved in the formation of a beneficial eggshell microbiome preventing trans-shell infection and subsequent hatching failure. Without classification to species level it is difficult to draw conclusions about the function of the elevated abundance of these taxa. Also, as DESeq2 has been shown to have compositionality-related assumptions which may make it less appropriate for microbiome data, potentially leading to more false positives, putatively differentially abundant taxa which are only supported by the DESeq2 method may be less reliable than those with support also from ANCOM (Gloor *et al.*, 2017; Weiss *et al.*, 2017). Further research comparing hatched and unhatched eggshell microbiomes across these and other species may yield results to verify and elucidate these relationships.

Does eggshell microbiome diversity or composition predict hatching success?

Along with a lack of differences between the eggshell microbiomes of hatched and unhatched eggs in terms of their alpha and beta diversity, no variables related to either eggshell microbiome alpha diversity or composition were found to be significant predictors of hatching for either hihi or kakī. One explanation is that trans-shell infection is not a major driver of hatching failure in these populations. The hihi population studied here has experienced multiple population bottlenecks, both historically, when they were reduced to a single remnant population following the arrival of Europeans to Aotearoa New Zealand, and recently, following translocations to Tiritiri Matangi Island (Brekke *et al.*, 2011; Duntsch *et al.*, 2023). There is evidence that the resulting inbreeding has led to inbreeding depression, which has previously been linked to embryo mortality (Brekke *et al.*, 2010) and negative effects on other components of reproduction (Duntsch *et al.*, 2023). Kakī have also experienced a recent severe bottleneck, being reduced to just 23 adult birds in 1981

(Maloney & Murray, 2001), with evidence of a significant negative relationship between inbreeding and fitness in the captive kakī population (Hagen *et al.*, 2011), although perhaps not across the whole population (Steeves *et al.*, 2010), and while inbreeding coefficient was found to be a significant predictor of kakī survivability in a recent analysis, it did not appear to influence hatchability (Bourke *et al.*, 2023).

Multiple studies have found a link between dam age and hatching success in hihi (Low *et al.*, 2007; Low & Pärt, 2009; de Villemereuil *et al.*, 2019b, 2019a; Mather *et al.*, 2020), which was supported by the finding here that dam age has a significant effect on whether or not an egg goes on to hatch. Previous work investigating the factors driving differences in the eggshell microbiome (**Chapter 4**) did not find any evidence of a relationship between dam age and eggshell microbiome diversity in hihi, and I did not find evidence that the interaction between these factors was significant in my models, indicating that aspects of female age other than any link to probability of trans-shell infection may be responsible for differences in hatching success.

Further evidence that trans-shell infection may not be major driver of hatching failure in hihi comes from research suggesting that eggs in cavity nests may suffer from lower levels of trans-shell infection and resulting embryo mortality than eggs in open-nests (Godard *et al.*, 2007), and as hihi are a cavity-nesting species this could explain the absence of an effect of eggshell microbiome on hatching success. However, Peralta-Sánchez *et al.* (2012) found a contrasting result of higher probability of microbial colonisation of eggshells in cavity nests compared to open nests. Furthermore, artificial nestboxes, which this hihi population uses almost exclusively, may provide a favourable environment for microorganisms (Baggott & Graeme-Cook, 2002; Goodenough & Hart, 2011; González-Braojos *et al.*, 2012; Devaynes *et al.*, 2018), and have been shown to contain higher loads of ectoparasites (Wesolowski & Stanska, 2001; Hebda *et al.*, 2017) than natural nests, with ectoparasite presence itself linked to higher eggshell bacterial loads (Tomás *et al.*, 2018). This is one possible explanation for populations provided with artificial nests experiencing higher rates of hatching failure than those without (Marshall *et al.*, 2023). However, on Tiritiri Matangi Island considerable attention is paid to the maintenance of artificial nestboxes (Ewen *et al.*, 2018), and this is likely to reduce the risk of infection

and should likely be adopted, where possible, as best practice in other nestbox-dependent populations of threatened species.

Aspects of current management may also help in limiting the incidence of trans-shell infection in kakī eggs. While almost every egg in this population is collected and hatched in captivity using artificial incubation, with artificial incubation generally being linked to reduced hatching success (Marshall *et al.*, 2023), this process is carefully monitored and great care is taken towards hygiene practices which could help reduce the risk to eggs from trans-shell infection. The use of disinfectant foot baths and antibacterial hand sanitiser by practitioners is standard practice, and all equipment is disinfected before and regularly during the breeding season (L. Brown, *pers. comm.*). Eggs from different clutches are kept separated from each other, and as cracks are a key route for trans-shell infection any eggs with minor damage when collected from nests are gently cleaned and sealed with clear nail varnish or micropore tape before being placed into artificial incubators (Brown *et al.*, 2020, 2021, 2022), as sealing cracks as soon as possible may be effective to reduce penetration of eggs by pathogens (Perić *et al.*, 2022). Many of these practices have been adapted from procedures in commercial hatcheries, aptly demonstrating how knowledge from poultry research can be used to inform conservation strategies for threatened species.

Bird populations in captivity generally experience higher rates of hatching failure than wild populations (Marshall *et al.*, 2023), and in kakī, hatchability (hatching success of ‘known fertile’ eggs) has previously been linked to whether an egg is captive- or wild-laid (van Heezik *et al.*, 2005), and to a number of parental characteristics such as clutch number, clutch size, dam age, and timing of collection for artificial incubation (van Heezik *et al.*, 2005; Bourke *et al.*, 2023). While clutch number, estimated days of parental incubation at swab (equivalent to the estimation of embryo age at collection for artificial incubation), and whether an egg was captive- or wild-laid were all found to be parameters in the top models in this study, which may seem to be consistent with this previous research, none had significant effects on hatching success. While this could be a consequence of analysing data from a single breeding season in this study, an aspect of the kakī population which should be considered is that hatching success has been seen to be gradually improving over time (Brown *et al.*,

2022), and while likely still a limiting factor in population recovery it may be the case that more could be gained in regards to conservation by focusing on other stages of their life history (Bourke *et al.*, 2023).

Conclusions and future directions

The field of avian eggshell microbiome research, and research into animal microbiomes in general, is quickly expanding and revealing previously unanticipated insights into many aspects of animal lives, including reproduction. The applications to conservation have so far been relatively limited, but this study has further demonstrated the feasibility of employing molecular methods to study the microbiomes of even highly threatened, free-living species, and has highlighted some of the potential questions which could be answered with such research. Detailed, long-term datasets such as those available for both hihi and kakī are invaluable both for conservation and for our understanding of many fundamental aspects of avian ecology, reproduction, and life histories. Microbiomes are another piece of the puzzle which can both help to answer ongoing questions, and open avenues for new areas of investigation, hence conducting this kind of study on the most vulnerable species may be essential. Future studies should focus on finding ways to implement repeated, or otherwise longitudinal, testing of the avian eggshell microbiome during incubation in relation to hatching success, which would help to expand our understanding of this relationship.

Chapter 6: Discussion

Throughout my PhD, I have focused on improving our understanding of avian hatching failure and examining some of the factors behind the often elevated rates of hatching failure experienced by managed populations and populations of threatened species. In this final chapter, I summarise my key findings and their potential implications for conservation, and discuss how they fit into the general body of knowledge in this field. I also identify some of the remaining questions which I could not address within the scope of my thesis and explore the opportunities for further research.

Hatching failure is higher in threatened and managed bird populations

Given that around half of bird species worldwide have declining populations (BirdLife International, 2022a), and the growing importance of conservation management and *ex situ* populations in the face of the escalating threats posed to many species by climate change, habitat loss, and invasive species, it appeared timely to conduct a systematic review and meta-analysis of the influence of threat status and management on hatching failure in **Chapter 2** (Marshall *et al.*, 2023). When multiple alternative management strategies are available for a struggling population, it is important that conservation practitioners are able to compare their potential impacts accurately and systematically (e.g. Ruiz *et al.*, 2021), following the principles of evidence-based conservation (Sutherland *et al.*, 2004, 2021). While post-hatch characteristics such as fledging and recruitment success are commonly used for such assessments, incorporating accurate measures of hatching failure for different species in the wild and in captivity, and having an appreciation of how various interventions may impact hatching rates, as outlined in **Chapter 2**, could add another dimension to decision-making.

While the meta-analysis in **Chapter 2** uncovered several moderators that influence the rate of hatching failure across populations, a significant amount of heterogeneity remained unexplained. This indicates that other factors influencing hatching failure were not included in the model. As phylogenetic history accounted for a large proportion of the overall heterogeneity, and as

more closely related species tend to share similar life history traits, this could indicate that certain traits influence hatching failure in different ways. These traits may also interact with the applicability or efficacy of different management interventions. Future research could build upon the results of **Chapter 2** by conducting a separate meta-analysis using the same dataset but with a focus on the effects of phylogeny, life history traits, and geographical variables to investigate these potential additional drivers. Identification of significant trends and patterns could help to fill current knowledge gaps surrounding the intrinsic causes of hatching failure, and is likely to have implications for future studies of birds within the fields of conservation biology, behavioural ecology, population biology, and evolutionary ecology. Further, it has been shown that threatened species are less likely to be held in zoos than non-threatened species, possibly due to the difficulty of breeding some species in captivity (Conde *et al.*, 2013; Martin *et al.*, 2014; Biega *et al.*, 2019). Improving our understanding of the relationships between hatching failure, life history traits, threat status, and management may enable captive-breeding practices to be applied to taxa where this has not previously been feasible, hence providing more options to protect some of the most vulnerable species from extinction.

Many of the gaps in our understanding of the underlying causes, drivers, and mechanisms of hatching failure, and issues with how it is assessed in managed bird populations, also apply to other taxa (Grueber *et al.*, 2015; Kaumanns *et al.*, 2020). In fact, as birds are one of the most well-studied taxa, it is likely that substantially less is known about early reproductive stage losses in relatively understudied taxa that are also managed in captivity such as amphibians (Troudet *et al.*, 2017; Womack *et al.*, 2022). While some of the lessons learned from the systematic review of avian hatching failure in **Chapter 2** may be transferable to such other taxonomic groups, it may be prudent for a similar analysis to be conducted in other taxa to explore any associations between threat status, management, and hatching failure.

Hatching failure lacks a consistent definition

The lack of consistency in hatching failure terminology identified in **Chapter 2**, combined with the frequent absence of stated definitions for terms used, or description of the relevant data collection, resulted in the exclusion of many

studies identified in the literature search from the meta-analysis, restricting its scale. The definition used throughout my thesis – i.e. hatching failure is the proportion of eggs present at the end of the incubation period that fail to hatch, excluding eggs lost due to predation, desertion, accident, extreme weather, or that disappeared during the incubation period due to unknown factors – removes any uncertainty surrounding which eggs to include in calculations, and is consistent with the definition used by earlier comparative analyses (Koenig, 1982; Morrow *et al.*, 2002; Briskie & Mackintosh, 2004; Spottiswoode & Møller, 2004; Heber & Briskie, 2010; Møller *et al.*, 2010; Soler *et al.*, 2012a; Reding, 2015). If this was to become the standardised definition of hatching failure it would facilitate better comparison of hatching failure across multiple studies, allowing for more robust comparative analyses in future. A standardised definition would also help to ensure that studies are reporting the most accurate hatching failure information from the population, which can be used to draw meaningful conclusions and apply appropriate management interventions.

Fertilisation failure is often overestimated in managed populations

As fertilisation failure and embryo mortality are likely to be driven by different factors, accurate differentiation between them can help to prevent misinterpretation of the pertinent reproductive problems in a population, as I demonstrated in **Chapter 3**. The finding that fertilisation failure likely plays a relatively minor role in determining hatching outcomes in birds suggests that the efforts of conservation programmes aiming to improve hatching success should mainly be directed towards actions to reduce embryo mortality, such as improving parental diet and managing genetic diversity. As incubation issues may play a critical role in embryo mortality, conservation managers could also consider the implementation of artificial incubation to eggs from pairs with recurrent hatching failure as this may indicate a problem with their natural incubation behaviour. However, given that populations in which eggs are artificially incubated have significantly higher hatching failure than populations where eggs are naturally incubated (**Chapter 2**; Marshall *et al.*, 2023), practitioners may only decide to implement this when other options have already been explored, or when there is supporting evidence from behavioural observations that there are issues in parental incubation. It is also important that practitioners consider that the optimum incubation parameters and required

weight loss during incubation likely vary between different species (Kuehler, 1993; Klimstra *et al.*, 2009), and seek out any prior knowledge of the parameters resulting in successful hatching for the species in question. A schematic diagram illustrating the steps a conservation practitioner may take in response to an unhatched egg is included in **Figure 6-1**.

The results of **Chapter 3** have already been used to support breeding decisions within the captive ZSL bird populations. Following dissemination of this work and the work of my collaborators via publications (including (Assersohn *et al.*, 2021b), a narrative review of hatching failure on which I was co-lead author), conference presentations, and social media, a number of other conservation programmes have expressed interest in applying fluorescence microscopy methods within their managed bird, reptile, and amphibian populations. While conducting fieldwork in Aotearoa New Zealand I was able to examine a small number of unhatched eggs from Endangered tuturuatu (shore plover; *Thinornis novaeseelandiae*) and Critically Endangered kākāriki karaka (orange-fronted parakeet; *Cyanoramphus malherbi*) using these methods, demonstrating their effectiveness and utility to conservation managers (**Table B14**, **Appendix B**). There are plans for collaborators to further apply these methods within tuturuatu and kākāriki karaka, as well within the Vulnerable black robin (*Petroica traversi*) population in the Chatham Islands. There are also projects in the early stages of development aiming to apply the fluorescence microscopy methods to the Endangered Mauritius kestrel (*Falco punctatus*), and potentially other species endemic to Mauritius. These plans have been facilitated by step-by-step protocols and video demonstrations of the methods available online as part of the supplementary material of Assersohn *et al.* (2021b), and through direct training conducted in-person and online by myself and my supervisory team.

Expanding accurate methods of differentiating between fertilisation failure and embryo mortality to more managed, threatened species will enable comparisons between species with different breeding and mating strategies, helping to identify any interactions between management, breeding behaviour, and fertilisation success. In particular, as several of the proposed projects include species with both captive and free-living populations, their results could help us ascertain whether captive-laid eggs experience elevated rates of fertilisation failure compared to wild-laid eggs (Hemmings *et al.*, 2012b).

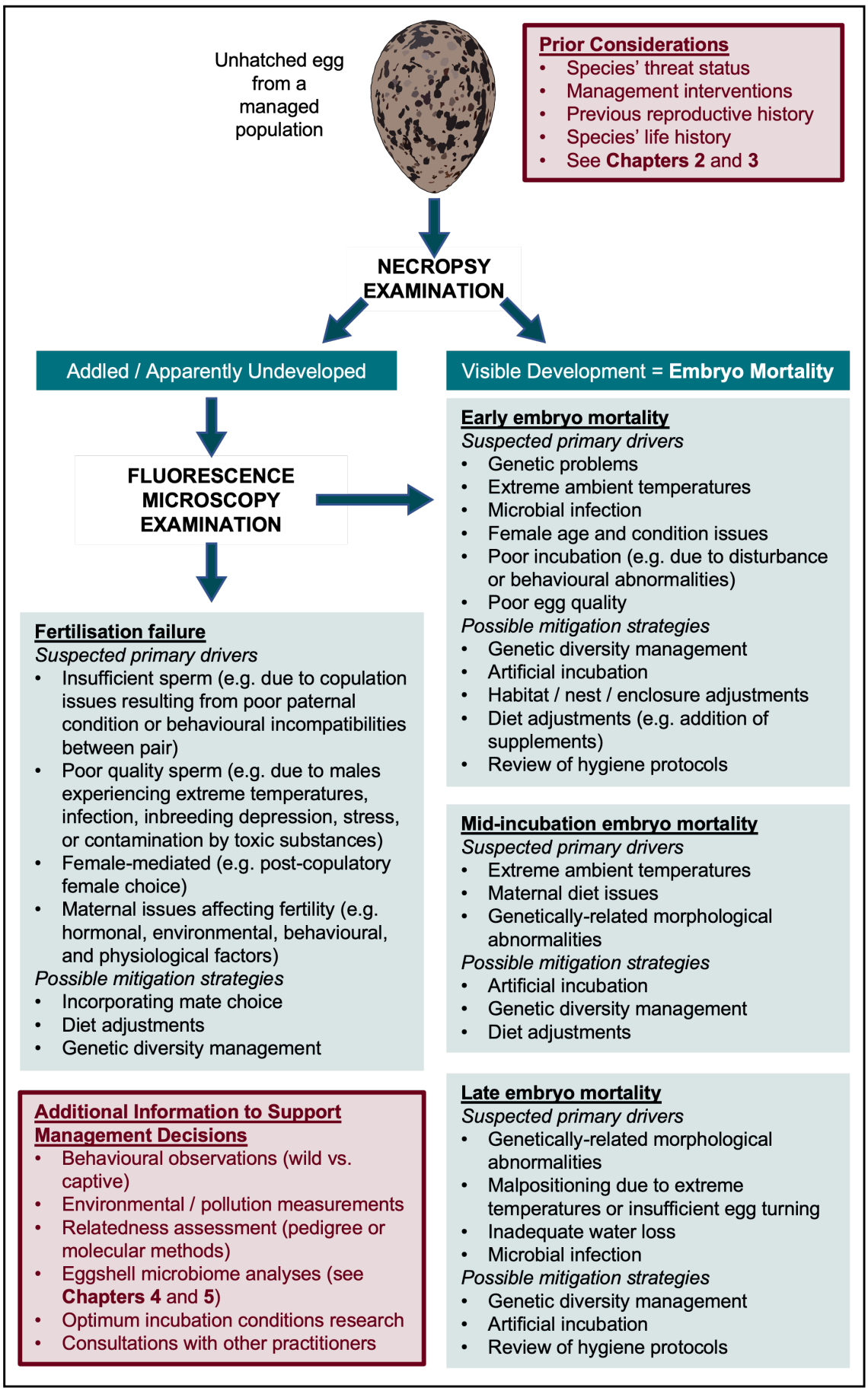

Figure 6-1. Schematic diagram illustrating the steps a conservation practitioner may take in response to an unhatched egg from a managed population.

Hatching failure rates do not differ between wild- and captive-laid kakī eggs

The finding of no difference in hatching failure rates between wild- and captive-laid kakī eggs in **Chapter 3**, in contradiction to the findings of **Chapter 2**, may be explained by adjustments to captive management following the findings of an earlier study by van Heezik *et al.* (2005), closing the gap between captive- and wild-laid egg quality and highlighting the value of using such studies to inform management. Alternatively, as both wild- and captive-laid eggs are artificially incubated, any differences in parental incubation or care between wild and captive parents which could be responsible for driving differences in hatching failure are eliminated. While differences in parental incubation behaviour between wild and captive breeding pairs of the same species have not received much attention in the published literature, there is some evidence of changes in incubation behaviour and hatching synchrony as a result of captivity in zebra finches (*Taeniopygia guttata*) (Gilby *et al.*, 2013). As changes in incubation behaviour due to captivity could be an underappreciated driver of higher hatching failure rates in captive populations, it may be useful to investigate this further. Although behavioural observations may be easier to achieve in captive populations, the increasing accessibility and affordability of remote nest cameras can also facilitate effective monitoring of behaviour in the wild (Caravaggi *et al.*, 2017; Hereward *et al.*, 2021).

Captivity has a significant effect on kakī eggshell microbiomes

Despite the growing appreciation of the importance of animal microbiomes in relation to reproduction, the eggshell microbiome and its role in hatching failure remains poorly understood in non-commercial species. The characterisation of the eggshell microbiomes of hihi and kakī in **Chapter 4** not only represents the first time the eggshell microbiome of these species have been investigated, but also represents one of the few examinations of the eggshell microbiomes of threatened species, and the first analysis of the effects of captivity.

The finding of significant differences in eggshell microbiome diversity between captive- and wild-laid kakī eggs is consistent with findings of an effect of captivity on other avian microbiomes (Xenoulis *et al.*, 2010; San Juan *et al.*,

2020; Alba *et al.*, 2022; Grieves *et al.*, 2022; Kelly *et al.*, 2022; West *et al.*, 2022). While these differences are likely to be driven by the different living conditions of captive and wild individuals, as well as by differential exposure to cleaning products and human microbiomes, the elements responsible for the differences are poorly understood. Additionally, we likely do not yet fully appreciate the potential consequences for survival, fitness, and reproductive success of any changes in the microbiomes of captive individuals, and whether this should be considered an adaptation to captivity and therefore limited as much as possible. Overall, this adds more support towards calls for microbiomes to be researched further and considered as part of threatened species management (Xie *et al.*, 2016; Trevelline *et al.*, 2019; Becker *et al.*, 2020; Oliveira *et al.*, 2020; Comizzoli *et al.*, 2021; Dallas & Warne, 2022; Matheen *et al.*, 2022; West *et al.*, 2022).

Many variables influencing the eggshell microbiome are unknown

Microbiomes are known to be incredibly complex, typically made up of an extremely high number of different bacterial and fungal species and influenced by many different independent and interacting factors related to the both the host and the environment (Berg *et al.*, 2020). The characterisation and analyses of hihi and kakī eggshell microbiomes in **Chapter 4** revealed that captivity, parental breeding experience, social sire age, and environmental variables including rainfall, temperature, and nest location drove differences in the microbiome diversity or composition. However, as these variables only accounted for a small proportion of the variation between samples, it is expected that other factors play a role in shaping the eggshell microbiome. Some factors which could not be explored within the scope of this study include the maternal diet, the condition of the parents, the nest materials included in the nest, the presence or absence of ectoparasites, and the nest microclimate, all of which could potentially be included in future studies of these species.

As the studies in **Chapter 4** and **Chapter 5** were restricted to samples obtained within a single breeding season, it was not possible to explore any potential inter-season differences, or to identify any patterns in the eggshell microbiome of eggs laid by females over sequential seasons. Studies conducted over multiple years can not only help to identify trends within individuals, but can also

be used to monitor changes in a population over time. Long-term studies can provide critical information on a population's ability to adapt to changes in their surroundings, including those driven by anthropogenic impacts such as habitat loss, climate change, and pollution. Given that many species are increasingly threatened by anthropogenic changes to their habitats, consideration of how these may impact the eggshell microbiome and hatching failure could be instrumental for future conservation planning.

Parental incubation influences the eggshell microbiome and hatching success

The finding in **Chapter 4** that kakī eggs receiving a longer period of parental incubation had a more diverse and even eggshell microbiome fits with the theory that parental incubation protects against trans-shell infection by promoting the relative abundance of harmless or antibiotic-producing bacteria and suppressing the growth of pathogenic bacteria (Shawkey *et al.*, 2009; Brandl *et al.*, 2014; Giraudeau *et al.*, 2014; Grizard *et al.*, 2014; Lee *et al.*, 2014, 2017; Song *et al.*, 2022). The acquisition of a beneficial microbiome from natural incubation could be one explanation for the finding in **Chapter 3** that kakī eggs containing older embryos at the time of collection for artificial incubation, i.e. that have experienced a longer period of parental incubation, were more likely to hatch than eggs collected sooner after laying, which is also consistent with findings from other studies (Burnham, 1983; Elliott *et al.*, 2001; Robertson *et al.*, 2006; Smith *et al.*, 2011; Hoeck *et al.*, 2015; Bourke *et al.*, 2023). While delaying the collection of eggs until partially though the incubation period may therefore reduce hatching failure, this conflicts with current management protocols recommending that kakī eggs are collected as soon as possible after laying due to the high level of risk posed by predators and flooding of nests. However, an improved understanding of how parental incubation, the eggshell microbiome, and hatching failure interact could potentially lead to the development of methods of recreating the bacteriostatic effect of parental incubation as part of artificial incubation, eliminating the need to leave eggs in nests where they may be in danger.

Critical to improving our understanding of the interactions between parental incubation and the eggshell microbiome is further investigation into how the

bacterial community composition on eggshell changes over time. I was only able to sample eggs at one timepoint, but by repeating sampling of eggs at multiple points during incubation in future studies it would be possible to identify any longitudinal trends in the eggshell microbiome composition, and in particular to identify if any taxa, whether beneficial or potentially pathogenic, are being promoted or suppressed by the mechanisms of parental incubation.

These mechanisms behind parental incubation's role in controlling microbial infection are so far unconfirmed. While the reduction of water on the egg surface has been hypothesised to be the main method by which parental incubation limits bacterial growth (Godard *et al.*, 2007; D'Alba *et al.*, 2010; Ruiz-de-Castañeda *et al.*, 2011a), another proposed mechanism is the inoculation of eggshells using antimicrobial compounds of specific waxes and fatty acids found in feathers (Sweeney *et al.*, 2004), the uropygial gland (Soler *et al.*, 2008; Moreno-Rueda, 2017) and the brood patch's epidermal layer (Menon & Menon, 2000). Also unclear is whether the eggshell microbiome originates via vertical transmission (Ruiz-de-Castañeda et al., 2011a, 2011b; Grizard et al., 2014), horizontal uptake (Godard et al., 2007; Martínez-García et al., 2016; van Veelen et al., 2018; Song et al., 2022), or a mixture of both. The findings of **Chapter 4** were generally ambiguous regarding this question. Incorporating samples of uropygial gland secretions and nest materials, as well as swabs of female and male cloacae, body feathers, and brood patch skin, alongside eggshell swabs in future studies could be key to answering many of the remaining questions around the mechanisms controlling bacterial growth on eggshell and the source of the eggshell microbiome.

Trans-shell infection is likely not a major threat to reproductive success in hihi and kakī

The control of disease and infection is often a top priority within managed populations, and particularly in captive-breeding scenarios where birds are typically kept in close proximity to each other and potentially to other species. Microbial infection of eggs is one of the most commonly suspected drivers of hatching failure in birds, but the findings of **Chapter 5** indicated that trans-shell infection may not be a major threat to the reproductive success of hihi and kakī. One explanation for this is that both the hihi and kakī conservation programmes

implement stringent hygiene practices to control disease, demonstrating the importance of such protocols to control infection in eggs, as well as reducing levels of disease in chicks, fledglings, and adults. Based on these findings, efforts to reduce hatching failure in these species should therefore be directed towards the other putative drivers, with inbreeding depression and maternal age suspected to be more prominent drivers of hatching failure in hihi, while the findings of **Chapter 3** indicated clutch size, egg weight, and length of parental incubation prior to collection for artificial incubation to be related to hatching failure in kakī.

The work in **Chapter 4** and **Chapter 5** has demonstrated the feasibility of applying molecular methods to analyse the eggshell microbiomes of highly threatened, free-living bird populations, opening the door to implementing similar methods across other managed threatened populations, some of which may experience a greater level of trans-shell infection. Expanding our understanding of the eggshell microbiomes of threatened, non-commercial species will help us answer some of the outstanding questions around the most influential drivers of eggshell microbiome diversity and composition, and the role of the microbiome in trans-shell infection and subsequent embryo mortality.

Concluding remarks

In this thesis, I have examined hatching failure both broadly and in-depth, and have demonstrated how an improved understanding of hatching failure in threatened bird species can be beneficial in their conservation management. I have been able to disseminate my research to diverse audiences within academia and beyond, and work directly with conservation practitioners to use my results to support real world conservation decisions, an aspect which was of great importance to me when undertaking a PhD. I hope that this research will continue to be of use to conservationists working diligently to preserve and restore threatened species, and ultimately bring the species most vulnerable to extinction “back from the brink”.

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Appendix A: Supplementary information for Chapter 2

Appendix A.1. Summary of previous comparative analyses

Table A1. Summary of previous comparative analyses of hatching success or failure across avian species. Hatching failure rates are as reported in the main text of the cited paper unless otherwise stated. The reported number of species are the species for which there is an associated hatching failure rate in the paper. Species were classified as threatened [Critically Endangered (CR), Endangered (EN), Vulnerable (VU)] or non-threatened [Least Concern (LC), Near Threatened (NT)] based on each species' current global IUCN Red List Classification (IUCN, 2021).

Reference and definition used	Data sources	Data description	Hatching failure rate	
			Range	Mean ± S.E.
Koenig (1982)* **Hatchability**: percentage of eggs surviving to the time of hatching that produce a chick, thus excluding eggs lost to predation, abandonment, accidental breakage, or any other unknown factor.	Literature search and author's own field data	113 species (threat status unclear) Natural populations	–	9.4%
Morrow *et al*. (2002)* **Hatching failure**: proportion of eggs that failed to hatch relative to the total number of eggs laid, but excluding those eggs lost to predation or desertion of the nest.	Electronic databases of published literature and directly contacting authors	64 species (6 threatened, 58 non-threatened) Natural populations	0–39%	12.35 ± 1.07%
Briskie & Mackintosh (2004)* **Hatching failure**: proportion of eggs in a nest that failed to hatch relative to the number present at hatching. Failed eggs include both unfertilised eggs as well as those that died as embryos. This measure excluded eggs that failed because of predation, desertion, or abiotic factors such as windstorms or floods.	Literature, personal communication with researchers, the New Zealand (NZ) Nest Record Scheme, authors' own field data	22 NZ native species (17 threatened, 5 non-threatened) Free-living populations	*Non-bottlenecked population*	
			0.3–7.0%	3.0 ± 0.6%
			Bottlenecked <150	
			2.4–64.6%	25.3 ± 5.0%
			Bottlenecked 300–5,500	
			0.1–12.5%	3.7 ± 1.2%
		15 introduced species (all non-threatened) Free-living populations	*Bottlenecked <150*	
			7.5–34.4%	21.6 ± 5.6%
			Bottlenecked >150	
			2.4–10.3%	4.9 ± 0.7%
Spottiswoode & Møller (2004) **Hatching success**: proportion of eggs incubated to term that hatched, thus excluding hatching failure owing to nest desertion and nest predation.	Literature search and author confirmation	99 species (5 threatened, 94 non-threatened) Free-living populations	0–55%	10.9 ± 0.86%
Heber & Briskie (2010) **Hatching failure**: proportion of eggs incubated to term that failed to hatch, excluding failure due to desertion, predation, or adverse weather. Under this definition, eggs that failed to	Literature, personal communication with researchers, and from authors' own field data	51 species (41 threatened, 10 non-threatened) Population type not stated	0.0–63.9%	23.51%†

hatch were either infertile or died during embryonic development.				
Møller *et al*. (2010) **Hatching success**: proportion of eggs incubated to term that hatched, thus excluding hatching failure owing to nest desertion and nest predation.	Spottiswoode & Møller (2004), Cramp & Simmons (1977, 1980, 1983), Cramp (1985, 1988, 1992) and Cramp & Perrins (1993, 1994a, 1994b)	74 species (6 threatened, 68 non-threatened) Natural populations	0.6–33.1%	8.27%†
Galván & Sanz (2011) **Hatching success**: definition not stated.	Cramp (1988, 1992) and Cramp & Perrins (1993, 1994a, 1994b)	90 species (1 threatened, 89 non-threatened) Natural populations	2–56.3%	25.4%†
Soler *et al*. (2012a) **Hatching success**: proportion of eggs incubated to term that hatched, thus excluding hatching failure owing to nest desertion and nest predation.	Møller *et al*. (2010)	18 species (all non-threatened) Natural populations	0.6–20.2%	7.25%†
Reding (2015) **Hatching success**: proportion of eggs laid that were incubated to hatching excluding nest predation and nest desertion.	Spottiswoode & Møller (2004) and Morrow *et al*. (2002)	113 species (8 threatened, 105 non-threatened) Natural populations	0–55%	11.2%†
Johnsen (2019) **Hatchability**: proportion of eggs hatching of those present in the nest at the end of incubation (Larsen *et al*., 2003). Eggs that were depredated, accidentally destroyed, or abandoned during incubation are excluded.	Spottiswoode & Møller (2004), Morrow *et al*. (2002), Møller *et al*. (2010), Hansen *et al*. (2011), Croxall *et al*. (1992), and The University Museum of Bergen nest card archive	174 species (15 threatened, 159 non-threatened) Natural and free-living populations	0–55%	10.4%†

* Dataset not publicly accessible.
† Calculated from data provided in text or in Supporting Information.

Deeming (2003) conducted a comparative review of hatchability across 231 species using data collected from Cramp & Simmons (1977, 1980, 1983), Cramp (1985, 1988, 1992) and Cramp & Perrins (1993, 1994a, 1994b) and found a mean hatching failure rate of 28.89 ± 0.93% (0–75.7%). However, egg losses were stated to be attributed to reasons including nest desertion, predation, and direct intervention (Deeming, 2003), therefore, this study was excluded from **Table A1**.

Table A2. Summary of variables investigated in previous comparative analyses of hatching success (HS%) or failure across avian species and the significance of their effects.

	Reference and focus of study								
Variable	**Koenig (1982)**	**Morrow *et al.* (2002)**	**Briskie & Mackintosh (2004)**	**Spottiswoode & Møller (2004)**	**Heber & Briskie (2010)**	**Møller *et al.* (2010)**	**Soler *et al.*** (2012a)	**Reding (2015)**	**Johnsen (2019)**
	Effects of different variables on HS%	*Coevolution of hatching rate and female polyandry*	*Fitness consequences of population bottlenecks*	*Detrimental fitness consequences of genetic similarity*	*Relationship between population bottleneck size and viability*	*Function of uropygial glands*	*Evolution of uropygial gland size*	*Benefits of polyandry; relationship between EPP and HS%*	*Effects of different variables on hatchability*
Study year	Not sig.	–	–	–	–	–	–	–	–
Taxon	Not sig.	–	–	–	–	–	–	–	–
Habitat	Not sig.	–	–	–	–	Control	Control	–	–
Diet	Sig. decrease in HS% in carnivorous spp. *vs.* herbivorous spp.	–	–	–	–	–	–	–	Sig. variation – lowest HS% in herbivorous/ granivorous spp.
Nest location /type	Sig. decrease in HS% in hole-nesting spp. *vs.* open-nesting spp.	Not sig.	–	–	–	–	–	–	Sig. higher HS% in open nests *vs.* closed nests
Latitude	Sig. increase in HS% along latitudinal gradient	–	–	–	Not sig.	–	–	–	Sig. increase in HS% with increasing latitude
Altitude	Not sig.	–	–	–	–	–	–	–	–
Mean clutch size	Near sig.	Not sig.	–	–	Not sig.	–	–	–	Not sig.
Egg size/mass	Near sig.	–	–	–	–	–	–	–	–

Coloniality/ spacing pattern	Colonial species have lowest HS% (not sig.)	–	–	Not sig.	–	–	–	–	–
Incubation type (female, male, shared)	Female-only incubation sig. higher HS% than shared incubation	Not sig.	–	–	–	–	–	–	Not sig.
Social mating/ organisation system	Monogamous species have sig. highest HS%, then polygynous, then cooperative breeders	Not sig.	–	Cooperative breeding sig. decreases HS%	–	–	–	–	Excluded from model due to missing data
Extrapair paternity (EPP)	–	Not sig.	–	–	–	–	–	–	–
Testes size	–	Not sig.	–	–	–	–	–	–	–
Body mass	–	Not sig.	Control	Sig. positive relationship with HS%	Not sig.	Control	–	–	–
Bottleneck size	–	–	Sig. decrease in HS% when bottleneck <150	–	Bottleneck size sig. inversely related to HS%	–	–	–	–
Time since bottleneck	–	–	–	–	Not sig.	–	–	–	–
Genetic similarity (band-sharing)	–	–	–	Band-sharing sig. negative correlation with HS%	–	Control	–	–	–
Island distribution	–	–	–	Control	Control	–	–	–	–

Uropygial gland size/mass	–	–	–	–	–	*(Controlled for body mass)* Sig. positive relationship with HS%	(*Controlled for body mass and effect of* Staphylo-coccus) Sig. negative relationship with HS%	–	–
Mean incubation period	–	–	–	–	–	–	–	–	Not sig. (but interaction with latitude)
Eggshell bacterial load	–	–	–	–	–	–	Sig. negative association with HS%	–	–
Incubation limitation (ratio of incubator body size and clutch mass)/ residual clutch mass	–	–	–	–	–	–	–	–	Sig. positive correlation with HS%. HS% increases as relative clutch mass increases
Incubator mass	–	–	–	–	–	–	–	–	Not sig.
Clutch mass	–	–	–	–	–	–	–	–	Not sig.
Phylogenetic correction?	NO	YES	YES	YES	YES	YES	YES	YES	YES

Galván & Sanz (2011) is included in **Table A1** but excluded here as hatching success was included in the study as a variable to explain the evolution of mate-feeding, but it was not the main effect of interest and the definition of hatching success was not explicitly stated.

Appendix A.2. Literature search and data compilation details

Table A3. The results of different searches conducted in *Web of Science*. All searches were conducted on 23.01.2020 with no year limitations (i.e. search included all years 1900–2020) and searching all document types. TS = Topic. Search set 18 was chosen as the final set of terms.

Search set	Search type	Search terms or search set	No. of results
1	Basic	"bird" AND "hatching failure"	38
2	Basic	"birds" AND "hatching failure"	94
3	Basic	bird AND hatching failure	392
4	Basic	birds AND hatching failure	393
5	Advanced	(TS=(bird AND hatching failure)) AND LANGUAGE: (English)	388
6	Advanced	(TS=(bird AND "hatching failure")) AND LANGUAGE: (English)	107
7	Advanced	(TS=(bird AND (hatching failure OR hatching success OR hatchability))) AND LANGUAGE: (English)	2585
8	Advanced	(TS=(bird AND ("hatching failure" OR "hatching success" OR "hatchability"))) AND LANGUAGE: (English)	1373
9	Advanced	((TS=("bird" AND ("hatching failure" OR "hatching success" OR "hatchability"))))) AND LANGUAGE: (English)	400
10	Advanced	((TS=("bird" AND ("hatching failure" OR "hatching success" OR "hatchability" OR "hatching rate")))) AND LANGUAGE: (English)	416
11	Advanced	((TS=(bird AND ("hatching failure" OR "hatching success" OR "hatchability" OR "hatching rate")))) AND LANGUAGE: (English)	1431
12	Advanced	((TS=(("hatching failure" OR "hatching success" OR "hatchability" OR "hatching rate")))) AND LANGUAGE: (English)	9142
13	Advanced	((TS=(("hatching failure" OR "hatching success" OR "hatchability")))) AND LANGUAGE: (English)	7576
14	Advanced	((TS=((egg AND ("hatching failure" OR "hatching success" OR "hatchability" OR "hatching rate")))))) AND LANGUAGE: (English)	6781
15	Advanced	((TS=(egg AND ("hatching failure" OR "hatching success" OR "hatchability" OR "hatching rate" OR "hatching")))) AND LANGUAGE: (English)	16,455
16	Advanced	((TS=(("hatching failure" OR "hatching success" OR "hatchability" OR "hatching rate") NOT broiler))) AND LANGUAGE: (English)	8199
17	Advanced	((TS=(("hatching failure" OR "hatching success" OR "hatchability" OR "hatching rate") NOT (broiler OR poultry)))) AND LANGUAGE: (English)	8016
18	**Advanced**	**((TS=(("hatching failure" OR "hatching success" OR "hatchability" OR "hatching rate") NOT (broiler OR poultry OR fish)))) AND LANGUAGE: (English)**	**6799**

Only the *Web of Science* Core Collection Database was searched, not All Databases. This was in the interest of feasibility, as a search for Search Set 18 (**Table A3**) across All Databases returned 14,265 results (search conducted 03.04.2020).

Justification for chosen search set (Search Set 18)

This search employs the Field Tag 'TS' for Topic. The fields mined for a Topic Search are the document Title, the Abstract, the Keywords and Keywords Plus fields (*https://clarivate.libguides.com/woscc/searchtips*). As papers investigating hatching failure in birds did not always include the word 'bird' in the title,

abstract, or key words, instead usually including the study species' common or scientific name, it was decided not to include 'bird' as one of the search terms to avoid unintentionally eliminating relevant studies. The definition of hatching failure is not universal and there are multiple terms used in the literature to describe what is defined in this review as hatching failure. To account for this, searches were made for papers including any of the most commonly used terms by grouping the phrases "hatching failure", "hatching success", "hatchability", and "hatching rate" within parentheses with the Boolean search operator 'OR'. It was decided to exclude studies on broiler hens and other poultry, as well as studies reporting hatching success in fish, hence the terms "broiler", "poultry", and "fish" were grouped within another set of parentheses preceded by the Boolean search operator 'NOT'. Results were limited to those written in English (see main text for considerations of bias due to language limitation and other potential sources of bias).

Assessments of study eligibility

All screening for inclusion of studies was conducted by A.F.M. in consultation with N.H. and P.B.

After the records were pooled into a large dataset, they were screened to remove duplicates and the compiled records were assessed for eligibility and inclusion in the final analyses. For purposes of increased efficiency, I removed all studies prior to 2004 which were not included within the previous comparative analyses as it could be assumed that the majority of eligible studies from this period would have been captured in these previous analyses. This cut-off led to the exclusion of the comparative analyses performed by Koenig (1982) and Morrow *et al*. (2002), but as the data from these studies could not be accessed they would have been ineligible for inclusion on this basis also. As Galván & Sanz (2011) did not state a definition for hatching success, the original data sources of Cramp (1988, 1992) and Cramp & Perrins (1993, 1994a, 1994b) were investigated to determine their eligibility. The majority of the included hatching success rates were found to be ineligible due to a lack of clarity around the causes of egg losses or the explicit inclusion of eggs lost to external factors such as predation. As a result all records from Galván & Sanz (2011) were excluded from the final dataset.

Extraction of data from eligible studies

All data collection from eligible studies was conducted by A.F.M. with extractions from around 10% of the included studies checked by P.B.

Each record in the final dataset was critically assessed to extract data for qualitative and quantitative syntheses of hatching failure, its influencing variables, and how it was reported. Relevant data were extracted from the text, figures, tables, or supplementary material of each study. Where relevant data were only available in the form of a figure the online tool *WebPlotDigitizer* (Rohatgi, 2020) was used to extract the raw data from the figures. This method was applied to the following studies: Briskie & Mackintosh (2004); Møller *et al*. (2005); Astié & Reboreda (2006); Casas *et al*. (2009); Fiorini *et al*. (2009); Wang *et al*. (2011); and Chin *et al*. (2017).

Where data were not readily available from the published paper, in-text citations and reference lists were searched to investigate original data sources. Where data for multiple species or independent populations of the same species were reported within the same study, the data were separated into individual population records. When available, life-history data for each species were taken from Cooney *et al.* (2020).

For records obtained from previous comparative analyses (**Table A1**) the original data sources were checked where possible to obtain additional information about the study. In some cases original sources were not accessible or it was not clear where the hatching failure rate had been taken from, in which case additional information was recorded as ‘NA’.

The chosen effect size was the proportion of hatching failure across the population, i.e. the proportion of eggs present at the end of the incubation period that fail to hatch (relative to all eggs present at the end of the incubation period), excluding eggs lost due to predation, desertion, accident, extreme weather, or that disappeared during the incubation period due to unknown factors. Where necessary, the absolute number of unhatched or surviving eggs was calculated or estimated using the information given in the study or supplementary material. Some caveats for this are that some studies may have classified all undamaged eggs remaining unhatched after the assumed end of

the incubation period to be ‘abandoned’ or ‘deserted’ (e.g. Chokri & Selmi, 2011), meaning they were excluded from my calculations of hatching failure. This may particularly be the case for studies that do not have accurate lay dates/incubation length data to verify egg fates. Another potential source of inaccuracy is that some studies only reported numbers of eggs hatched or unhatched in ‘successful’ nests, i.e. nests in which at least one egg hatched. While this eliminated nests which were lost to whole-clutch predation or abandonment, in keeping with my definition, this may have also excluded some nests in which the whole clutch failed due to fertilisation failure or embryo mortality.

Geographic breakdown

Where a site location and Global Positioning System (GPS) coordinates were provided within the text of the paper these were converted to Decimal Degrees Latitude and Longitude using *Google Maps* (*www.google.co.uk/maps*). Where only a site location was provided within the text this was used to find Decimal Degrees Latitude and Longitude using *Google Maps*. Where a range of GPS coordinates were given, for example when a study site covered a large area or when data were collected from multiple study sites, a central point equidistant from each of the given points was used where possible. In some cases no location or GPS information was available, in which case the latitude and longitude was marked as “NA” unless the subject species had such a limited or restricted distribution that it was possible to approximate the study’s location, e.g. Mauritius Fody (*Foudia rubra*) is only found in Mauritius. In such cases the species’ distributions were verified using information from the *IUCN Red List* (IUCN, 2021) and *Birds of the World* (Billerman *et al.*, 2020).

Appendix A.3. PRISMA-EcoEvo checklist

Checklist item	Sub-item number	Sub-item	Reported by authors?	Notes
Title and abstract	1.1	Identify the review as a systematic review, meta-analysis, or both	Yes	
	1.2	Summarise the aims and scope of the review	Yes	
	1.3	Describe the data set	Yes	
	1.4	State the results of the primary outcome	Yes	
	1.5	State conclusions	Yes	
	1.6	State limitations	Yes	
Aims and questions	2.1	Provide a rationale for the review	Yes	
	2.2	Reference any previous reviews or meta-analyses on the topic	Yes	
	2.3	State the aims and scope of the review (including its generality)	Yes	
	2.4	State the primary questions the review addresses (e.g. which moderators were tested)	Yes	
	2.5	Describe whether effect sizes were derived from experimental and/or observational comparisons	Yes	
Review registration	3.1	Register review aims, hypotheses (if applicable), and methods in a time-stamped and publicly accessible archive and provide a link to the registration in the methods section of the manuscript. Ideally registration occurs before the search, but it can be done at any stage before data analysis.	No	
	3.2	Describe deviations from the registered aims and methods	NA: see 'Notes' for why	The review was not registered
	3.3	Justify deviations from the registered aims and methods	NA: see 'Notes' for why	The review was not registered
Eligibility criteria	4.1	Report the specific criteria used for including or excluding studies when screening titles and/or abstracts, and full texts, according to the aims of the systematic review (e.g. study design, taxa, data availability)	Yes	
	4.2	Justify criteria, if necessary (i.e. not obvious from aims and scope)	Yes	
Finding studies	5.1	Define the type of search (e.g. comprehensive search, representative sample)	Yes	
	5.2	State what sources of information were sought (e.g. published and unpublished studies, personal communications)	Yes	
	5.3	Include, for each database searched, the exact search strings used, with keyword combinations and Boolean operators	Yes	In supplementary material
	5.4	Provide enough information to repeat the equivalent search (if possible), including the timespan covered (start and end dates)	Yes	
Study selection	6.1	Describe how studies were selected for inclusion at each stage of the screening process (e.g. use of decision trees, screening software)	Yes	
	6.2	Report the number of people involved and how they contributed (e.g. independent parallel screening)	Yes	In supplementary material
Data collection process	7.1	Describe where in the reports data were collected from (e.g. text or figures)	Yes	
	7.2	Describe how data were collected (e.g. software used to digitize figures, external data sources)	Yes	In supplementary material
	7.3	Describe moderator variables that were constructed from collected data (e.g. number of generations calculated from years and average generation time)	Yes	
	7.4	Report how missing or ambiguous information was dealt with during data collection (e.g. authors of original studies were contacted for missing descriptive statistics, and/or effect sizes were calculated from test statistics)	Yes	
	7.5	Report who collected data	Yes	
	7.6	State the number of extractions that were checked for accuracy by co-authors	Yes	
Data items	8.1	Describe the key data sought from each study	Yes	
	8.2	Describe items that do not appear in the main results, or which could not be extracted due to insufficient information	Yes	
	8.3	Describe main assumptions or simplifications that were made (e.g. categorising both 'length' and 'mass' as 'morphology')	Yes	
	8.4	Describe the type of replication unit (e.g. individuals, broods, study sites)	Yes	
Assessment of individual study quality	9.1	Describe whether the quality of studies included in the systematic review or meta-analysis was assessed (e.g. blinded data collection, reporting quality, experimental *versus* observational)	Yes	
	9.2	Describe how information about study quality was incorporated into analyses (e.g. meta-regression and/or sensitivity analysis)	Yes	
Effect size measures	10.1	Describe effect size(s) used	Yes	
	10.2	Provide a reference to the equation of each calculated effect size (e.g. standardised mean difference, log response ratio) and (if applicable) its sampling variance	NA: the effect size was extracted without calculation (e.g., mean, proportion, regression coefficient)	
	10.3	If no reference exists, derive the equations for each effect size and state the assumed sampling distribution(s)	NA: the effect size was extracted without calculation (e.g., mean, proportion, regression coefficient)	
Missing data	11.1	Describe any steps taken to deal with missing data during analysis (e.g. imputation, complete case, subset analysis)	Yes	
	11.2	Justify the decisions made to deal with missing data	Yes	
Meta-analytic model description	12.1	Describe the models used for synthesis of effect sizes	Yes	
	12.2	The most common approach in ecology and evolution will be a random-effects model, often with a hierarchical/multilevel structure. If other types of models are chosen (e.g. common/fixed effects model, unweighted model), provide justification for this choice	NA: only (weighted) random-effects models were used	
Software	13.1	Describe the statistical platform used for inference (e.g. *R*)	Yes	
	13.2	Describe the packages used to run models	Yes	
	13.3	Describe the functions used to run models	Yes	
	13.4	Describe any arguments that differed from the default settings	Yes	
	13.5	Describe the version numbers of all software used	Yes	
Non-independence	14.1	Describe the types of non-independence encountered (e.g. phylogenetic, spatial, multiple measurements over time)	Yes	
	14.2	Describe how non-independence has been handled	Yes	
	14.3	Justify decisions made	Yes	
	15.1	Provide a rationale for the inclusion of moderators (covariates) that were evaluated in meta-regression models	Yes	

Meta-regression and model selection	15.2	Justify the number of parameters estimated in models, in relation to the number of effect sizes and studies (e.g. interaction terms were not included due to insufficient sample sizes)	Yes	
	15.3	Describe any process of model selection	Yes	In supplementary material
Publication bias and sensitivity analyses	16.1	Describe assessments of the risk of bias due to missing results (e.g. publication, time-lag, and taxonomic biases)	Yes	
	16.2	Describe any steps taken to investigate the effects of such biases (if present)	Yes	
	16.3	Describe any other analyses of robustness of the results, e.g. due to effect size choice, weighting or analytical model assumptions, inclusion or exclusion of subsets of the data, or the inclusion of alternative moderator variables in meta-regressions	Yes	
Clarification of *post hoc* analyses	17.1	When hypotheses were formulated after data analysis, this should be acknowledged.	NA: only exploratory analyses were conducted	
Metadata, data, and code	18.1	Share metadata (i.e. data descriptions)	Yes	
	18.2	Share data required to reproduce the results presented in the manuscript	Yes	
	18.3	Share additional data, including information that was not presented in the manuscript (e.g. raw data used to calculate effect sizes, descriptions of where data were located in papers)	Yes	
	18.4	Share analysis scripts (or, if a software package with graphical user interface (GUI) was used, then describe full model specification and fully specify choices)	Yes	
Results of study selection process	19.1	Report the number of studies screened	Yes	
	19.2	Report the number of studies excluded at each stage of screening	Yes	
	19.3	Report brief reasons for exclusion from the full text stage	Yes	
	19.4	Present a Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA)-like flowchart (www.prisma-statement.org).	Yes	
Sample sizes and study characteristics	20.1	Report the number of studies and effect sizes for data included in meta-analyses	Yes	
	20.2	Report the number of studies and effect sizes for subsets of data included in meta-regressions	Yes	
	20.3	Provide a summary of key characteristics for reported outcomes (either in text or figures; e.g. one quarter of effect sizes reported for vertebrates and the rest invertebrates)	Yes	
	20.4	Provide a summary of limitations of included moderators (e.g. collinearity and overlap between moderators)	Yes	
	20.5	Provide a summary of characteristics related to individual study quality (risk of bias)	Yes	
Meta-analysis	21.1	Provide a quantitative synthesis of results across studies, including estimates for the mean effect size, with confidence/credible intervals	Yes	
Heterogeneity	22.1	Report indicators of heterogeneity in the estimated effect (e.g. I^2, tau^2 and other variance components)	Yes	
Meta-regression	23.1	Provide estimates of meta-regression slopes (i.e. regression coefficients) and confidence/credible intervals	Yes	
	23.2	Include estimates and confidence/credible intervals for all moderator variables that were assessed (i.e. complete reporting)	Yes	
	23.3	Report interactions, if they were included	Yes	
	23.4	Describe outcomes from model selection, if done (e.g. R2 and AIC)	Yes	
Outcomes of publication bias and sensitivity analyses	24.1	Provide results for the assessments of the risks of bias (e.g. Egger's regression, funnel plots)	NA: see 'Notes' for why	Not appropriate for the included data (proportions)
	24.2	Provide results for the robustness of the review's results (e.g. subgroup analyses, meta-regression of study quality, results from alternative methods of analysis, and temporal trends)	Yes	
Results of synthesis	25.1	Summarise the main findings in terms of the magnitude of effect	Yes	
	25.2	Summarise the main findings in terms of the precision of effects (e.g. size of confidence intervals, statistical significance)	Yes	
	25.3	Summarise the main findings in terms of their heterogeneity	Yes	
Discussion	25.4	Summarise the main findings in terms of their biological/practical relevance	Yes	
	25.5	Compare results with previous reviews on the topic, if available	Yes	
	25.6	Consider limitations and their influence on the generality of conclusions, such as gaps in the available evidence (e.g. taxonomic and geographical research biases)	Yes	
Contributions and funding	26.1	Provide names, affiliations, and funding sources of all co-authors	NA: see 'Notes' for why	Not applicable
	26.2	List the contributions of each co-author	NA: see 'Notes' for why	Not applicable
	26.3	Provide contact details for the corresponding author	NA: see 'Notes' for why	Not applicable
	26.4	Disclose any conflicts of interest	NA: there were no conflicts of interest	
References	27.1	Provide a reference list of all studies included in the systematic review or meta-analysis	Yes	
	27.2	List included studies as referenced sources (e.g. rather than listing them in a table or supplement)	Yes	

Appendix A.4. Coding of variables and additional category details

For each effect size the following key variables were coded:

(1) Study name
(2) Year of publication
(3) Study start
(4) Study end
(5) Order
(6) Species name (as listed on BirdLife International (*www.birdlife.org*))
(7) Common name
(8) *IUCN Red List* classification (IUCN, 2021): (*a*) Least Concern (LC); (*b*) Near Threatened (NT); (*c*) Vulnerable (VU); (*d*) Endangered (EN); (*e*) Critically Endangered (CR); (*f*) Not Recognised (NR).
(9) Threat status: (*a*) Threatened – species classified as Vulnerable (VU), Endangered (EN), or Critically Endangered (CR); (*b*) Non-threatened – species classified as Least Concern (LC) or Near Threatened (NT).
(10) Management level: (*a*) Wild – the study was conducted on a wild population; (*b*) Captive – the study was conducted on a captive population; (*c*) Wild managed – the study was conducted on a population living in the wild with some management applied, e.g. nestbox provision, or the eggs examined were taken from a wild population and hatched in captivity, e.g. using artificial incubation or fostering.
(11) Incubation type – the type of incubation experienced by the eggs: (*a*) Natural – eggs are incubated by adult birds either in the wild or in captivity, by natal or foster parents; (*b*) Artificial – eggs are incubated in captivity in artificial incubators.
(12) Supplementary feeding – whether or not a population was provided with supplementary food: (*a*) Fed – population was provided with supplementary food; included captive populations which do not source their own food; (*b*) Not fed – population was not provided with any supplementary food.
(13) Artificial nest provision – whether a population was provided with an artificial nest site in the form of a nestbox or other species-specific nesting site: (*a*) Provided – population was provided with artificial nest sites which were used by all, or some, of the studied population; (*b*) Not provided – the population was not provided with any form of artificial nest site.

Threat status classifications

For each species both the current *IUCN Red List* Classification (IUCN, 2021) and the classification at the time of the study was recorded. As some records did not include details of the study period the current *IUCN Red List* Classification was used for all analyses. While this may lead to some inaccuracies due to a species' threat status being up- or down-graded in the period between the study's completion and the time of this analysis, this was considered preferable to the alternative of excluding these records. Where both global and local (e.g. Europe) assessments were available, the global assessment was used.

Subspecies threat status

For populations of subspecies threat status was taken from the relevant regional or national *Red List* (www.nationalredlist.org), *Federal Endangered Species List* (www.fws.gov/endangered), or *European Red List* (https://eunis.eea.europa.eu/species.jsp) where available. In cases where taxa classified as subspecies at the time of the study have since been promoted to full species level [i.e. *Charadrius obscurus aquilonius* to *Charadrius aquilonius*, *Synthliboramphus hypoleucus scrippsi* to *Synthliboramphus scrippsi*, and *Tockus erythrorhynchus damarensis* to *Tockus damarensis* (del Hoyo *et al.*, 2014)] the current *IUCN Red List* category for the species was used. In some cases subspecies have been promoted to species level according to some taxonomic classifications [e.g. *Corvus corone cornix* to *Corvus cornix* (Knox *et al.*, 2002; Parkin *et al.*, 2003; Poelstra *et al.*, 2014)], but for consistency only species recognised by BirdLife International (www.birdlife.org) and listed on the *IUCN Red List of Threatened Species* (www.iucnredlist.org) were used. Where there was no threat status available for the subspecies the threat status of the parent species was used. While this has potential downfalls (Gippoliti & Amori, 2007; Scherz *et al.*, 2019) these were deemed acceptable compared to the alternative of excluding the record from the dataset.

Classification of population type as 'captive', 'wild', or 'wild managed'

Populations were classified according to the management applied to both the breeding population and the eggs. For example, wild populations from which

eggs were removed for artificial incubation and/or captive-rearing before returning to the population were classified as ‘wild managed’ even if no other direct management was applied to the adult population. In general:

- **Captive** – populations in which the adults were maintained in captivity and eggs were laid, hatched, and reared in captivity, inclusive of any other management interventions such as artificial insemination, artificial incubation, or fostering of eggs.
- **Wild** – populations for which the adults live in the wild with no management interventions applied, with the exception of monitoring methods (e.g. nest checks, geologgers, etc.).
- **Wild managed** – populations for which the adults live in the wild with management interventions applied, such as predator control/exclusion, nestbox provision, supplemental feeding, and egg manipulations such as removal of eggs for artificial incubation (whether or not this is specifically for the purposes of forced re-clutching). Any population provided with nestboxes was classified as ‘wild managed’ even if not all individuals in the population used the nestboxes. Note that in some cases management interventions applied to the population were not clearly reported and hence some ‘wild managed’ records may have been misclassified as ‘wild’.

In the case of previous comparative analyses, the original sources of the data were checked where possible in order to classify the population as ‘wild’, ‘wild managed’, or ‘captive’. Where the original source could not be checked, the classification was either entered as ‘NA’ or was assumed based on the context of the comparative analysis itself. Explicitly:

- **Briskie & Mackintosh** (2004) – hatching data taken from free-living populations, assumed to equate to ‘wild’ in this context. There is a caveat that this may misclassify some populations which would be classified as ‘wild managed’ according to my definition (e.g. kakī, kākāpō and others are known to be managed in the wild). Sensitivity analyses were conducted with and without these records to assess the robustness of the results.
- **Spottiswoode & Møller** (2004) – implied a focus on free-living populations but do not state the type of populations included, hence records without verification from original sources were entered as ‘NA’.

- **Heber & Briskie** (2010) – do not specify the type of populations included, hence records without verification from original sources were entered as 'NA'.
- **Møller *et al*.** (2010) – data mainly taken from Cramp & Simmons (1977, 1980, 1983), Cramp (1985, 1988, 1992) and Cramp & Perrins (1993, 1994a, 1994b) and are assumed to equate to 'wild' populations. There is a caveat that this may include some populations that use nestboxes.
- **Reding** (2015) – data taken from Morrow *et al*. (2002) which looked at natural populations, and are assumed to equate to 'wild' according to my definition.
- **Johnsen** (2019) – some data taken from Morrow *et al*. (2002) which looked at natural populations and are assumed to equate to 'wild' according to my definition. Other records were from nest card archives and could be either natural nests or nestboxes so are entered as 'NA'.

Management interventions classifications

For many studies it was not possible to extract information regarding the specific management interventions applied due to a lack of detail in the text, and it is likely that some have been unintentionally excluded, in particular those considered to be less intensive such as nest-box provision. It was also necessary for some assumptions to be made. Firstly, all captive populations were assumed to be supplementary fed and not provided with an artificial nest unless explicitly stated otherwise in the paper. Secondly, all non-captive populations were assumed to have no management interventions applied unless they were explicitly mentioned in the text of the study. Finally, in cases where at least part of the population was subject to a management intervention such as supplementary feeding or artificial nest provision, and the results could not be separated into subpopulations with and without the intervention, it was assumed to have been applied to the whole population.

Appendix A.5. Full bibliography for final dataset

The final dataset is available at: https://github.com/ashleigh-f-marshall/hatch-fail-meta.

Ackerman, J.T., Herzog, M.P., Takekawa, J.Y. & Hartman, C.A. 2014. Comparative reproductive biology of sympatric species: nest and chick survival of American Avocets and Black-necked Stilts. J. Avian Biol. **45**, 609–623.

Aldredge, R.A. 2017. Incubation onset maintains survival of most embryos and growth and survival of late-hatched young. Anim. Behav. **134**, 35–43.

Aldredge, R.A., Leclair, S.C. & Bowman, R. 2012. Declining egg viability explains higher hatching failure in a suburban population of the threatened Florida Scrub-jay *Aphelocoma coerulescens*. J. Avian Biol. **43**, 369–375.

Álvarez, E. & Barba, E. 2011. Nest characteristics and reproductive performance in Great Tits *Parus major*. Ardeola **58**, 125–136.

Amininasab, S.M., Birker, M., Kingma, S.A., Hildenbrandt, H. & Komdeur, J. 2017. The effect of male incubation feeding on female nest attendance and reproductive performance in a socially monogamous bird. J. Ornithol. **158**, 687–696.

Angulo-Gastélum, U.T., Castillo-Guerrero, J.A. & Mellink, E. 2011. Breeding ecology of the Royal Tern (*Thalasseus maximus*) at Isla el Rancho, Mexico: colony size and nest location affect predation. Ornitol. Neotrop. **22**, 131–142.

Antczak, M., Hromada, M., Grzybek, J. & Tryjanowski, P. 2004. Breeding biology of the Great Grey Shrike *Lanius excubitor* in W Poland. Acta Ornithol. **39**, 9–14.

Aourir, M., Znari, M., El Abbassi, A., Radi, M. & Melin, J.-M. 2008. Reproductive parameters in captive hand-reared Black-bellied Sandgrouse. Zoo Biol. **27**, 269–281.

Aourir, M., Znari, M., Radi, M. & Melin, J.-M. 2013. Wild-laid versus captive-laid eggs in the Black-bellied Sandgrouse: is there any effect on chick productivity? Zoo Biol. **32**, 592–599.

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Astié, A.A. & Reboreda, J.C. 2006. Costs of egg punctures and parasitism by Shiny Cowbirds (*Molothrus Bonariensis*) at Creamy-Bellied Thrush (*Turdus Amaurochalinus*) nests. Auk **123**, 23–32.

Ballantyne, K. & Nol, E. 2011. Nesting habitat selection and hatching success of Whimbrels near Churchill, Manitoba, Canada. Waterbirds **34**, 151–159.

Bank, L., Haraszthy, L., Horváth, A. & Horváth, G.F. 2019. Nesting success and productivity of the Common Barn-owl *Tyto alba*: results from a nest box installation and long-term breeding monitoring program in Southern Hungary. Ornis Hung. **27**, 1–31.

Bao, X., Zhao, W., Liu, F., Li, J. & Ma, D. 2020. Egg investment strategies adopted by a desertic passerine, the Saxaul Sparrow (*Passer ammodendri*). Avian Res. **11**, 15.

Barri, F.R., Martella, M.B. & Navarro, J.L. 2009. Reproductive success of wild Lesser Rheas (*Pterocnemia-Rhea-pennata pennata*) in north-western Patagonia, Argentina. J. Ornithol. **150**, 127–132.

Bartlett, T.L., Mock, D.W. & Schwagmeyer, P.L. 2005. Division of labor: incubation and biparental care in House Sparrows (*Passer domesticus*). Auk **122**, 835–842.

Beier, C. & Fontana, C.S. 2019. Breeding biology of the endangered Yellow Cardinal *Gubernatrix cristata* in Brazil. Ornithol. Res. **27**, 44–52.

Beissinger, S.R., Cook, M.I. & Arendt, W.J. 2005. The shelf life of bird eggs: testing egg viability using a tropical climate gradient. Ecology **86**, 2164–2175.

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Berkunsky, I., Segura, L.N., Ruggera, R.A., Faegre, S.I.K., Trofino-Falasco, C., López, F.G., Velasco, M.A., Kacoliris, F.P., Aramburú, R.M. & Reboreda, J.C. 2017. Reproductive parameters of the Turquoise-fronted Parrot (*Amazona aestiva*) in the dry Chaco forest.

Avian Conserv. Ecol. **12**, 6.
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Appendix A.6. Definitions of hatching failure/success and alternative terms

For each study/record, data was extracted on:

- Whether or not the study/record used the terms “hatching success” or “hatching failure” within the text
- Whether or not the study/record used the term “hatchability” within the text
- The definition of hatching success, hatching failure, or hatchability given. If a definition was not given, it was categorised as ‘Not stated’. If no term was given, it was categorised as ‘NA’
- If another term was used and defined in place of the above terms, this was extracted and recorded.

After revising the wording to use consistent phrasing and terminology, inverting definitions of hatching failure to hatching success, and reassigning definitions of alternative terms to define hatching success where appropriate (e.g. if a study used hatching success and the alternative term synonymously) a total of 51 different definitions of hatching success (**Table A4**) and 12 definitions of hatchability (**Table A5**) remained across the final dataset. Please see **Appendix A.5** for full list of references included in the meta-analysis.

Table A4. Definitions for hatching success used across the final dataset. Any alternative terms (excluding ‘Hatchability’) used synonymously with hatching success are noted in square brackets.

Definition	Reference
An unabandoned egg in the nest for the length of the incubation period that hatches	Aldredge, Leclair & Bowman (2012)
Brood size on day 11 of nestling period relative to clutch size	Higgott (2019)
Egg viability	Wang *et al.* (2011)
Mayfield method (Mayfield, 1961, 1975)	Hothem & Hatch (2004); Ferreira-Rodríguez & Pombal (2018)
Mayfield method (Mayfield, 1961, 1975) AND Percentage of eggs that hatched out of eggs laid	Pardo-Cervera *et al.* (2010)
Mayfield method (Mayfield, 1975) – Number of eggs hatched by number of eggs incubated per nest [Hatching rate]	Lobo & Ângelo Marini (2013)
Nest success × hatchability × survival of female during June	Kobayashi & Nakamura (2013)
Number of eggs that hatched per clutch/nest	Ludlow *et al.* (2014)
Number of eggs that hatched relative to clutch size, excluding depredated nests	Wiebe (2018)
Percentage/Proportion of eggs that hatched	Aragonés (2003); Custer *et al.* (2005); Wilcoxen *et al.* (2011); Álvarez & Barba (2011); Santos & Nakagawa (2013); Tobolka *et al.* (2015); Amininasab *et al.* (2017); Berkunsky *et al.* (2017); Lee *et al.* (2018); Hurley *et al.* (2020)

Percentage/Proportion of eggs that hatched [Apparent hatching success] AND Mayfield Method (Johnson, 1979)	Taylor *et al.* (2017)
Percentage/Proportion of eggs that hatched in successful clutches/nests (at least one egg hatched)	Barri *et al.* (2009); Whittaker *et al.* (2009); Casas *et al.* (2009); Dieter & Anderson (2009); van Oosten (2016); Bernard-Laurent *et al.* (2017); Clauser & McRae (2017); Ludwig *et al.* (2018); van Oosten *et al.* (2019)
Percentage/Proportion of eggs that hatched in successful clutches/nests (at least one egg hatched) [% eggs hatched]	Winkler *et al.* (2011)
Percentage/Proportion of eggs that hatched in successful clutches/nests (at least one egg hatched) [Hatching rate]	Fu *et al.* (2017)
Percentage/Proportion of eggs that hatched out of eggs available	Custer *et al.* (2014); Chin *et al.* (2017)
Percentage/Proportion of eggs that hatched out of eggs available, excluding predation	Neigh *et al.* (2006)
Percentage/Proportion of eggs that hatched out of eggs available, excluding predation [Hatching Rate/Percentage]	Rosoni *et al.* (2019)
Percentage/Proportion of eggs that hatched out of eggs detected in the nests 2–3 days before hatching (i.e. after possible partial predation events) in non-predated clutches	Peralta-Sánchez *et al.* (2018)
Percentage/Proportion of eggs that hatched out of eggs laid	Antczak *et al.* (2004); Beissinger *et al.* (2005); Wellicome (2005); Sanz & Rodríguez-Ferraro (2006); Schwarzbach *et al.* (2006); Cooper *et al.* (2006); Elliott *et al.* (2006); Hernández *et al.* (2008); García-Navas *et al.* (2008); Sklepowicz & Halupka (2010); Sethi *et al.* (2011); Lara *et al.* (2011); Custer *et al.* (2012); Morosinotto *et al.* (2013); Khan *et al.* (2016*b*); Thongcharoen *et al.* (2018); Sievwright *et al.* (2019); Bollinger (2020)
Percentage/Proportion of eggs that hatched out of eggs laid [Hatching probability]	Eeva *et al.* (2009)
Percentage/Proportion of eggs that hatched out of eggs laid [Hatching rate]	Hammond *et al.* (2015); Mohamadian *et al.* (2019)
Percentage/Proportion of eggs that hatched out of eggs laid [Apparent hatching success]	Doherty & Heath (2011)
Percentage/Proportion of eggs that hatched out of eggs laid in successful clutches/nests (at least one egg hatched) [Hatching rate]	Sawa *et al.* (2011)
Percentage/Proportion of eggs that hatched out of eggs laid minus lost eggs	Zuarth *et al.* (2016)
Percentage/Proportion of eggs that hatched out of eggs laid per nest	Wolfaardt *et al.* (2008)
Percentage/Proportion of eggs that hatched out of eggs laid, excluding failure due to predation	Covas *et al.* (2008)
Percentage/Proportion of eggs that hatched out of eggs laid, excluding failure due to predation or overwashing	Schneider & McWilliams (2007)
Percentage/Proportion of eggs that hatched out of eggs that survived the entire incubation period	Astié & Reboreda (2006); Massoni *et al.* (2007); Tuero *et al.* (2007); De Mársico & Reboreda (2008); Klimstra *et al.* (2009); Liljesthröm *et al.* (2009); Di Giacomo *et al.* (2011*a*,*b*); Stager *et al.* (2012); Astié & Luchesi (2012); Stewart & Westneat (2013); Congdon & Briskie (2014); Berkunsky *et al.* (2014); Segura *et al.*

	(2015); Di Giacomo & Reboreda (2015); Domínguez *et al.* (2015); Orłowski *et al.* (2016); Proctor (2016); Dorset *et al.* (2017); Wilson (2019); Reséndiz-Infante *et al.* (2020); Wilson & Walters (2020)
Percentage/Proportion of eggs that hatched out of eggs that survived the entire incubation period [Hatching rate]	Bouakkaz *et al.* (2017)
Percentage/Proportion of eggs that hatched out of eggs that survived the entire incubation period [Hatching rate]	Beier & Fontana (2019)
Percentage/Proportion of eggs that hatched out of eggs that survived the entire incubation period in nests that survived until the nestling stage	De Márs ico *et al.* (2010)
Percentage/Proportion of eggs that hatched out of eggs that survived the entire incubation period in successful clutches/nests (at least one egg hatched)	Herzog *et al.* (2020)
Percentage/Proportion of eggs that hatched out of eggs that survived the entire incubation period, excluding failure due to desertion and predation	Spottiswoode & Møller (2004); Møller *et al.* (2010)
Percentage/Proportion of eggs that hatched out of eggs that survived the entire incubation period, excluding failure due to desertion and predation [Hatching rate]	Chiarani & Fontana (2015)
Percentage/Proportion of eggs that hatched out of eggs that survived the entire incubation period, excluding failure due to desertion, predation, accident, or severe weather	Mackintosh & Briskie (2005)
Percentage/Proportion of eggs that hatched out of eggs that survived the entire incubation period, excluding failure due to desertion, predation, or abiotic factors such as windstorms or floods	Briskie & Mackintosh (2004)
Percentage/Proportion of eggs that hatched out of eggs that survived the entire incubation period, excluding failure due to desertion, predation, or accident	Serrano *et al.* (2005); Boessenkool *et al.* (2007)
Percentage/Proportion of eggs that hatched out of eggs that survived the entire incubation period, excluding failure due to desertion, predation, or adverse weather	Heber & Briskie (2010)
Percentage/Proportion of eggs that hatched out of eggs that survived the entire incubation period, excluding failure due to desertion, predation, or disappearance	Ortego *et al.* (2010)
Percentage/Proportion of eggs that hatched out of total hatched and unhatched eggs present in the nest after brood departure in successful clutches/nests (at least one egg hatched)	Stiver *et al.* (2008)
Percentage/Proportion of eggs that hatched per clutch/nest	Bartlett *et al.* (2005); Djerdali *et al.* (2008); Kim *et al.* (2010*a*); Podofillini *et al.* (2018); Vuarin *et al.* (2018); Bank *et al.* (2019)
Percentage/Proportion of eggs that hatched per clutch/nest excluding predation	de Zwaan & Martin (2018)
Percentage/Proportion of eggs that hatched relative to clutch size	Tomás *et al.* (2012); Glądalski *et al.* (2017); Diez-Méndez *et al.* (2020); Metallaoui *et al.* (2020)
Percentage/Proportion of eggs that hatched relative to clutch size for nests that survived to hatching [Hatching Rate]	Manica & Marini (2012)
Percentage/Proportion of eggs that hatched relative to eggs monitored	Jakubas & Wojczulanis-Jakubas (2011)

Percentage/Proportion of successful clutches/nests (at least one egg hatched) AND Number of eggs that hatched	Lengyel *et al.* (2009)
Percentage/Proportion of successful clutches/nests (at least one egg hatched) out of total monitored clutches/nests	Ballantyne & Nol (2011); Debata *et al.* (2019)
Probability of an egg hatching	Ihle *et al.* (2017)
Probability of partial hatching failure AND Proportion of unhatched eggs in individual clutches	Burton (2006)
Probability that an egg hatched [Embryo survival]	Aldredge (2017)
Probability that at least one egg of a clutch/nest hatches	Van de Loock *et al.* (2020)

Table A5. Definitions for hatchability used across the final dataset. Any alternative terms (excluding 'Hatching success') used synonymously with hatchability are noted in square brackets.

Definition	Articles
Percentage/Proportion of eggs that hatched out of eggs available	Yamak (2015)
Percentage/Proportion of eggs that hatched out of eggs laid	Zduniak & Kuczyński (2003); Zimmerling & Ankney (2005)
Percentage/Proportion of eggs that hatched out of eggs laid, excluding failure due to desertion or predation	Yuta *et al.* (2018)
Percentage/Proportion of eggs that hatched out of eggs that survived the entire incubation period	Norment & Green (2004); Toschik *et al.* (2005); Schwarzbach *et al.* (2006); Mallory *et al.* (2009); Rolland *et al.* (2011); Bao *et al.* (2020)
Percentage/Proportion of eggs that hatched out of eggs that survived the entire incubation period after pooling all eggs laid per clutch and pair	Cordero *et al.* (2004)
Percentage/Proportion of eggs that hatched out of eggs that survived the entire incubation period in complete clutches	Praus & Weidinger (2015)
Percentage/Proportion of eggs that hatched out of eggs that survived the entire incubation period, excluding failure due to predation	Lengyel *et al.* (2009); Kim *et al.* (2010*a*)
Percentage/Proportion of eggs that hatched out of eggs that survived the entire incubation period, excluding failure due to predation or physical destruction	Doherty & Heath (2011)
Percentage/Proportion of eggs that hatched out of incubated eggs [Hatchability of incubated eggs]); Percentage/Proportion of eggs that hatched out of fertilised eggs [Hatchability of fertile eggs]	Aourir *et al.* (2013)
Percentage/Proportion of eggs that hatched per clutch in successful clutches/nests (at least one egg hatched)	Kobayashi & Nakamura (2013)
Percentage/Proportion of eggs that hatched relative to clutch size in successful clutches/nests (at least one egg hatched), excluding failure due to desertion, predation, disappearance, collected, or unknown) [Egg success]	Hothem & Hatch (2004); Hothem *et al.* (2006)
Percentage/Proportion of eggs that hatched, excluding failure due to desertion, predation, accident, or flooding	Beissinger *et al.* (2008)

Appendix A.7. Additional information for meta-analysis model selection

Transformations and continuity corrections

As the restriction of the range of proportions to values between 0 and 1 can be problematic (Lipsey & Wilson, 2001) due to the potential to overestimate the precision of the proportion estimate, the proportions required transformation before being pooled. The most commonly used and often default transformation for proportions is the logit transformation, however, recommendations on the use of transformations for proportions in the published literature are contradictory (Schwarzer, 2021). Barendregt *et al*. (2013) advocate the use of the Freeman–Tukey double arcsine transformation (Freeman & Tukey, 1950; Miller, 1978) in place of the logit transformation due to its putatively superior variance stabilisation. However, critics of this method have described issues including potentially seriously misleading results due to the use of the harmonic mean in calculating the back-transformation of the Freeman–Tukey transformation (Schwarzer *et al.*, 2019; Lin & Xu, 2020). Instead, a growing number of studies recommend using generalised linear mixed models (GLMMs) with a logit transformation for the meta-analysis of single proportions (Warton & Hui, 2011; Schwarzer *et al.*, 2019; e.g. Lin & Xu, 2020). However, GLMMs are not universally recommended for meta-analyses of binary outcome data (Bakbergenuly & Kulinskaya, 2018) and a known limitation of this method is that individual study weights are not available.

While none of the included studies had a hatching failure proportion of 1, several studies had reported a proportion of 0 (i.e. 100% hatching success). Depending on how the proportions are transformed, this necessitates a continuity correction to be applied. The continuity correction for logit-transformed values involves adding an increment of 0.5 to any study with an event probability of 0 or 1. For Freeman–Tukey double arcsine transformed data and GLMMs no continuity correction is required. Continuity corrections are not universally supported due to concerns that they may lead to biased results (Efthimiou, 2018; Harrer *et al.*, 2021).

Estimators

Another important consideration is the estimator used to estimate the between-study heterogeneity, τ^2, with the optimal estimator dependent on parameters including the total number of studies (*k*), the study size (*n*), the variation in study size among studies, and the size of τ^2 itself (Harrer *et al.*, 2021). The most commonly used estimators are: the DerSimonian–Laird estimator (DerSimonian & Laird, 1986); the restricted maximum likelihood (REML) or maximum likelihood (ML) procedures (Viechtbauer, 2005); the Paule–Mandel procedure (Paule & Mandel, 1982); the empirical Bayes procedure (Sidik & Jonkman, 2019); and the Sidik–Jonkman estimator (aka the model error variance method) (Sidik & Jonkman, 2005).

The DerSimonian–Laird estimator is frequently used and is often the default option in meta-analysis software (Harrer *et al.*, 2021). Despite this, it has been shown that the DerSimonian–Laird estimator can be biased, especially in cases of small study number and high heterogeneity (Follmann & Proschan, 1999; Hartung, 1999; Hartung & Knapp, 2001a, 2001b; Makambi, 2004; Langan *et al.*, 2019; Harrer *et al.*, 2021). While there is expected to be high heterogeneity in this meta-analysis, the number of included studies is relatively high, hence this is not considered to be of serious concern. The REML estimator is often preferred to the ML estimator due to the latter's potential for large biases (Viechtbauer, 2005; Veroniki *et al.*, 2016; Langan *et al.*, 2019). REML is widely used and recommended for meta-analyses (Langan *et al.*, 2019), although it has been particularly promoted for use with data with continuous outcomes (Veroniki *et al.*, 2016). It is the default method used within the *metafor* package in R and is frequently used as a method for fitting linear mixed models. Although the Paule–Mandel estimator has been recommended by some as a good choice for binary effect size data (Veroniki *et al.*, 2016), it has been shown potentially to be suboptimal when there is large variation in sample sizes across the studies (Langan *et al.*, 2019), hence making it unsuitable for this meta-analysis. As the Empirical Bayes procedure is almost identical to the Paule–Mandel procedure it was also ruled out as an option. Use of the Sidik–Jonkman estimator is only recommended when τ^2 is very large (Sidik & Jonkman, 2007; Harrer *et al.*, 2021), and was hence considered as an option as it was

reasonable to expect a large amount of between-study heterogeneity in this meta-analysis.

Adjustments

The Knapp–Hartung adjustment [aka Hartung–Knapp adjustment or the Hartung–Knapp–Sidik–Jonkman (HKSJ) method] (Sidik & Jonkman, 2002; Knapp & Hartung, 2003) is often recommended to calculate the confidence interval around the pooled effect with the aim of controlling for the uncertainty in estimates of the between-study heterogeneity (Harrer *et al.*, 2021). The Knapp–Hartung method is based on a *t*-distribution and has been shown to reduce the chance of false positives (Inthout *et al.*, 2014; Langan *et al.*, 2019).

Model selection

As several studies in the final dataset contributed multiple effect size estimates this required a meta-analysis model with a hierarchical structure, while the inclusion of a large number of different species presented the need to account for phylogenetic dependence, hence a multilevel meta-analytic model was required (Konstantopoulos, 2011; Nakagawa & Santos, 2012; Cinar *et al.*, 2022). Based on the nature of the dataset and consideration of the various transformation, estimators, and adjustments, and their respective downfalls and benefits, I decided to apply a Freeman–Tukey double arcsine transformation, a REML estimation, and the Knapp–Hartung adjustment. It is noted that one of the main issues with the Freeman–Tukey double arcsine transformation is potential misinterpretation as a result of the back-transformation calculation, as reported by Schwarzer *et al*. (2019), however the anomaly seen in their study does not seem to be replicated here. This method also benefits from not requiring a continuity correction which has been suggested to lead to biases in results. The REML estimator was chosen due to broad recommendations for usage and apparent lack of any major drawbacks. The Knapp–Hartung adjustment was applied due to its potential benefits and lack of issues providing effect sizes are not strongly homogeneous (Wiksten *et al.*, 2016), which is not the case in this meta-analysis.

Appendix A.8. Resolution of taxonomical differences for phylogenetic tree construction

Table A6. Resolution of taxonomical name differences between the final dataset (based on the taxonomy used by the *IUCN Red List* and BirdLife International) and the taxonomy used by BirdTree. Also listed are the subspecies with parent species substitutions used for construction of the phylogeny.

Common name	IUCN scientific name	BirdTree scientific name	Name used for phylogeny
Taxonomical differences			
Redpoll/common redpoll	*Acanthis flammea* **Synonym**: *Carduelis flammea*	*Carduelis flammea*	*Carduelis flammea*
Snow goose	*Anser caerulescens* **Synonym**: *Chen caerulescens*	*Chen caerulescens*	*Chen caerulescens*
Ross's goose	*Anser rossii* **Synonym**: *Chen rossii*	*Chen rossii*	*Chen rossii*
Short-tailed shearwater	*Ardenna tenuirostris* **Synonym***: Puffinus tenuirostris*	*Puffinus tenuirostris*	*Puffinus tenuirostris*
Brown skua	*Catharacta antarctica* **Synonym***: Catharacta lonnbergi*	*Catharacta antarctica* and *Catharacta lonnbergi*	*Catharacta antarctica*
Northern red-breasted plover	*Charadrius aquilonius* **Formerly***: Charadrius obscurus aquilonius*	*Charadrius obscurus*	*Charadrius obscurus*
Palestine sunbird	*Cinnyris osea* **Synonym***: Nectarinina osea*	*Nectarinina osea*	*Nectarinina osea*
Eurasian blue tit	*Cyanistes caeruleus* **Synonym***: Parus caeruleus*	*Parus caeruleus*	*Parus caeruleus*
Corn bunting	*Emberiza calandra* **Synonym***: Miliaria calandra*	*Miliaria calandra*	*Miliaria calandra*
Tasmanian native-hen	*Tribonyx mortierii* **Synonym***: Gallinula mortieri*	*Gallinula mortieri*	*Gallinula mortieri*
Leach's storm-petrel	*Hydrobates leucorhous* **Synonyms***: Hydrobates leucorhoa, Oceanodroma leucorhoa*	*Oceanodroma leucorhoa*	*Oceanodroma leucorhoa*
Middle spotted woodpecker	*Leiopicus medius* **Synonym***: Dendrocopos medius*	*Dendrocopos medius*	*Dendrocopos medius*
Double-crested cormorant	*Nannopterum auritus* **Synonym***: Phalacrocorax auritus*	*Phalacrocorax auritus*	*Phalacrocorax auritus*

Bridled tern	*Onychoprion anaethetus* **Synonym***: Sterna anaethetus*	*Sterna anaethetus*	*Sterna anaethetus*
Coal tit	*Peroparus ater* **Synonym***: Parus ater*	*Parus ater*	*Parus ater*
Willow tit	*Poecile montanus*	*Parus montanus*	*Parus montanus*
Marsh tit	*Poecile palustris* **Synonym***: Parus palustris*	*Parus palustris*	*Parus palustris*
Black-throated blue warbler	*Setophaga caerulescens* **Synonym***: Dendroica caerulescens*	*Dendroica caerulescens*	*Dendroica caerulescens*
Golden-cheeked warbler	*Setophaga chrysoparia* **Synonym***: Dendroica chrysoparia*	*Dendroica chrysoparia*	*Dendroica chrysoparia*
Hooded warbler	*Setophaga citrina* **Synonym***: Wilsonia citrina*	*Wilsonia citrina*	*Wilsonia citrina*
Kirtland's warbler	*Setophaga kirtlandii* **Synonym***: Dendroica kirtlandii*	*Dendroica kirtlandii*	*Dendroica kirtlandii*
American yellow warbler	*Setophaga petechia* **Synonym***: Dendroica petechia*	*Dendroica petechia*	*Dendroica petechia*
Fairy tern	*Sternula nereis* **Synonym***: Sterna nereis*	*Sterna nereis*	*Sterna nereis*
Scripps's murrelet	*Synthliboramphus scrippsi* **Formerly***: Synthliboramphus hypoleucus scrippsi*	*Synthliboramphus hypoleucus*	*Synthliboramphus hypoleucus*
Royal tern	*Thalasseus maximus* **Synonym***: Sterna maxima*	*Sterna maxima*	*Sterna maxima*
Damara hornbill	*Tockus damarensis* **Formerly***: Tockus erythrorhynchus damarensis*	*Tockus erythrorhynchus*	*Tockus erythrorhynchus*
Subspecies substitutions			
Rock partridge [*saxatilis*]	*Alectoris graeca saxatilis*	*Alectoris graeca*	*Alectoris graeca*
Greater snow goose	*Anser caerulescens atlanticus* **Synonym:** *Chen caerulescens atlantica*	*Chen caerulescens*	*Chen caerulescens*
Giant Canada goose	*Branta canadensis maxima*	*Branta canadensis*	*Branta canadensis*
North African houbara bustard	*Chlamydotis undulata undulata*	*Chlamydotis undulata*	*Chlamydotis undulata*
Hooded crow	*Corvus corone cornix*	*Corvus corone*	*Corvus corone*
Asian black francolin	*Francolinus francolinus asiae*	*Francolinus francolinus*	*Francolinus francolinus*
Hawaiian stilt	*Himantopus mexicanus knudseni*	*Himantopus mexicanus*	*Himantopus mexicanus*
Red grouse	*Lagopus lagopus scotica*	*Lagopus lagopus*	*Lagopus lagopus*

Japanese rock ptarmigan	*Lagopus muta japonica*	*Lagopus muta*	*Lagopus muta*
Amur wagtail	*Motacilla alba leucopsis*	*Motacilla alba*	*Motacilla alba*
South Island robin [*australis*]	*Petroica australis australis*	*Petroica australis*	*Petroica australis*
Chatham tomtit/Chatham Island tomtit	*Petroica macrocephala chathamensis*	*Petroica macrocephala*	*Petroica macrocephala*
South Island saddleback [*carunculatus*]	*Philesturnus carunculatus carunculatus*	*Philesturnus carunculatus*	*Philesturnus carunculatus*
Ridgeway's rail, formerly clapper rail	*Rallus crepitans crepitans, Rallus obsoletus levipes, Rallus obsoletus obsoletus* **Formerly***: Rallus longirostris crepitans, Rallus longirostris levipes, Rallus longirostris obsoletus*	*Rallus longirostris*	*Rallus longirostris*
Lesser rhea (Darwin's)	*Rhea pennata pennata*	*Rhea pennata*	*Rhea pennata*
Atlantic least tern	*Sternula antillarum antillarum* **Synonym***: Sterna antillarum antillarum*	*Sterna antillarum*	*Sterna antillarum*
Southern ostrich	*Struthio camelus australis*	*Struthio camelus*	*Struthio camelus*
Southern African red-billed hornbill	*Tockus erythrorhynchus rufirostris*	*Tockus erythrorhynchus*	*Tockus erythrorhynchus*
Andalusian hemipode/buttonquail	*Turnix sylvaticus sylvaticus*	*Turnix sylvaticus*	*Turnix sylvaticus*
Greater prairie chicken	*Tympanuchus cupido pinnatus*	*Tympanuchus cupido*	*Tympanuchus cupido*

Three species (*Parus minor*, *Philesturnus rufusater*, and *Phyllastrephus placidus*) from the final dataset were not available on BirdTree and were hence excluded from the phylogenetic tree and all subsequent analysis.

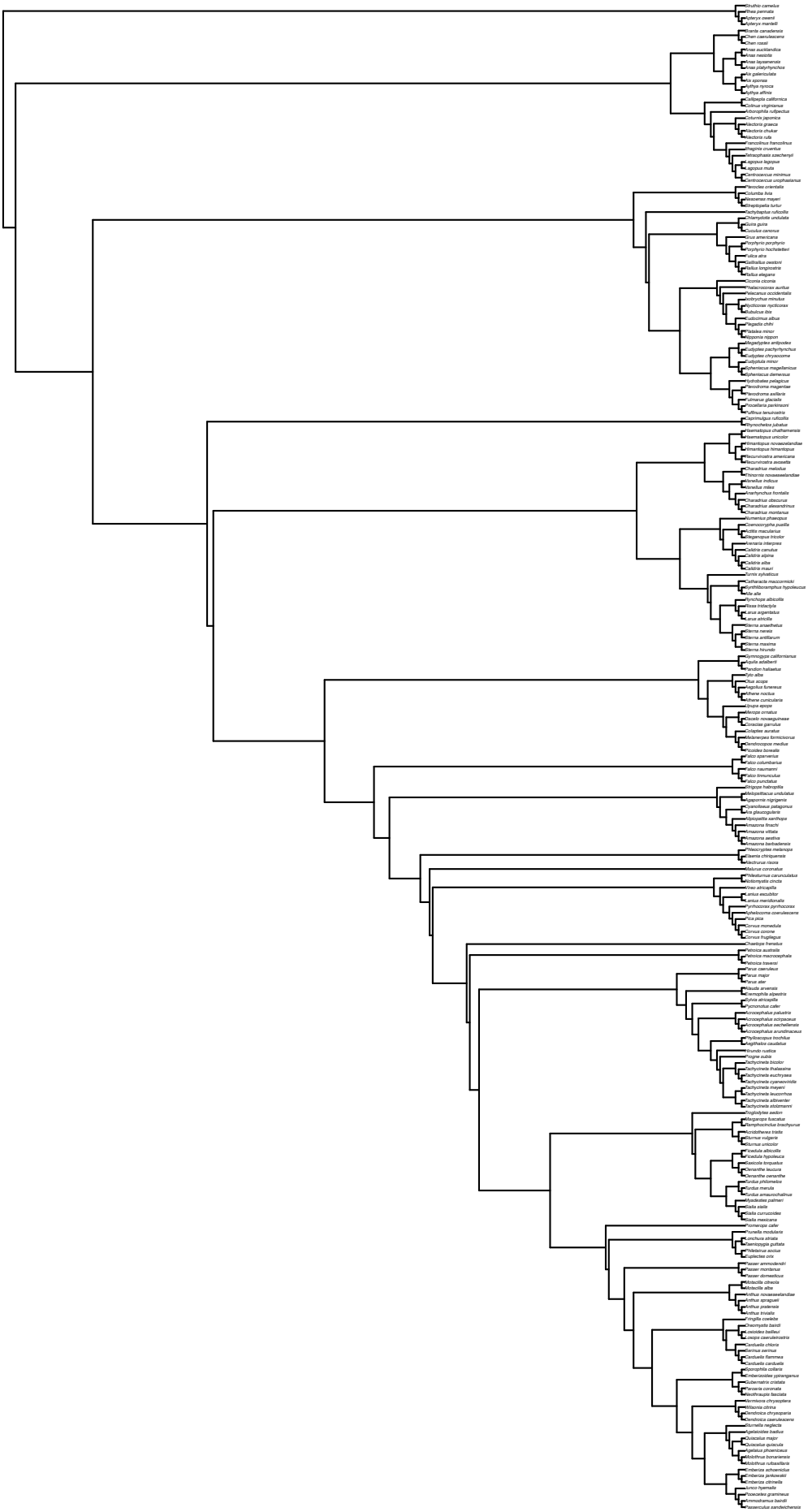


Figure A1. The final ultrametric tree of 241 species used to compute the correlation matrix included as a random effect in the multilevel meta-analyses.

Appendix A.9. Additional meta-analysis results, sensitivity analyses and publication bias information

Profile likelihood plots

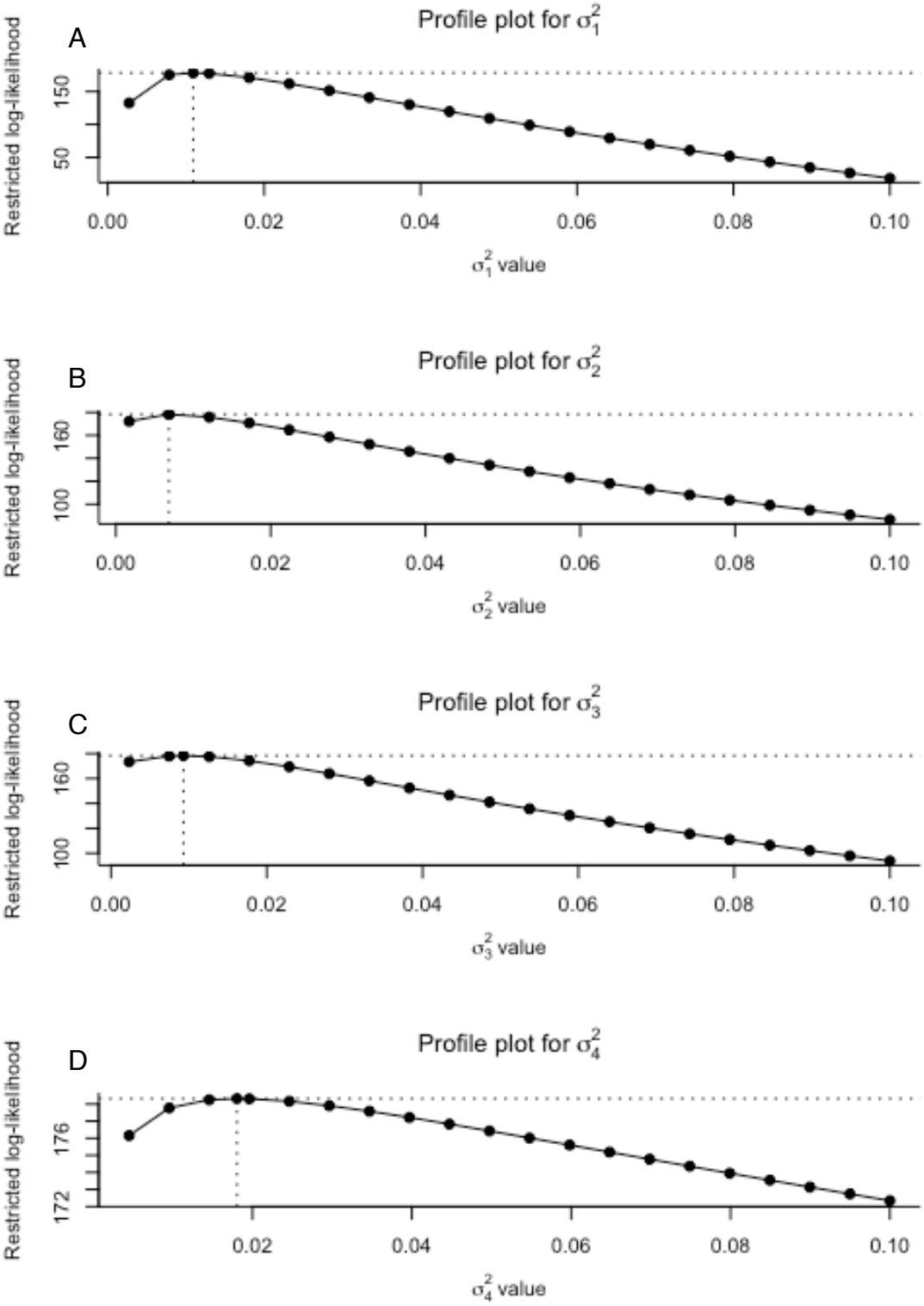


Figure A2. Profile likelihood plots for the four variance components [random effects of effect size ID (A), study ID (B), species ID (C), and phylogenetic history (D)] showing that each component was identifiable in the multilevel meta-analytical model.

Outliers and influential studies

Table A7. Summary of the potential outliers and influential studies from the multilevel meta-analysis with four random effects (effect size ID, study ID, species, phylogeny) identified using externally studentised residuals and Cook's Distances. HF% = hatching failure percentage. $\overline{x}$, mean of all distances; *N*, number of records.

Record	HF%	Externally studentised residual ±1.96	Cook's Distance		Record description		
			>3$\overline{x}$	>4/*N*	Threat status	Management level	Incubation type
Aourir *et al*. 2008 (01)	62.50	X			Non-threatened	Captive	Artificial
Barri *et al*. 2009	25.60		X		Non-threatened	Wild	Natural
Briskie & Mackintosh 2004 (33)	64.46	X			Threatened	Wild	Natural
Harvey *et al*. 2004 (01)	66.96	X			Threatened	Captive	Artificial
Hawke's Bay Today 2019	27.42		X		Threatened	Wild managed	Artificial
Heber & Briskie 2010 (17)	57.80	X			Threatened	Captive	Artificial
Heber & Briskie 2010 (28)	63.90	X	X		Threatened	Wild managed	Natural
Heber & Briskie 2010 (41)	0.00		X	X	Threatened	Wild	Natural
Hernandez *et al*. 2008 (01)	58.11	X			Threatened	Wild	Natural
Klimstra *et al*. 2009 (08)	67.00	X			Non-threatened	Wild managed	Artificial
Klimstra *et al*. 2009 (09)	71.43	X			Non-threatened	Wild managed	Artificial
Kumar *et al*. 2020	0.00		X		Non-threatened	Wild	Natural
Mansouri *et al*. 2020	6.06		X		Threatened	Wild	Natural
Oswald *et al*. 2020	0.00		X		Non-threatened	Wild	Natural
Sebei *et al*. 2009	58.17	X	X	X	Non-threatened	Wild	Natural
Taylor *et al*. 2017 (01)	65.28	X	X	X	Non-threatened	Wild	Natural
Taylor *et al*. 2017 (02)	32.27		X		Non-threatened	Wild	Natural
Thongcharoen *et al*. 2018	3.30		X		Non-threatened	Wild	Natural
Zhao *et al*. 2017 (01)	70.59	X			Non-threatened	Captive	Artificial
Zhao *et al*. 2017 (02)	67.50	X			Non-threatened	Captive	Artificial

Full model results

Table A8. Mean effect size [hatching failure percentage (HF%)] estimates with 95% confidence intervals (CI) for all moderator levels from the univariate meta-regression models. Random effects included were effect size ID, study ID, species name, and phylogenetic history. Proportions were transformed using a Freeman–Tukey double arcsine transformation, and all models were fitted using a restricted maximum likelihood (REML) estimation with a Knapp–Hartung adjustment. A significant *F* statistic indicates that at least one of the regression coefficients of the moderator categories significantly deviates from zero, indicating that that moderator influences hatching failure. Significant differences between levels in each category can be seen in **Figure 2-3**. Estimates were back-transformed with the inverse of the Freeman–Tukey transformation using the harmonic mean of the sample sizes and are presented here in the natural scale.

Moderator and categories	*N* effect sizes	*N* studies	*N* species	Mean HF%	Lower 95% CI	Upper 95% CI	*F*	*P*
None								
- All	478	231	241	16.79	8.28	27.40	–	–
Threat status								
- Threatened	96	51	62	21.02	11.62	32.22	$F_{1,476}$ = 9.025	0.0028
- Non-threatened	382	186	181	15.24	7.42	25.07		
IUCN Red List classification								
- Least Concern	347	168	158	15.13	7.16	25.24	$F_{4,473}$ = 4.236	0.0022
- Near Threatened	35	23	23	17.57	8.22	29.35		
- Vulnerable	37	24	25	16.60	7.61	28.02		
- Endangered	39	21	25	23.41	12.83	35.93		
- Critically Endangered	20	10	13	27.95	15.26	42.67		
Management level								
- Wild	265	134	178	13.78	7.25	21.87	$F_{2,475}$ = 32.181	<0.0001
- Wild managed	172	85	74	20.05	12.02	29.46		
- Captive	41	21	16	38.07	26.60	50.23		
Incubation type								
- Artificial	53	23	28	37.38	26.04	49.45	$F_{1,476}$ = 78.053	<0.0001
- Natural	425	210	226	14.38	7.37	23.09		
Supplementary feeding								
- Fed	51	27	23	33.47	23.03	44.77	$F_{1,476}$ = 51.945	<0.0001
- Not fed	427	211	230	15.37	8.57	23.62		
Artificial nest provision								
- Provided	161	79	56	21.34	11.34	33.37	$F_{1,476}$ = 7.152	0.0077
- Not provided	317	156	197	15.89	7.57	26.38		

Sensitivity analyses

Table A9. Comparison of the overall and partitioned heterogeneity (I^2) across the included random effects across the full dataset, the 'outliers removed' dataset, the 'Briskie & Mackintosh (2004) removed' dataset, and the 'sample size estimated from Cooney *et al*. (2020) removed' dataset.

Dataset	Overall effect size (95% CI)	Total heterogeneity (I^2)	Random effect	Heterogeneity (I^2)
Full dataset (*n* = 478; species = 241)	0.1679 (0.0828–0.2740)	99.23%	Effect size ID	24.02%
			Study ID	15.04%
			Species ID	20.46%
			Phylogenetic history	39.70%
All outliers removed (*n* = 458; species = 228)	0.1380 (0.0870–0.1979)	98.85%	Effect size ID	33.06%
			Study ID	21.40%
			Species ID	25.58%
			Phylogenetic history	18.81%
Sample size estimated from Cooney *et al*. (2020) removed (*n* = 418; species = 210)	0.1700 (0.0822–0.2799)	99.29%	Effect size ID	23.27%
			Study ID	15.39%
			Species ID	19.00%
			Phylogenetic history	41.63%
Briskie & Mackintosh (2004) removed (*n* = 442; species = 219)	0.1669 (0.0891–0.2618)	99.18%	Effect size ID	25.86%
			Study ID	19.87%
			Species ID	18.49%
			Phylogenetic history	34.95%

Table A10. Mean hatching failure percentage (HF%) estimates with 95% confidence intervals (CI) for all moderator levels from the univariate meta-regression models for the outliers removed dataset. Random effects included were effect size ID, study ID, species name, and phylogenetic history. Proportions were transformed using a Freeman–Tukey double arcsine transformation, and all models were fitted using a restricted maximum likelihood (REML) estimation with a Knapp–Hartung adjustment. A significant *F* statistic indicates that at least one of the regression coefficients of the moderator categories significantly deviates from zero, indicating that that moderator influences hatching failure. Estimates were back-transformed with the inverse of the Freeman–Tukey transformation using the harmonic mean of the sample sizes and are presented here in the natural scale.

Moderator and categories	*N* effect sizes	*N* studies	*N* species	Mean HF%	Lower 95% CI	Upper 95% CI	*F*	*P*
None								
- All	458	222	228	13.80	8.70	19.79	–	–
Threat status								
- Threatened	88	49	58	17.78	12.45	23.78	$F_{1,456}$ = 10.573	0.0012
- Non-threatened	370	179	172	12.29	8.19	17.04		
IUCN Red List classification								
- Least Concern	339	163	152	12.10	8.10	16.73	$F_{4,453}$ = 5.300	0.0004
- Near Threatened	31	20	20	14.24	8.52	21.06		
- Vulnerable	33	22	22	12.74	7.51	19.03		
- Endangered	37	21	24	20.34	14.00	27.49		
- Critically Endangered	18	10	13	24.18	15.72	33.76		
Management level								
- Wild	254	127	169	10.97	6.85	15.84	$F_{2,455}$ = 26.970	<0.0001
- Wild managed	168	84	71	16.33	11.12	22.28		
- Captive	36	20	13	31.27	22.92	40.27		
Incubation type								
- Artificial	45	21	22	32.31	23.72	41.52	$F_{1,456}$ = 66.430	<0.0001
- Natural	413	203	216	11.80	7.02	17.54		
Supplementary feeding								
- Fed	45	26	19	27.27	20.56	34.51	$F_{1,456}$ = 40.722	<0.0001
- Not fed	413	203	219	12.43	8.70	16.69		
Artificial nest provision								
- Provided	158	78	54	17.05	10.73	24.43	$F_{1,456}$ = 5.374	0.0209
- Not provided	300	148	186	12.97	7.82	19.12		

Moderator multi-collinearity

Table A11. Summary of pairwise chi-squared test and Cramer's V values for the proposed moderators. Values given in the table are the Cramer's V values which were interpreted as: 0.1–0.3 = weak association; 0.3–0.5 = moderate association; and >0.5 = strong association. Levels of significance are: ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

	Threat status	Management level	Incubation type	Supplementary feeding	Artificial nest provision
Threat status		0.15**	0.072	0.047	0.24***
Management level	0.15**		0.53***	0.89***	0.82***
Incubation type	0.072	0.53***		0.44***	0.05
Supplementary feeding	0.047	0.89***	0.44***		0.13**
Artificial nest provision	0.24***	0.82***	0.05	0.13**	

Table A12. The variance inflation factor (VIF) and generalised variance inflation factor (GVIF) for each proposed moderator when they are all combined into one multivariate mixed-effects multilevel meta-analytical model.

Moderator	Variance inflation factor (VIF)	Generalised variance inflation factor (GVIF)	$(GVIF^{1/(2\times df)})^2$
Threat status	1.0167	–	–
Management level	–	12.0335	3.468934
Incubation type	2.0270	–	–
Supplementary feeding	3.1982	–	–
Artificial nest provision	3.2989	–	–

Threat status and management interactions

Table A13. Estimated average effects and 95% confidence intervals of threat status and management level based on an additive and interactive model. Random effects included were effect size ID, study ID, species name, and phylogenetic history. Proportions were transformed using a Freeman–Tukey double arcsine transformation, and all models were fitted using a restricted maximum likelihood (REML) estimation with a Knapp–Hartung adjustment. Estimates were back-transformed with the inverse of the Freeman–Tukey transformation using the harmonic mean of the sample sizes and are presented here in the natural scale.

	Wild	Wild managed	Captive
Threat status + Management level			
Threatened	**17.39%** (10.26–25.85%)	**24.06%** (15.58–33.67%)	**42.84%** (31.28–54.80%)
Non-threatened	**12.40%** (6.59–19.60%)	**18.36%** (11.10–26.89%)	**36.06%** (25.35–47.51%)
Threat status × Management level			
Threatened	**17.14%** (10.05–25.58%)	**24.99%** (15.36–36.01%)	**42.19%** (27.82–57.25%)
Non-threatened	**12.38%** (6.65–19.46%)	**18.04%** (10.85–26.51%)	**36.43%** (25.00–48.68%)

Testing for presence of a significant interaction:

Likelihood ratio test (LRT): $\chi^2 = 0.2186$, $df = 2$, $p = 0.8965$

Without KH adjustment: Wald chi-squared test: $Q_M = 0.1734$, $df = 2$, $p = 0.9170$

With KH adjustment: ANOVA: $F = 0.0867$, $df_1 = 2$, $df_2 = 472$, $p = 0.9170$

Table A14. Estimated average effects and 95% confidence intervals of threat status and incubation type based on an additive and interactive model. Random effects included were effect size ID, study ID, species name, and phylogenetic history. Proportions were transformed using a Freeman–Tukey double arcsine transformation, and all models were fitted using a restricted maximum likelihood (REML) estimation with a Knapp–Hartung adjustment. Estimates were back-transformed with the inverse of the Freeman–Tukey transformation using the harmonic mean of the sample sizes and are presented here in the natural scale.

	Artificial	Natural
Threat status + Incubation type		
Threatened	**41.46%** (29.80–53.61%)	**17.71%** (9.94–27.07%)
Non-threatened	**35.51%** (24.62–47.19%)	**13.24%** (6.71–21.42%)
Threat status × Incubation type		
Threatened	**35.51%** (22.45–49.73%)	**18.46%** (10.26–28.31%)
Non-threatened	**38.93%** (26.81–51.78%)	**13.10%** (6.40–21.59%)

Testing for presence of a significant interaction:

Likelihood ratio test (LRT): $\chi^2 = 2.7029$, $df = 1$, $p = 0.1002$

Without KH adjustment: Wald chi-squared test: $Q_M = 2.8270$, $df = 1$, $p = 0.0927$

With KH adjustment: ANOVA: $F = 2.8270$, $df_1 = 1$, $df_2 = 474$, $p = 0.0934$

Table A15. Estimated average effects and 95% confidence intervals of threat status and supplementary feeding based on an additive and interactive model. Random effects included were effect size ID, study ID, species name, and phylogenetic history. Proportions were transformed using a Freeman–Tukey double arcsine transformation, and all models were fitted using a restricted maximum likelihood (REML) estimation with a Knapp–Hartung adjustment. Estimates were back-transformed with the inverse of the Freeman–Tukey transformation using the harmonic mean of the sample sizes and are presented here in the natural scale.

	Fed	Not fed
Threat status + Supplementary feeding		
Threatened	**37.16%** (26.50–48.48%)	**18.72%** (11.27–27.46%)
Non-threatened	**31.37%** (21.51–42.13%)	**14.15%** (7.90–21.76%)
Threat status × Supplementary feeding		
Threatened	**41.83%** (29.12–55.10%)	**18.29%** (11.07–26.78%)
Non-threatened	**28.88%** (18.97–39.88%)	**14.23%** (8.10–21.64%)

Testing for presence of a significant interaction:

Likelihood ratio test (LRT): $\chi^2 = 1.7543$, $df = 1$, $p = 0.1853$

Without KH adjustment: Wald chi-squared test: $Q_M = 1.7593$, $df = 1$, $p = 0.1847$

With KH adjustment: ANOVA: $F = 1.7593$, $df_1 = 1$, $df_2 = 474$, $p = 0.1853$

Table A16. Estimated average effects and 95% confidence intervals of threat status and artificial nest provision based on an additive and interactive model. Random effects included were effect size ID, study ID, species name, and phylogenetic history. Proportions were transformed using a Freeman–Tukey double arcsine transformation, and all models were fitted using a restricted maximum likelihood (REML) estimation with a Knapp–Hartung adjustment. Estimates were back-transformed with the inverse of the Freeman–Tukey transformation using the harmonic mean of the sample sizes and are presented here in the natural scale.

	Provided	**Not provided**
Threat status + Artificial nest provision		
Threatened	**26.39%** (15.55–38.84%)	**20.16%** (10.99–31.15%)
Non-threatened	**19.78%** (10.55–30.94%)	**14.22%** (6.71–21.78%)
Threat status × Artificial nest provision		
Threatened	**26.81%** (13.52–42.56%)	**20.11%** (10.95–31.10%)
Non-threatened	**19.72%** (10.47–30.90%)	**14.22%** (6.73–23.75%)

Testing for presence of a significant interaction:

Likelihood ratio test (LRT): $\chi^2 = 0.0150$, $df = 1$, $p = 0.9026$

Without KH adjustment: Wald chi-squared test: $Q_M = 0.0088$, $df = 1$, $p = 0.9253$

With KH adjustment: ANOVA: $F = 0.0088$, $df_1 = 1$, $df_2 = 474$, $p = 0.9253$

Table A17. Estimated average effects and 95% confidence intervals of *IUCN Red List* classification and management level based on an additive and interactive model. Random effects included were effect size ID, study ID, species name, and phylogenetic history. Proportions were transformed using a Freeman–Tukey double arcsine transformation, and all models were fitted using a restricted maximum likelihood (REML) estimation with a Knapp–Hartung adjustment. Estimates were back-transformed with the inverse of the Freeman–Tukey transformation using the harmonic mean of the sample sizes and are presented here in the natural scale.

	Wild	**Wild managed**	**Captive**
IUCN Red List classification + Management level			
Critically Endangered	**23.46%** (13.35–35.31%)	**31.19%** (19.82–43.80%)	**50.86%** (36.62–65.02%)
Endangered	**20.41%** (12.14–30.09%)	**27.83%** (17.99–38.85%)	**47.19%** (34.60–59.96%)
Vulnerable	**12.53%** (5.95–20.94%)	**18.88%** (10.73–28.61%)	**36.79%** (25.08–49.31%)
Near Threatened	**15.20%** (7.90–24.22%)	**21.98%** (12.97–32.50%)	**40.50%** (28.16–53.46%)
Least Concern	**12.07%** (6.23–19.37%)	**18.34%** (10.98–27.01%)	**36.12%** (25.31–47.68%)
IUCN Red List classification × Management level			
Critically Endangered	**26.32%** (14.33–40.35%)	**28.78%** (15.70–43.88%)	**42.71%** (20.14–66.96%)
Endangered	**20.12%** (11.85–29.85%)	**32.12%** (10.99–57.92%)	**49.57%** (28.51–70.70%)
Vulnerable	**10.93%** (4.46–19.57%)	**22.22%** (11.77–34.74%)	**40.04%** (20.35–61.52%)
Near Threatened	**14.24%** (6.98–23.37%)	**25.27%** (10.64–43.42%)	**48.74%** (26.31–71.43%)
Least Concern	**12.31%** (6.39–19.69%)	**17.63%** (10.29–26.36%)	**34.59%** (22.82–47.38%)

Testing for presence of a significant interaction:

Likelihood ratio test (LRT): $\chi^2 = 3.9489$, $df = 8$, $p = 0.8617$

Without KH adjustment: Wald chi-squared test: $Q_M = 3.7987$, $df = 8$, $p = 0.8748$

With KH adjustment: ANOVA: $F = 0.4748$, $df_1 = 8$, $df_2 = 463$, $p = 0.8740$

Table A18. Estimated average effects and 95% confidence intervals of *IUCN Red List* classification and incubation type based on an additive and interactive model. Random effects included were effect size ID, study ID, species name, and phylogenetic history. Proportions were transformed using a Freeman–Tukey double arcsine transformation, and all models were fitted using a restricted maximum likelihood (REML) estimation with a Knapp–Hartung adjustment. Estimates were back-transformed with the inverse of the Freeman–Tukey transformation using the harmonic mean of the sample sizes and are presented here in the natural scale.

	Artificial	Natural
IUCN Red List classification + Incubation type		
Critically Endangered	**45.65%** (31.84–59.81%)	**21.54%** (11.33–33.83%)
Endangered	**43.96%** (31.34–56.97%)	**20.14%** (11.36–30.58%)
Vulnerable	**36.49%** (24.52–49.34%)	**14.34%** (6.90–23.76%)
Near Threatened	**37.20%** (25.09–50.15%)	**14.87%** (7.20–24.54%)
Least Concern	**34.86%** (23.91–46.65%)	**13.15%** (6.58–21.42%)
IUCN Red List classification × Incubation type		
Critically Endangered	**35.83%** (18.85–54.82%)	**24.92%** (13.14–38.88%)
Endangered	**46.08%** (25.97–66.86%)	**20.49%** (11.19–31.64%)
Vulnerable	**28.45%** (12.67–47.44%)	**15.37%** (7.20–25.75%)
Near Threatened	**47.86%** (29.31–66.71%)	**14.04%** (6.16–24.28%)
Least Concern	**37.13%** (24.53–50.66%)	**13.27%** (6.29–22.19%)

Testing for presence of a significant interaction:

Likelihood ratio test (LRT): $\chi^2 = 5.7682$, $df = 4$, $p = 0.2171$

Without KH adjustment: Wald chi-squared test: $Q_M = 5.9145$, $df = 4$, $p = 0.2056$

With KH adjustment: ANOVA: $F = 1.4786$, $df_1 = 4$, $df_2 = 468$, $p = 0.2075$

Table A19. Estimated average effects and 95% confidence intervals of *IUCN Red List* classification and supplementary feeding based on an additive and interactive model. Random effects included were effect size ID, study ID, species name, and phylogenetic history. Proportions were transformed using a Freeman–Tukey double arcsine transformation, and all models were fitted using a restricted maximum likelihood (REML) estimation with a Knapp–Hartung adjustment. Estimates were back-transformed with the inverse of the Freeman–Tukey transformation using the harmonic mean of the sample sizes and are presented here in the natural scale.

	Fed	Not fed
IUCN Red List classification + Supplementary feeding		
Critically Endangered	**47.63%** (34.05–61.39%)	**26.89%** (16.29–38.98%)
Endangered	**41.05%** (29.33–53.30%)	**21.21%** (12.77–31.05%)
Vulnerable	**31.65%** (21.04–43.29%)	**13.74%** (6.86–22.38%)
Near Threatened	**35.39%** (23.95–47.71%)	**16.61%** (8.98–25.90%)
Least Concern	**32.00%** (22.03–42.84%)	**14.00%** (7.72–21.67%)
IUCN Red List classification × Supplementary feeding		
Critically Endangered	**43.15%** (23.91–63.49%)	**28.11%** (17.33–40.29%)
Endangered	**48.89%** (29.69–68.26%)	**20.96%** (12.71–30.57%)
Vulnerable	**43.33%** (27.89–59.45%)	**12.13%** (5.68–20.41%)
Near Threatened	**48.23%** (26.09–70.74%)	**15.93%** (8.53–25.00%)
Least Concern	**27.01%** (17.22–38.04%)	**14.20%** (8.03–21.67%)

Testing for presence of a significant interaction:

Likelihood ratio test (LRT): $\chi^2 = 9.0040$, $df = 4$, $p = 0.0610$

Without KH adjustment: Wald chi-squared test: $Q_M = 9.0327$, $df = 4$, $p = 0.0603$

With KH adjustment: ANOVA: $F = 2.2582$, $df_1 = 4$, $df_2 = 468$, $p = 0.0620$

Table A20. Estimated average effects and 95% confidence intervals of *IUCN Red List* classification and artificial nest provision based on an additive and interactive model. Random effects included were effect size ID, study ID, species name, and phylogenetic history. Proportions were transformed using a Freeman–Tukey double arcsine transformation, and all models were fitted using a restricted maximum likelihood (REML) estimation with a Knapp–Hartung adjustment. Estimates were back-transformed with the inverse of the Freeman–Tukey transformation using the harmonic mean of the sample sizes and are presented here in the natural scale.

	Provided	**Not provided**
IUCN Red List classification + Artificial nest provision		
Critically Endangered	**34.43%** (20.25–50.14%)	**26.94%** (14.52–41.46%)
Endangered	**30.16%** (17.87–44.04%)	**22.98%** (12.56–35.34%)
Vulnerable	**21.57%** (11.07–34.27%)	**15.24%** (6.67–26.33%)
Near Threatened	**23.58%** (12.38–36.96%)	**17.02%** (7.87–28.61%)
Least Concern	**20.07%** (10.58–31.54%)	**13.93%** (6.32–23.72%)
IUCN Red List classification × Artificial nest provision		
Critically Endangered	**40.94%** (19.78–63.97%)	**26.07%** (13.78–40.55%)
Endangered	**29.95%** (13.16–50.01%)	**22.91%** (12.61–35.10%)
Vulnerable	**21.42%** (8.27–38.39%)	**15.22%** (6.60–26.38%)
Near Threatened	**23.48%** (3.27–53.64%)	**16.95%** (7.87–28.44%)
Least Concern	**19.68%** (10.33–31.01%)	**13.92%** (6.41–23.57%)

Testing for presence of a significant interaction:

Likelihood ratio test (LRT): $\chi^2 = 0.6238$, $df = 3$, $p = 0.8910$

Without KH adjustment: Wald chi-squared test: $Q_M = 0.6035$, $df = 3$, $p = 0.8956$

With KH adjustment: ANOVA: $F = 0.2012$, $df_1 = 3$, $df_2 = 469$, $p = 0.8956$

Publication bias information

Taxonomy

Table A21. Comparison of the orders of the population records from the final dataset and the global representation of bird orders according to the *IUCN Red List* (www.iucnredlist.org/statistics; 27.10.21).

Order	Included population records				Global *IUCN* records	
	Total records	*%*	*Total species*	*%*	*Total species*	*%*
Accipitriformes	3	0.63%	2	0.83%	251	2.28%
Anseriformes	24	5.02%	11	4.56%	170	1.55%
Bucerotiformes	2	0.42%	1	0.41%	72	0.65%
Caprimulgiformes	1	0.21%	1	0.41%	597	5.43%
Cariamiformes	0	0.00%	0	0.00%	2	0.02%
Cathartiformes	6	1.26%	1	0.41%	7	0.06%
Charadriiformes	58	12.13%	36	14.94%	377	3.43%
Ciconiiformes	3	0.63%	1	0.41%	20	0.18%
Coliiformes	0	0.00%	0	0.00%	6	0.05%
Columbiformes	3	0.63%	3	1.24%	352	3.20%
Coraciiformes	3	0.63%	3	1.24%	187	1.70%
Cuculiformes	2	0.42%	2	0.83%	150	1.36%
Eurypygiformes	1	0.21%	1	0.41%	2	0.02%
Falconiformes	14	2.93%	5	2.07%	64	0.58%
Galliformes	25	5.23%	14	5.81%	306	2.78%
Gaviiformes	0	0.00%	0	0.00%	5	0.05%
Gruiformes	13	2.72%	7	2.90%	169	1.54%
Leptosomiformes	0	0.00%	0	0.00%	1	0.01%
Mesitornithiformes	0	0.00%	0	0.00%	3	0.03%
Musophagiformes	0	0.00%	0	0.00%	24	0.22%
Opisthocomiformes	0	0.00%	0	0.00%	1	0.01%
Otidiformes	2	0.42%	1	0.41%	26	0.24%
Passeriformes	245	51.26%	106	43.98%	6602	60.05%
Pelecaniformes	12	2.51%	8	3.32%	110	1.00%
Phaethontiformes	0	0.00%	0	0.00%	3	0.03%
Phoenicopteriformes	0	0.00%	0	0.00%	6	0.05%
Piciformes	4	0.84%	4	1.66%	484	4.40%
Podicipediformes	1	0.21%	1	0.41%	20	0.18%
Procellariiformes	10	2.09%	6	2.49%	145	1.32%
Psittaciformes	12	2.51%	10	4.15%	403	3.67%
Pterocliformes	6	1.26%	1	0.41%	16	0.15%
Sphenisciformes	11	2.30%	6	2.49%	18	0.16%
Strigiformes	11	2.30%	5	2.07%	238	2.16%
Struthioniformes	5	1.05%	4	1.66%	61	0.55%
Suliformes	1	0.21%	1	0.41%	53	0.48%
Trogoniformes	0	0.00%	0	0.00%	43	0.39%
Total	478		241		10994	

Chi-squared goodness of fit test

Comparing global distribution to number of records in final dataset

$\chi^2 = 586.83$, df = 35, $p < 2.2e\text{-}16$

Comparing global distribution to number of species in final dataset

$\chi^2 = 290.25$, df = 35, $p < 2.2e\text{-}16$

Threat status

Table A22. Comparison of the *IUCN Red List* classification of the population records from the included dataset and the global representation of bird orders according to the *IUCN Red List* (www.iucnredlist.org/statistics; 27.10.21).

Threat status	Included population records				Global *IUCN* records	
	Total records	*%*	*Total species*	*%*	*Total species*	*%*
Critically Endangered	20	4.18%	13	5.33%	223	2.03%
Endangered	39	8.16%	25	10.25%	460	4.18%
Vulnerable	37	7.74%	25	10.25%	798	7.26%
Near Threatened	35	7.32%	23	9.43%	1001	9.10%
Least Concern	347	72.59%	158	64.75%	8460	76.95%
Data Deficient	0	0.00%	0	0.00%	52	0.47%
Total	478		244		10,994	

Note: total number of unique species across all threat categories is 244 rather than 241 because for some subspecies the parent species was used for the phylogenetic correction but the subspecies had a different threat status.

Chi-squared goodness of fit test

Comparing global distribution to number of records in final dataset

$\chi^2 = 31.84$, df = 4, $p = 2.063e\text{-}06$

Comparing global distribution to number of species in final dataset

$\chi^2 = 42.064$, df = 4, $p = 1.618e\text{-}08$

Publication year

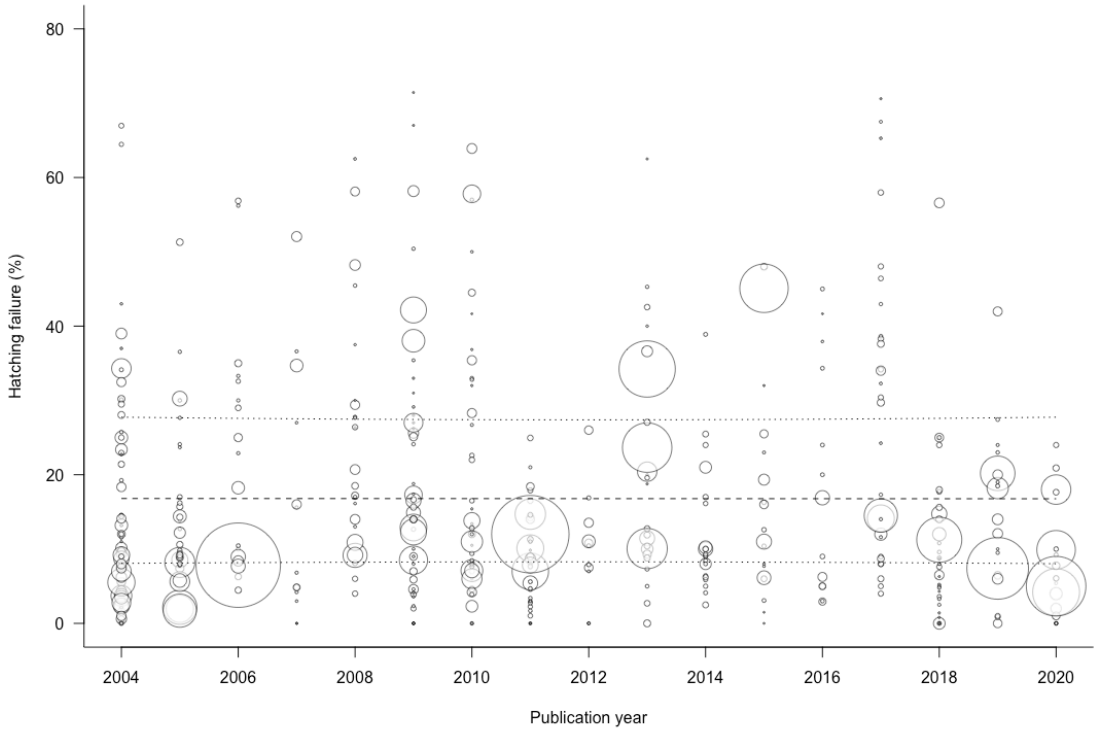


Figure A3. Bubble plot showing the relationship between hatching failure percentage and publication year. The dashed line represents the line of fit from a meta-regression including publication year as a fixed effect (moderator) and effect size ID, study ID, species name, and phylogenetic history as random effects. Dotted lines represent the 95% confidence interval around the fitted line. Points are scaled according to the inverse of their variance meaning that larger points represent more reliable estimates and are given greater weights in the model. There was no significant relationship between hatching success proportion and publication year (p = 0.9897). Slope estimate for the line of fit is –0.002.

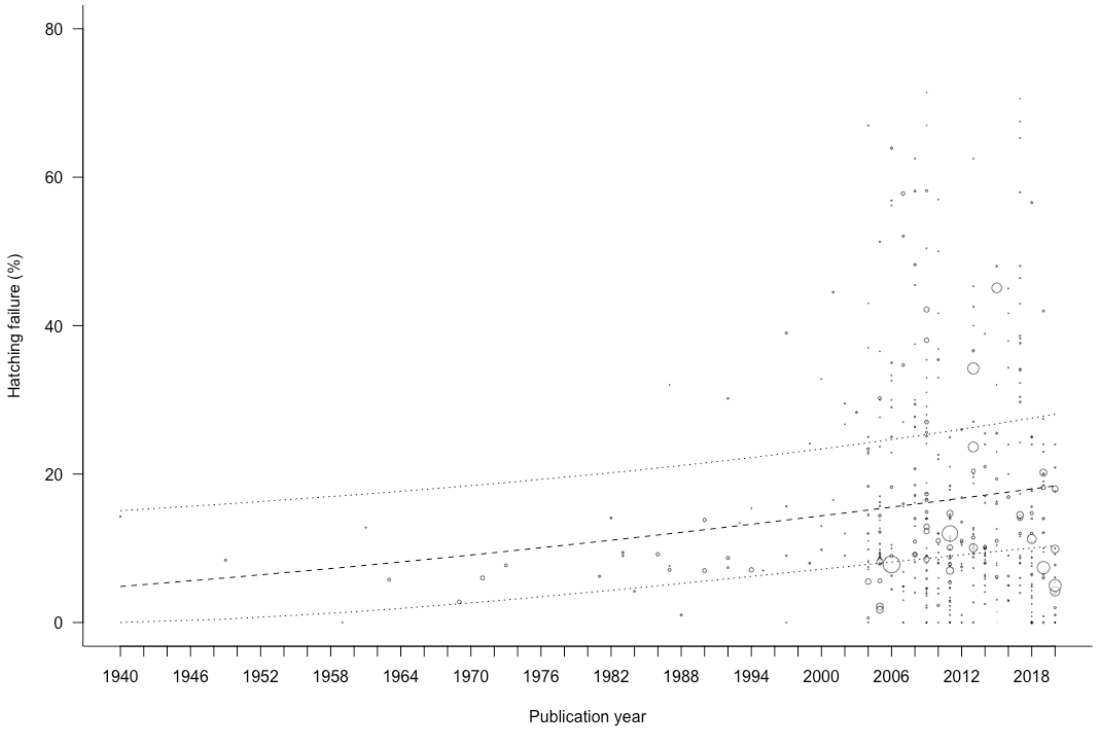


Figure A4. Bubble plot showing the relationship between hatching failure percentage and original publication year where available. The dashed line represents the line of fit from a meta-regression including original publication year as a fixed effect (moderator) and effect size ID, study ID, species name, and phylogenetic history as random effects. Dotted lines represent the 95% confidence interval around the fitted line. Points are scaled according to the inverse of their variance meaning that larger points represent more reliable estimates and are given greater weights in the model. There was a significant relationship between hatching success proportion and original publication year (p = 0.0037). Slope estimate for the line of fit is 0.176.

Additional sensitivity analyses

Table A23. Mean hatching failure percentage (HF%) estimates with 95% confidence intervals (CI) from the univariate models with incubation type, supplementary feeding, and artificial nest provision as moderators, excluding captive populations. Random effects for each model were effect size ID, study ID, species name, and phylogenetic history. Proportions were transformed using a Freeman–Tukey double arcsine transformation, and all models were fitted using a restricted maximum likelihood (REML) estimation with a Knapp–Hartung adjustment. A significant F statistic indicates that at least one of the regression coefficients of the moderator categories significantly deviates from zero, indicating that that moderator influences hatching failure. Estimates were back-transformed with the inverse of the Freeman–Tukey transformation using the harmonic mean of the sample sizes and are presented here in the natural scale.

Moderator and categories	*N* effect sizes	*N* studies	*N* species	Mean HF%	Lower 95% CI	Upper 95% CI	*F*	*P*
Incubation type								
- Artificial	29	15	19	31.43	20.87	43.03	$F_{1,435}$ =	<0.0001
- Natural	408	201	223	13.91	7.67	21.53	30.133	
Supplementary feeding								
- Fed	10	7	7	24.39	13.46	37.25	$F_{1,435}$ =	0.0166
- Not fed	427	211	230	15.11	8.34	23.35	5.784	
Artificial nest provision								
- Provided	141	68	50	17.74	9.63	27.57	$F_{1,435}$ =	0.1340
- Not provided	296	149	193	14.83	7.82	23.48	2.254	

Table A24. Mean hatching failure percentage (HF%) estimates with 95% confidence intervals (CI) from the univariate models with incubation type, supplementary feeding, and artificial nest provision as moderators, where only the intervention included as a moderator was being applied to the studied population. Random effects for each model were effect size ID, study ID, species name, and phylogenetic history. Proportions were transformed using a Freeman–Tukey double arcsine transformation, and all models were fitted using a restricted maximum likelihood (REML) estimation with a Knapp–Hartung adjustment. A significant *F* statistic indicates that at least one of the regression coefficients of the moderator categories significantly deviates from zero, indicating that that moderator influences hatching failure. Estimates were back-transformed with the inverse of the Freeman–Tukey transformation using the harmonic mean of the sample sizes and are presented here in the natural scale.

Moderator and categories	*N* effect sizes	*N* studies	*N* species	Mean HF%	Lower 95% CI	Upper 95% CI	*F*	*P*
Incubation type								
- Artificial	19	10	15	34.31	23.49	46.00	$F_{1,282}$ =	<0.0001
- Natural	265	134	178	13.25	7.46	20.28	36.696	
Supplementary feeding								
- Fed	5	3	3	36.30	19.76	54.66	$F_{1,268}$ =	0.0014
- Not fed	265	134	178	14.64	7.70	23.22	10.411	
Artificial nest provision								
- Provided	127	63	49	16.54	9.30	25.27	$F_{1,390}$ =	0.1374
- Not provided	265	134	178	13.84	7.64	21.43	2.216	

Table A25. Mean hatching failure percentage (HF%) estimates with 95% confidence intervals (CI) for all moderator levels from the univariate meta-regression models when records for which the sample size was estimated based on the mean clutch size from Cooney *et al*. (2020) were removed. Random effects for each model were effect size ID, study ID, species name, and phylogenetic history. Proportions were transformed using a Freeman–Tukey double arcsine transformation, and all models were fitted using a restricted maximum likelihood (REML) estimation with a Knapp–Hartung adjustment. A significant *F* statistic indicates that at least one of the regression coefficients of the moderator categories significantly deviates from zero, indicating that that moderator influences hatching failure. Estimates were back-transformed with the inverse of the Freeman–Tukey transformation using the harmonic mean of the sample sizes and are presented here in the natural scale.

Moderator and categories	*N* effect sizes	*N* studies	*N* species	Mean HF%	Lower 95% CI	Upper 95% CI	*F*	*P*
None								
- All	418	226	210	17.00	8.22	27.99	–	–
Threat status								
- Threatened	82	50	52	21.48	11.73	33.12	$F_{1,416}$ = 8.101	0.0046
- Non-threatened	336	181	159	15.46	7.43	25.58		
IUCN Red List classification								
- Least Concern	302	162	137	15.42	7.02	26.17	$F_{1,413}$ = 3.141	0.0146
- Near Threatened	34	23	22	17.22	7.61	29.53		
- Vulnerable	30	23	20	17.68	7.91	30.12		
- Endangered	34	21	21	23.63	12.44	36.97		
- Critically Endangered	18	10	12	26.98	13.81	42.55		
Management level								
- Wild	230	133	154	13.90	7.63	21.56	$F_{1,415}$ = 33.613	<0.0001
- Wild managed	147	81	61	20.47	12.69	29.50		
- Captive	41	21	16	38.11	27.13	49.73		
Incubation type								
- Artificial	53	23	28	37.97	26.99	49.60	$F_{1,416}$ = 85.497	<0.0001
- Natural	365	205	194	14.35	7.59	22.69		
Supplementary feeding								
- Fed	51	27	23	34.07	24.19	0.4469	$F_{1,416}$ = 58.048	<0.0001
- Not fed	367	206	198	15.34	8.96	0.2297		
Artificial nest provision								
- Provided	136	75	43	21.46	11.20	33.84	$F_{1,416}$ = 5.926	0.0153
- Not provided	282	155	175	16.27	7.71	27.08		

Table A26. Mean hatching failure percentage (HF%) estimates with 95% confidence intervals (CI) for all moderator levels from the univariate meta-regression models for the Briskie & Mackintosh (2004) removed dataset. Random effects for each model were effect size ID, study ID, species name, and phylogenetic history. Proportions were transformed using a Freeman–Tukey double arcsine transformation, and all models were fitted using a restricted maximum likelihood (REML) estimation with a Knapp–Hartung adjustment. A significant *F* statistic indicates that at least one of the regression coefficients of the moderator categories significantly deviates from zero, indicating that that moderator influences hatching failure. Estimates were back-transformed with the inverse of the Freeman–Tukey transformation using the harmonic mean of the sample sizes and are presented here in the natural scale.

Moderator and categories	*N* effect sizes	*N* studies	*N* species	Mean HF%	Lower 95% CI	Upper 95% CI	*F*	*P*
None								
- All	442	230	219	16.69	8.91	26.18	–	–
Threat status								
- Threatened	79	50	50	21.74	13.19	31.66	$F_{1,440}$ = 10.301	0.0014
- Non-threatened	363	185	170	15.00	8.18	23.33		
IUCN Red List classification								
- Least Concern	330	167	149	14.87	8.03	23.25	$F_{4,437}$ = 3.711	0.0055
- Near Threatened	33	22	21	16.87	8.61	27.06		
- Vulnerable	30	23	20	18.37	9.78	28.80		
- Endangered	32	20	20	22.86	13.33	34.00		
- Critically Endangered	17	9	11	28.81	16.61	42.74		
Management level								
- Wild	229	133	151	13.52	7.81	20.41	$F_{2,439}$ = 34.055	<0.0001
- Wild managed	172	85	74	19.86	12.76	28.01		
- Captive	41	21	16	37.84	27.55	48.69		
Incubation type								
- Artificial	53	23	28	37.70	27.36	48.62	$F_{1,440}$ = 82.621	<0.0001
- Natural	389	209	203	14.12	7.86	21.73		
Supplementary feeding								
- Fed	51	27	23	34.10	24.92	43.90	$F_{1,440}$ = 61.234	<0.0001
- Not fed	391	210	207	15.03	9.25	21.86		
Artificial nest provision								
- Provided	161	79	56	20.86	11.72	31.71	$F_{1,440}$ = 5.893	0.0156
- Not provided	281	155	172	15.87	8.26	25.25		

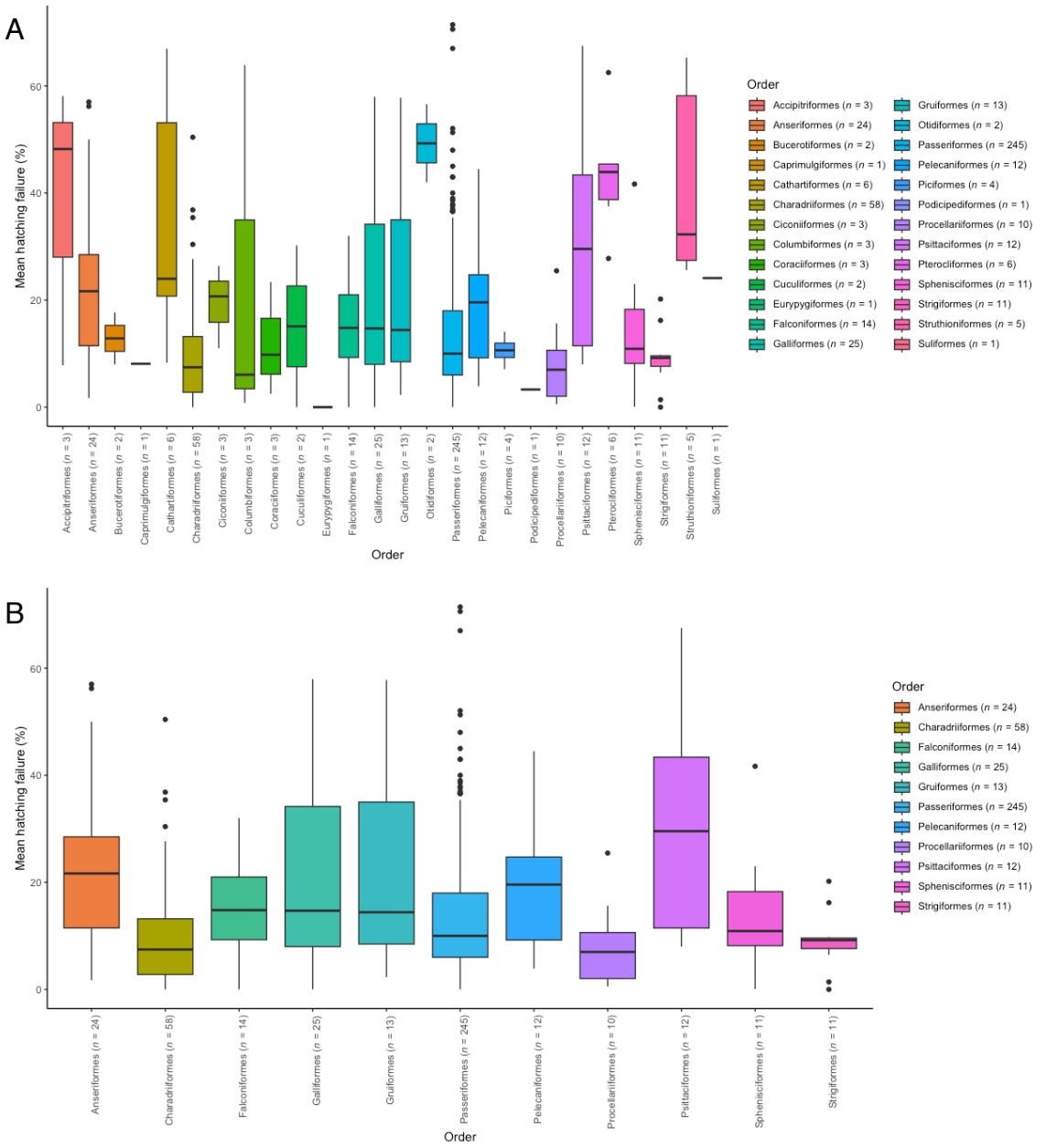


Figure A5. Mean hatching failure (%) across: (A) all effect sizes in the final dataset grouped by taxonomic order; (B) effect sizes in the final dataset grouped by taxonomic order, limited to orders containing at least 10 effect sizes. Each box shows the interquartile range (IQR) of hatching failure values, extending from the first quartile (Q_1) to the third quartile (Q_3), with an intermediate line representing the median (Q_2). The lower and upper whiskers extend to the smallest value at most 1.5 × IQR from the first quartile [Q_1 – 1.5(IQR)] and the largest value no further than 1.5 × IQR from the third quartile [Q_3 + 1.5(IQR)] respectively. Individual points represent 'outlying' data beyond this range.

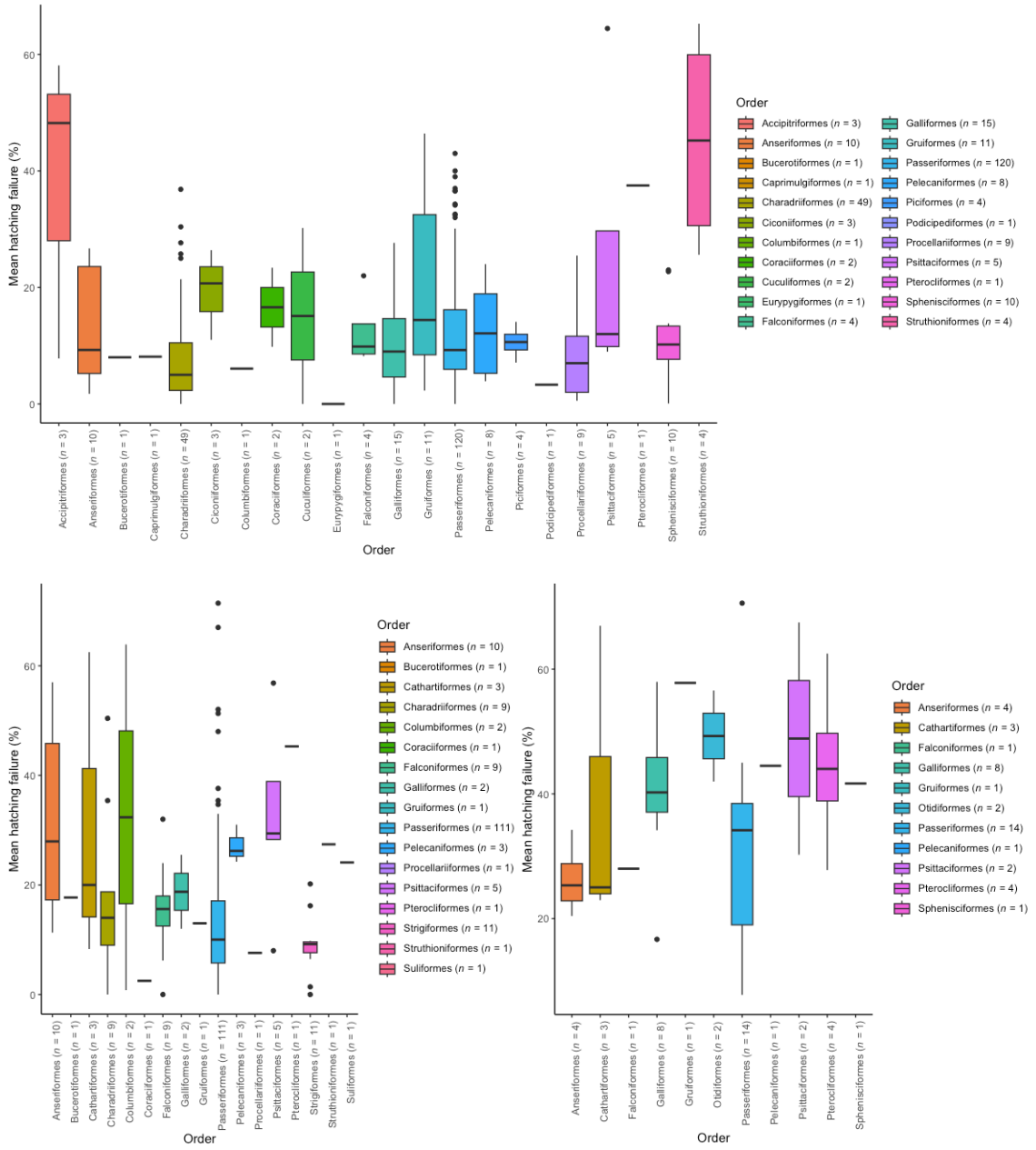


Figure A6. Mean hatching failure (%) across: (A) effect sizes in the final dataset for wild populations only, grouped by taxonomic order; (B) effect sizes in the final dataset for wild managed populations only, grouped by taxonomic order; (C) effect sizes in the final dataset for captive populations only, grouped by taxonomic order. Each box shows the interquartile range (IQR) of hatching failure values, extending from the first quartile (Q_1) to the third quartile (Q_3), with an intermediate line representing the median (Q_2). The lower and upper whiskers extend to the smallest value at most 1.5 × IQR from the first quartile [Q_1 – 1.5(IQR)] and the largest value no further than 1.5 × IQR from the third quartile [Q_3 + 1.5(IQR)] respectively. Individual points represent 'outlying' data beyond this range.

Appendix B: Supplementary information for Chapter 3

Summary of previous studies using fluorescence microscopy

Table B1. Summary of studies which have used fluorescence microscopy methods to assess egg fertility status. 'Unhatched' refers to all eggs failing to hatch at the end of the incubation period, while 'undeveloped' refers only to failed eggs with no apparent signs of embryo development. Asterisk (*) indicates studies included in the meta-analysis in **Chapter 2** and in **Figure 3-1.** *IUCN Red List Classification* at the time of the study is as follows: LC, Least Concern; NT, Near Threatened; VU, Vulnerable; CR, Critically Endangered; NA, Not Assessed. Where a species had not yet been assessed at the time of the study the first assessment after the study's completion is included in parentheses.

Reference	Study Species	Population	*IUCN Red List* Classification	Findings		
				% unhatched eggs unfertilised	% undeveloped eggs unfertilised	% population fertilisation failure rate
(Birkhead *et al.*, 1995)	House sparrow (*Passer domesticus*)	Wild	LC	–	27.3% (*n* = 22)	~1.2% (*n* = 518)
(Kempenaers *et al.*, 1999)	Tree swallow (*Tachycineta bicolor*)	Wild (nestbox)	LC	0-25.6% (*n* = 43)†	0% (*n* = 8)	0-4.0% (*n* = 272)†
(Birkhead *et al.*, 2008)	Tree sparrow (*P. montanus*)	Wild (nestbox)	LC	0% (*n* = 36)	–	–
*(White *et al.*, 2008)	Black-legged kittiwake (*Rissa tridactyla*)	Wild	LC	Sperm aging negatively affected fertilisation potential.		
*(Heber, 2012)	South Island robin (*Petroica australis*)	Wild	NA (LC)	10.4% (*n* = 77)	–	–
(Hemmings *et al.*, 2012a)	Zebra finch (*Taeniopygia guttata*)	Captive	NA (LC)	Sib-sib and unrelated pairs produced similar proportions of unfertilised eggs.		
(Hemmings *et al.*, 2012b)	Hihi (stitchbird; *Notiomystis cincta*)	Wild	VU	–	12% (*n* = 120)	–
	Yellow-shouldered Amazon parrot (*Amazona barbadensis*)	Wild	VU	–	0% (*n* = 10)	–
	Helmeted honeyeater (*Lichenostomus melanops cassidix*)	Wild	CR	–	0% (*n* = 4)	–
		Captive	CR		32% (*n* = 19)	
	Orange-bellied parrot (*Neophema chrysogaster*)	Captive	CR	–	60% (*n* = 146)	–
	Spix's macaw (*Cyanopsitta spixii*)	Captive	CR	–	55% (*n* = 40)	–
(Croyle *et al.*, 2015)	72 exotic species	Captive	–	Sperm detected in perivitelline layer of 54.2% of species.		
(Hemmings & Birkhead, 2015)	Zebra finch (*T. guttata*)	Captive	NA (LC)	Experimental sperm limitation resulted in high proportion of unfertilised eggs in fowl, but not zebra finch.		
	Domestic fowl (*Gallus gallus*)	Captive	LC			
*(O'Brien *et al.*, 2016)	Magellanic penguin (*Spheniscus magellanicus*)	Captive	NT	20% (*n* = 5)	–	8.3% (*n* = 12)
*(Kato *et al.*, 2017)	Tree sparrow (*P. montanus*)	Wild (nestbox)	LC	9.8% (*n* = 82)	17.8% (*n* = 45)	3.7% (*n* = 218)
*(Vuarin *et al.*, 2018)	Houbara bustard (*Chlamydotis undulata undulata*)	Captive	VU	2.4-15.9% (*n* = 245)‡	15.4-100% (*n* = 39)‡	1.4-9.0% (*n* = 433)‡

(Girndt *et al.*, 2019)	House sparrow (*P. domesticus*)	Captive	LC	–	4.9% (*n* = 41)§	–
*(van Oosten *et al.*, 2019)	Northern wheatear (*Oenanthe oenanthe*)	Wild	LC	0% (*n* = 103)	0% (*n* = 37)	–
*(Vuarin *et al.*, 2019)	Houbara bustard (*C. undulata undulata*)	Captive	VU	16.6% (*n* = 157)	–	7.0% (*n* = 374)
*(Hemmings & Evans, 2020)	Great tit (*Parus major*)	Wild (nestbox)	LC	1.3% (*n* = 375)	–	~0.1% (*n* = 6788)
	Blue tit (*Cyanistes caeruleus*)	Wild (nestbox)	LC	2.2% (*n* = 416)	–	~0.1% (*n* = 10,047)
*(Hurley *et al.*, 2020)	Zebra finch (*T. guttata*)	Captive	LC	10.5% (*n* = 20)	–	0.8% (*n* = 258)
(Savage *et al.*, 2021)	Kākāpō (*Strigops habroptilus*)	Wild managed	CR	21.7% (n = 157)	27.4% (*n* = 124)	13.7% (*n* = 248)
This study	Kakī (black stilt; *Himantopus novaezelandiae*)	Wild managed	CR	0-2.9% (*n* = 35)‡	0-16.7% (*n* = 6)‡	0-0.82% (*n* = 122)‡
		Captive	CR	0% (*n* = 8)	0% (*n* = 2)	0% (*n* = 43)

†Not all undeveloped eggs were able to be examined, hence a range is estimated with the lower value calculated assuming all unexamined eggs were fertilised, and the upper value assuming all unexamined eggs were unfertilised.

‡Not all eggs could be unambiguously assigned as fertility or not, hence a range is estimated with the lower value calculated assuming all ambiguous eggs were fertilised, and the upper value assuming all ambiguous eggs were unfertilised.

§Eggs were experimentally removed before the onset of incubation and immediately examined, hence were equivalent to undeveloped eggs but were not truly eggs that had failed to hatch.

Assessment of usage of egg examination methods in records from systematic review

Table B2. The reported methods for determination of fertility status of unhatched eggs in each record.

Determination of Unhatched Egg Fertility	Number of Records
Examined	
Candled only	19 (3.3%)
Methods not stated	33 (6.9%)
Floating	1 (0.2%)
Fluorescence microscopy	12 (2.5%)
Necropsy and visual examination	97 (20.3%)
Shaking	2 (0.4%)
Not Examined or Unknown	
NA	47 (9.8%)
None	267 (55.9%)
Total	**478**

For each population record the type of examination conducted on unhatched eggs was recorded from the text or supplementary material and categorised into the following:

- "Candled only" – Unhatched eggs were examined using a candling procedure to detect blood vessels and/or embryo presence (e.g. Aourir et al., 2008).
- "Methods not stated" – Eggs are described as being infertile or having experienced embryo mortality, but the method used to determine this is not stated (e.g. Wu et al., 2017).
- "Floating" – Unhatched eggs were floated to determine when the embryo development progress had ceased (e.g. Herzog et al., 2020).
- "Fluorescence microscopy" – Unhatched eggs were examined using a fluorescence microscopy method as described by Birkhead *et al.* (2008) or similar (e.g. O'Brien et al., 2016).
- "NA" – It was not possible to determine if unhatched eggs were examined as the original source of the reported hatching failure rate was 'personal communications' or otherwise could not be accessed. In cases where there was 100% hatching success there were no unhatched eggs to be examined hence the record was also marked as NA (e.g. Williams & DeLeon, 2020).
- "Necropsy and visual examination" – Unhatched eggs were opened and contents were visually examined to detect the presence or absence of an embryo or other evidence of development (e.g. Taylor *et al.*, 2017).

- “None” – Unhatched eggs were not differentiated as being infertile or having experienced embryo mortality, and there was no mention of examination of the eggs. In some cases unhatched eggs were collected to be measured or for contaminant analyses, which may have involved opening eggs and examining contents, but any determination of fertility during these analyses was not mentioned (e.g. Friedl & Klump, 2005).
- “Shaking” – Unhatched eggs were shaken to determine whether or not they were addled (e.g. Kim *et al.*, 2010).

Summary of eggs collected from the ZSL Living Bird Collection

Table B3. Summary of study species from the ZSL Living Bird Collection, their IUCN Red List classification (LC = Least Concern, NT = Near Threatened, VU = Vulnerable, CR = Critically Endangered), reported hatching failure rate (where available), and the total number of eggs examined in this study. Asterisk (*) indicates studies in which the definition of hatching failure does not match that used throughout the thesis (e.g. includes eggs lost to predation).

Species	*IUCN Red List* Classification	Reported Hatching Failure Rate	Elective Removal	Failed	Total
Abdim's stork (*Ciconia abdimii*)	LC	23.5% * (Adjakpa, 2000) – wild	0	1	1
African comb duck (*Sarkidiornis melanotos*)	LC	–	0	1	1
Black-cheeked lovebird (*Agapornis nigrigenis*)	VU	30.2% (Warburton & Perrin, 2005) – captive	3	12	15
Blue-crowned laughingthrush (*Garrulax courtoisi*)	CR	5.3% (Liao *et al.*, 2007; Zhang *et al.*, 2017) – wild	1	2	3
Blue-throated macaw (*Ara glaucogularis*)	CR	38.9% (Berkunsky *et al.*, 2014) – wild managed	1	2	3
Collared hill partridge (*Arborophila gingica*)	NT	–	0	3	3
Crested wood partridge (*Rollulus rouloul*)	NT	–	0	6	6
Grey-capped emerald dove (*Chalcophaps indica*)	LC	–	0	4	4
Helmeted guineafowl (*Numida meleagris*)	LC	28%* (Koné *et al.*, 2018); 36.2%* (Zvakare *et al.*, 2018) – captive	0	1	1
Hottentot teal (*Spatula hottentota*)	LC	–	0	5	5
Humboldt penguin (*Spheniscus humboldti*)	VU	30.0% (Scholten, 1987) – captive	58	20	78
Magpie goose (*Anseranas semipalmata*)	LC		0	1	1
Mount Omei babbler (*Liocichla omeiensis*)	VU	41.4%* (Fu *et al.*, 2011) – wild	0	6	6
Orange-headed thrush (*Geokichla citrina*)	LC	–	0	12	12
Red-cheeked cordon-bleu (*Uraeginthus bengalus*)	LC	–	0	3	3
Red-whiskered bulbul (*Pycnonotus jocosus*)	LC	21.4% (Khan & Naher, 2020); 9.0% (Li *et al.*, 2015) – wild	0	3	3
Stanley crane (*Anthropoides paradiseus*)	VU	–	0	3	3
Sulawesi hornbill (*Rhabdotorrhinus exarhatus sanfordi*)	VU	–	0	3	3
Superb fruit dove (*Ptilinopus superbus*)	LC	–	0	3	3
Tricoloured parrot finch (*Erythrura tricolor*)	LC	–	0	6	6
Ultramarine grosbeak (*Passerina brissonii*)	LC	–	0	2	2
Victoria crowned pigeon (*Goura victoria*)	VU	–	0	1	1
Violet turaco (*Musophaga violacea*)	LC	–	0	5	5
Waldrapp ibis (*Geronticus eremita*)	CR	82.1% (Bowden *et al.*, 2003) – wild; 75.7% (Yeniyurt *et al.*, 2017) – wild managed	0	1	1
Wattled crane (*Bugeranus carunculatus*)	VU	–	0	1	1
White-faced whistling duck (*Dendrocygna viduata*)	LC	–	3	3	6
Zebra finch (*Taeniopygia guttata*)	LC	17.1% (Zann, 1996) – wild	0	1	1
			66	111	177

Fluorescence microscopy fertility diagnoses criteria

Table B4. Criteria for diagnosing fertility using Hoechst 33342 DNA staining and fluorescence microscopy methods.

	Diagnosis	Criteria
	Fertilised	Located embryo. *OR* Located germinal disc: embryonic cells present, sperm cells present/absent. *OR* Didn't locate germinal disc: embryonic cells present, sperm cells present/absent.
	Unfertilised	Located germinal disc: embryonic cells absent, sperm cell absent.
Undetermined	**Inconclusive**	Didn't locate germinal disc: embryonic cells absent, sperm cells present/absent. *OR* Located germinal disc: embryonic cells absent, sperm cells present. *OR* Unsure if germinal disc located: embryonic cells absent, sperm cells present/absent. *OR* Unidentified cells present = potentially sperm/embryonic cells or contamination.
	Unknown	No examinable material.

Checks for multicollinearity

Table B5. Assessment of correlations between continuous variables using Pearson correlation coefficients. Cut-offs for interpretation were as follows: ±1.0-0.9 = very high correlation; ±0.9-0.7 = high correlation; ±0.7-0.5 = moderate correlation; ±0.5-0.3 = low correlation; ±0.3-0.1 = very low correlation; ±0.1-0.0 = negligible correlation.

Parameter 1	Parameter 2	*r*	df	*p*-value	Correlation Interpretation
Dam age	Sire age	0.3262	1139	**< 0.001**	low
Dam age^2	Sire age^2	0.2866	1139	**< 0.001**	very low
Dam age	Clutch number	0.0540	1139	0.0680	negligible
Dam age^2	Clutch number	0.0276	1139	0.3524	negligible
Dam age	Clutch size	-0.0524	1139	0.0767	negligible
Dam age^2	Clutch size	-0.0815	1139	**0.0059**	negligible
Dam age	Estimated embryo age	0.0875	1139	**0.0031**	negligible
Dam age^2	Estimated embryo age	0.1237	1139	**< 0.001**	very low
Dam age	Egg weight	-0.1032	1139	**< 0.001**	very low
Dam age^2	Egg weight	-0.1308	1139	**< 0.001**	very low
Sire age	Clutch number	0.0852	1139	**0.0040**	negligible
Sire age^2	Clutch number	0.0756	1139	**0.0106**	negligible
Sire age	Clutch size	0.0303	1139	0.3072	negligible
Sire age^2	Clutch size	0.0129	1139	0.6632	negligible
Sire age	Estimated embryo age	0.1995	1139	**< 0.001**	very low
Sire age^2	Estimated embryo age	0.1929	1139	**< 0.001**	very low
Sire age	Egg weight	0.0079	1139	0.7899	negligible
Sire age^2	Egg weight	-0.0126	1139	0.6713	negligible
Egg weight	Estimated embryo age	-0.3502	1139	**< 0.001**	low

Table B6. Assessment of correlations between categorical variables using pairwise chi-squared tests of independence and Cramér's V. Interpretation of Cramér's V values were as follows: 0.1–0.3 = weak association; 0.3–0.5 = moderate association; and >0.5 = strong association.

Parameter 1	Parameter 2	*Cramér's V*	df	*p*-value	Correlation Interpretation
Nest location	Egg captive- vs. wild-laid	0.9980	27	**< 0.001**	strong

Table B7. Assessment of correlations between categorical and continuous variables using point biserial correlation coefficients. Cut-offs for interpretation were as follows: ±1.0-0.9 = very high correlation; ±0.9-0.7 = high correlation; ±0.7-0.5 = moderate correlation; ±0.5-0.3 = low correlation; ±0.3-0.1 = very low correlation; ±0.1-0.0 = negligible correlation.

Parameter 1	Parameter 2	r_{pb}	df	*p*-value	Correlation Interpretation
Dam age	Egg captive- vs. wild-laid	-0.1295	1139	**< 0.001**	very low
Dam age^2	Egg captive- vs. wild-laid	-0.0864	1139	**0.0035**	negligible
Egg weight	Egg captive- vs. wild-laid	0.1617	1139	**< 0.001**	very low
Estimated embryo age	Egg captive- vs. wild-laid	0.0712	1139	**0.0161**	negligible
Clutch number	Egg captive- vs. wild-laid	-0.2656	1139	**< 0.001**	very low
Clutch size	Egg captive- vs. wild-laid	0.0500	1139	0.0916	negligible

Table B8. The fertility diagnosis based on necropsy examination and the results of Hoechst 33342 DNA staining and fluorescence microscopy fertility examinations of undeveloped eggs from ZSL Living Bird Collection collected between January 2019 and May 2021. GD = germinal disc, ECN = embryonic cell nuclei, TS = trapped sperm, Emb. = embryo. An asterisk (*) indicates embryos that were detected during the necropsy examination.

Egg ID	Species	Necropsy Fertility Diagnosis	Addled	Fertility Examination			Microscopy Fertility Diagnosis
				GD found	ECN	TS	
W19E131	*Agapornis nigrigenis*	Fertilised	Yes	Emb.	NA	NA	Embryo (24-25)*
W19E133	*Agapornis nigrigenis*	Fertilised	No	Emb.	NA	NA	Embryo (~21)
W19E137	*Agapornis nigrigenis*	Unfertilised	No	No	Yes	Yes	Fertilised
W19E138	*Agapornis nigrigenis*	Unfertilised	No	No	Yes	No	Fertilised
W19E139	*Agapornis nigrigenis*	Unfertilised	No	No	Yes	Yes	Fertilised
W19E140	*Agapornis nigrigenis*	Unfertilised	No	No	Yes	Yes	Fertilised
W19E141	*Agapornis nigrigenis*	Unfertilised	No	No	Yes	Yes	Fertilised
W19E142	*Agapornis nigrigenis*	Unfertilised	No	No	Yes	No	Fertilised
W19E176	*Agapornis nigrigenis*	NA	Yes	Emb.	NA	NA	Embryo (21-22)
ZB906/19	*Agapornis nigrigenis*	Undetermined	No	Yes	Yes	Yes	Fertilised
ZB907/19	*Agapornis nigrigenis*	Undetermined	No	Yes	Yes	Yes	Fertilised
ZB908/19	*Agapornis nigrigenis*	Undetermined	No	Yes	Yes	Yes	Fertilised
W19E180	*Agapornis nigrigenis*	Unfertilised	No	Yes	Yes	Yes	Fertilised
W19E181	*Agapornis nigrigenis*	Unfertilised	No	Yes	?	No	Inconclusive
W19E182	*Agapornis nigrigenis*	Unfertilised	No	Yes	Yes	No	Fertilised
W21B192	*Anseranas semipalmata*	NA	No	No	No	Yes	Inconclusive
W20E190	*Anthropoides paradiseus*	Unfertilised	No	No	Yes	No	Fertilised
W21E192	*Anthropoides paradiseus*	Unfertilised	Yes	No	Yes	No	Fertilised
W21E193	*Anthropoides paradiseus*	Unfertilised	Yes	No	Yes	No	Fertilised
ZB327/19	*Ara glaucogularis*	Fertilised	No	Yes	Yes	Yes	Fertilised
ZB337/19	*Ara glaucogularis*	Undetermined	No	No	Yes	No	Fertilised
ZB345/19	*Ara glaucogularis*	Unfertilised	No	Yes	Yes	No	Fertilised
ZB445/21	*Arborophila gingica*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB446/21	*Arborophila gingica*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB447/21	*Arborophila gingica*	Unfertilised	No	Yes	Yes	No	Fertilised
W21B142	*Bugeranus carunculatus*	Unfertilised	No	No	Yes	No	Fertilised
ZB217/19	*Chalcophaps indica*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB251/19	*Chalcophaps indica*	Undetermined	No	No	Yes	Yes	Fertilised
ZB464/19	*Chalcophaps indica*	Unfertilised	No	No	Yes	Yes	Fertilised
ZB801/19	*Chalcophaps indica*	Unfertilised	No	No	Yes	Yes	Fertilised
ZB492/20	*Ciconia abdimii*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB502/19	*Dendrocygna viduata*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB541/19	*Dendrocygna viduata*	Indeterminate	No	Yes	Yes	Yes	Fertilised
ZB665/19	*Dendrocygna viduata*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB267/20	*Dendrocygna viduata*	Unfertilised	No	No	Yes	No	Fertilised
ZB268/20	*Dendrocygna viduata*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB270/20	*Dendrocygna viduata*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB687/19	*Erythrura tricolor*	Unfertilised	No	No	No	No	Unknown
ZB688/19	*Erythrura tricolor*	Unfertilised	No	No	No	No	Unknown
ZB689/19	*Erythrura tricolor*	Unfertilised	No	No	Yes	No	Fertilised
ZB383/21	*Erythrura tricolor*	Unfertilised	Yes	Emb.	NA	NA	Embryo (?)
ZB384/21	*Erythrura tricolor*	Unfertilised	No	Emb.	NA	NA	Embryo (25)
ZB433/21	*Erythrura tricolor*	Unfertilised	No	Yes	Yes	Yes	Fertilised
W19E117	*Garrulax courtoisi*	Fertilised	No	No	Yes	Yes	Fertilised
W19E118	*Garrulax courtoisi*	Fertilised	No	Yes	Yes	Yes	Fertilised
W19E119	*Garrulax courtoisi*	Fertilised	No	Yes	Yes	Yes	Fertilised
ZB307/19	*Geokichla citrina*	Undetermined	No	No	?	No	Inconclusive
ZB308/19	*Geokichla citrina*	Undetermined	No	No	?	No	Inconclusive
ZB309/19	*Geokichla citrina*	Undetermined	No	No	?	No	Inconclusive
ZB455/19	*Geokichla citrina*	Unfertilised	No	No	No	No	Unknown
ZB456/19	*Geokichla citrina*	Fertilised	No	Emb.	NA	NA	Embryo (25-26)*
ZB607/19	*Geokichla citrina*	Unfertilised	Yes	Emb.	NA	NA	Embryo (<25)
ZB803/19	*Geokichla citrina*	Unfertilised	Yes	No	?	?	Inconclusive
ZB804/19	*Geokichla citrina*	Unfertilised	Yes	No	?	?	Inconclusive
ZB977/19	*Geokichla citrina*	Undetermined	No	Emb.	NA	NA	Embryo (<25)
ZB493/20	*Geokichla citrina*	Unfertilised	No	Emb.	NA	NA	Embryo (21-23)
ZB494/20	*Geokichla citrina*	Unfertilised	No	No	No	Yes	Inconclusive
ZB495/20	*Geokichla citrina*	Unfertilised	No	Emb.	NA	NA	Embryo (<25)
ZB485/21	*Geronticus eremita*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB364/19	*Goura victoria*	Unfertilised	No	No	No	Yes	Inconclusive
ZB351/19	*Liocichla omeiensis*	Unfertilised	Yes	No	Yes	No	Fertilised
ZB352/19	*Liocichla omeiensis*	Fertilised	Yes	Emb.	NA	NA	Embryo (<25)
ZB353/19	*Liocichla omeiensis*	Fertilised	Yes	No	Yes	Yes	Fertilised
ZB690/19	*Liocichla omeiensis*	Unfertilised	Yes	No	Yes	No	Fertilised
ZB274/20	*Liocichla omeiensis*	Unfertilised	Yes	No	Yes	No	Fertilised

ZB275/20	*Liocichla omeiensis*	Unfertilised	Yes	No	Yes	No	Fertilised
ZB465/19	*Musophaga violacea*	Unfertilised	Yes	No	Yes	No	Fertilised
ZB466/19	*Musophaga violacea*	Unfertilised	Yes	No	Yes	No	Fertilised
ZB724/19	*Musophaga violacea*	Unfertilised	No	Yes	?	No	Inconclusive
ZB725/19	*Musophaga violacea*	Unfertilised	No	Yes	Yes	No	Fertilised
ZB1115/19	*Musophaga violacea*	Unfertilised	No	Yes	Yes	No	Fertilised
W20E108	*Numida meleagris*	Fertilised	Yes	Emb.	NA	NA	Embryo (<24)*
ZB435/21	*Passerina brissonii*	Unfertilised	Yes	Emb.	NA	NA	Embryo (<25)
ZB436/21	*Passerina brissonii*	Unfertilised	Yes	Emb.	NA	NA	Embryo (<25)
ZB654/19	*Ptilinopus superbus*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB691/19	*Ptilinopus superbus*	Unfertilised	No	No	No	Yes	Inconclusive
ZB483/21	*Ptilinopus superbus*	Unfertilised	Yes	No	Yes	Yes	Fertilised
ZB464/21	*Pycnonotus jocosus*	Unfertilised	No	No	Yes	No	Fertilised
ZB466/21	*Pycnonotus jocosus*	Unfertilised	No	No	No	No	Inconclusive
ZB538/21	*Pycnonotus jocosus*	Unfertilised	Yes	No	?	Yes	Inconclusive
W19E120	*Rhabdotorrhinus exarhatus sanfordi*	Unfertilised	Yes	No	No	No	Inconclusive
W19E121	*Rhabdotorrhinus exarhatus sanfordi*	Unfertilised	Yes	No	No	No	Inconclusive
W20B179	*Rhabdotorrhinus exarhatus sanfordi*	Unfertilised	No	No	Yes	Yes	Fertilised
ZB151/21	*Rollulus roulroul*	Unfertilised	No	No	Yes	Yes	Fertilised
ZB152/21	*Rollulus roulroul*	Unfertilised	No	No	Yes	Yes	Fertilised
ZB153/21	*Rollulus roulroul*	Unfertilised	No	No	Yes	Yes	Fertilised
ZB322/21	*Rollulus roulroul*	Unfertilised	No	Yes	Yes	No	Fertilised
ZB323/21	*Rollulus roulroul*	Unfertilised	No	Yes	Yes	No	Fertilised
ZB324/21	*Rollulus roulroul*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB488/20	*Sarkidiornis melanotos*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB445/20	*Spatula hottentota*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB446/20	*Spatula hottentota*	Unfertilised	No	Yes	No	No	Unfertilised
ZB447/20	*Spatula hottentota*	Unfertilised	No	Yes	No	No	Unfertilised
ZB448/20	*Spatula hottentota*	Unfertilised	No	Yes	Yes	No	Fertilised
ZB449/20	*Spatula hottentota*	Unfertilised	No	Yes	No	No	Unfertilised
ZB045/19	*Spheniscus humboldti*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB046/19	*Spheniscus humboldti*	Fertilised	No	Yes	Yes	Yes	Fertilised
ZB047/19	*Spheniscus humboldti*	Unfertilised	No	No	No	No	Inconclusive
ZB048/19	*Spheniscus humboldti*	Unfertilised	No	Yes	No	Yes	Inconclusive
ZB049/19	*Spheniscus humboldti*	Undetermined	No	Yes	Yes	Yes	Fertilised
ZB060/19	*Spheniscus humboldti*	Undetermined	No	Yes	No	No	Unfertilised
ZB061/19	*Spheniscus humboldti*	Fertilised	No	Yes	No	No	Unfertilised
ZB062/19	*Spheniscus humboldti*	Undetermined	No	Yes	Yes	Yes	Fertilised
ZB063/19	*Spheniscus humboldti*	Undetermined	No	Yes	Yes	Yes	Fertilised
ZB064/19	*Spheniscus humboldti*	Undetermined	No	Yes	No	No	Unfertilised
ZB072/19	*Spheniscus humboldti*	Unfertilised	No	Yes	No	No	Unfertilised
ZB073/19	*Spheniscus humboldti*	Fertilised	No	Yes	No	Yes	Inconclusive
ZB074/19	*Spheniscus humboldti*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB075/19	*Spheniscus humboldti*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB076/19	*Spheniscus humboldti*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB085/19	*Spheniscus humboldti*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB095/19	*Spheniscus humboldti*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB096/19	*Spheniscus humboldti*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB103/19	*Spheniscus humboldti*	Unfertilised	No	No	No	Yes	Inconclusive
ZB104/19	*Spheniscus humboldti*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB105/19	*Spheniscus humboldti*	Undetermined	No	Yes	No	No	Unfertilised
ZB106/19	*Spheniscus humboldti*	Undetermined	No	No	No	Yes	Inconclusive
ZB113/19	*Spheniscus humboldti*	Unfertilised	No	Yes	Yes	No	Fertilised
ZB114/19	*Spheniscus humboldti*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB115/19	*Spheniscus humboldti*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB116/19	*Spheniscus humboldti*	Unfertilised	No	Yes	No	Yes	Inconclusive
ZB117/19	*Spheniscus humboldti*	Unfertilised	No	Yes	?	No	Inconclusive
ZB134/19	*Spheniscus humboldti*	Undetermined	No	Yes	No	No	Unfertilised
ZB135/19	*Spheniscus humboldti*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB136/19	*Spheniscus humboldti*	Undetermined	No	Yes	No	No	Unfertilised
ZB137/19	*Spheniscus humboldti*	Undetermined	No	No	?	No	Inconclusive
ZB138/19	*Spheniscus humboldti*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB139/19	*Spheniscus humboldti*	Unfertilised	No	No	No	Yes	Inconclusive
ZB140/19	*Spheniscus humboldti*	Undetermined	No	Yes	No	No	Unfertilised
ZB141/19	*Spheniscus humboldti*	Unfertilised	No	No	No	No	Inconclusive
ZB165/19	*Spheniscus humboldti*	Undetermined	No	Yes	No	Yes	Inconclusive
ZB242/19	*Spheniscus humboldti*	Unfertilised	No	No	No	No	Inconclusive
ZB249/19	*Spheniscus humboldti*	Unfertilised	Yes	No	No	No	Inconclusive
ZB258/19	*Spheniscus humboldti*	Unfertilised	No	No	Yes	No	Fertilised
ZB259/19	*Spheniscus humboldti*	Unfertilised	No	No	No	No	Inconclusive
ZB291/19	*Spheniscus humboldti*	Unfertilised	Yes	No	No	Yes	Inconclusive
ZB322/19	*Spheniscus humboldti*	Unfertilised	Yes	No	Yes	No	Fertilised
ZB323/19	*Spheniscus humboldti*	Unfertilised	Yes	No	No	No	Inconclusive
ZB336/19	*Spheniscus humboldti*	Unfertilised	No	Yes	?	No	Inconclusive
ZB342/19	*Spheniscus humboldti*	Unfertilised	No	Yes	Yes	No	Fertilised
ZB389/19	*Spheniscus humboldti*	Undetermined	Yes	No	No	No	Unknown

ZB421/19	*Spheniscus humboldti*	Fertilised	Yes	No	Yes	Yes	Fertilised
ZB422/19	*Spheniscus humboldti*	Unfertilised	Yes	No	No	No	Unknown
ZB497/19	*Spheniscus humboldti*	Unfertilised	Yes	No	Yes	Yes	Fertilised
ZB503/19	*Spheniscus humboldti*	Unfertilised	No	No	Yes	Yes	Fertilised
ZB523/19	*Spheniscus humboldti*	Unfertilised	Yes	No	Yes	No	Fertilised
ZB524/19	*Spheniscus humboldti*	Unfertilised	Yes	No	Yes	No	Fertilised
ZB569/19	*Spheniscus humboldti*	Unfertilised	No	No	Yes	Yes	Fertilised
ZB570/19	*Spheniscus humboldti*	Unfertilised	No	No	Yes	No	Fertilised
ZB608/19	*Spheniscus humboldti*	Unfertilised	No	No	Yes	No	Fertilised
ZB609/19	*Spheniscus humboldti*	Unfertilised	No	No	No	No	Inconclusive
ZB610/19	*Spheniscus humboldti*	Unfertilised	Yes	Emb.	NA	NA	Embryo (<24)
ZB611/19	*Spheniscus humboldti*	Unfertilised	Yes	No	No	No	Unknown
ZB1335/19	*Spheniscus humboldti*	Undetermined	No	Yes	Yes	Yes	Fertilised
ZB130/20	*Spheniscus humboldti*	Undetermined	No	No	Yes	Yes	Fertilised
ZB151/20	*Spheniscus humboldti*	Undetermined	No	Yes	Yes	Yes	Fertilised
ZB152/20	*Spheniscus humboldti*	Undetermined	No	Yes	Yes	Yes	Fertilised
ZB153/20	*Spheniscus humboldti*	Undetermined	No	Yes	Yes	Yes	Fertilised
ZB165/20	*Spheniscus humboldti*	Undetermined	No	Yes	Yes	No	Fertilised
ZB166/20	*Spheniscus humboldti*	Undetermined	No	No	Yes	No	Fertilised
ZB167/20	*Spheniscus humboldti*	Undetermined	No	Yes	Yes	Yes	Fertilised
ZB172/20	*Spheniscus humboldti*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB767/20	*Spheniscus humboldti*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB768/20	*Spheniscus humboldti*	Unfertilised	Yes	No	Yes	Yes	Fertilised
ZB769/20	*Spheniscus humboldti*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB770/20	*Spheniscus humboldti*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB771/20	*Spheniscus humboldti*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB779/20	*Spheniscus humboldti*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB780/20	*Spheniscus humboldti*	Unfertilised	No	No	Yes	Yes	Fertilised
ZB781/20	*Spheniscus humboldti*	Unfertilised	No	Yes	Yes	No	Fertilised
ZB783/20	*Spheniscus humboldti*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB784/20	*Spheniscus humboldti*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB825/20	*Spheniscus humboldti*	Unfertilised	No	Yes	Yes	Yes	Fertilised
ZB545/19	*Taeniopygia guttata*	Undetermined	No	Yes	Yes	Yes	Fertilised
ZB401/19	*Uraeginthus bengalus*	Unfertilised	No	No	No	No	Inconclusive
ZB402/19	*Uraeginthus bengalus*	Unfertilised	No	No	No	No	Unknown
ZB403/19	*Uraeginthus bengalus*	Unfertilised	No	No	No	No	Unknown

Table B9. Summary of unhatched kakī egg samples, their egg necropsy results, and embryo staging (where relevant). Fertility statuses and staging in bold were determined using Hoechst 33342 DNA staining and fluorescence microscopy methods (see **Table B10**).

Egg ID	Wild/ Captive	Embryo Stage	Description	Fertility Status
19/04	Wild	34	Died mid	Fertilised
19/05	Wild	40-44	Died late – Malpositioned (severe)	Fertilised
19/08	Wild	~26	Died mid	Fertilised
19/12	Wild	40-44	Died late – Malpositioned (severe)	Fertilised
19/31	Wild	**<4**	Addled – **Died pre-incubation**	**Fertilised**
19/33	Wild	25	Died early	Fertilised
19/34	Wild	18	Died early	Fertilised
19/39	Wild	24-25	Died early	Fertilised
19/40	Wild	39-40	Died late	Fertilised
19/41	Wild	**<4**	No visible development (damaged) – **Died pre-incubation**	**Fertilised**
19/45	Wild	43-44	Died hatching – Malpositioned (mild)	Fertilised
19/47	Wild	32-34	Died mid	Fertilised
19/48	Wild	**<4**	No visible development (damaged) – **Died pre-incubation**	**Fertilised**
19/49	Wild	25	Died early	Fertilised
19/50	Wild	40-44	Died late – Malpositioned (severe)	Fertilised
19/51	Captive	–	Broken – not kept for examination	NA
19/53	Captive	26-30	Died mid	Fertilised
19/54	Captive	<20	Died early	Fertilised
19/56	Captive	<25	Died early	Fertilised
19/57	Captive	**<4**	Addled – **Died pre-incubation**	**Fertilised**
19/62	Wild	44-45	Died hatching – Malpositioned (severe) + Deformed	Fertilised
19/63	Wild	43-44	Died hatching – Malpositioned (severe) + Deformed	Fertilised
19/64	Wild	45	Died hatching – Malpositioned (severe) + Deformed	Fertilised
19/65	Wild	~24	Died early	Fertilised
19/73	Wild	28-29	Died mid	Fertilised
19/75	Wild	40-44	Died hatching	Fertilised
19/80	Wild	45-46	Died hatching – Malpositioned (severe)	Fertilised
19/87	Wild	~40	Died late	Fertilised
19/91	Wild	24-25	Died early	Fertilised
19/92	Wild	45	Died hatching – Damaged during incubation	Fertilised
19/100	Wild	**<4**	No visible development – **Died pre-incubation**	**Fertilised**
19/101	Wild	17-21	Died early	Fertilised
19/110	Captive	~34	Died mid	Fertilised
19/120	Wild	45-46	Died hatching	Fertilised
19/127	Wild	40-44	Died late – Malpositioned (severe)	Fertilised
19/128	Wild	~30	Died mid	Fertilised
19/132	Wild	40-44	Died late – Malpositioned (severe)	Fertilised
19/135	Wild	~24	Died early	Fertilised
19/138	Wild	45	Died hatching – Malpositioned (minor)	Fertilised
19/143	Captive	**<4**	Addled – **Died pre-incubation**	**Fertilised**
19/147	Wild	–	No visible development	**Inconclusive**
19/150	Wild	**<4**	No visible development – **Died pre-incubation**	**Fertilised**
19/166	Captive	~34/35	Died mid	Fertilised

Table B10. Results of fertility examination using Hoechst 33342 fluorescent DNA microscopy performed on 3 addled kakī eggs and 5 which lacked visible signs of development.

Egg ID	Wild/ Captive	Necropsy Results	Embryonic Cell Nuclei	Trapped Sperm	Penetration Holes	Suspected Fertility Status
19/31	Wild	Addled	Present	Absent	Absent	Fertilised
19/41*	Wild	No development	Present	Absent	Absent	Fertilised
19/48*	Wild	No development	Present	Absent	Absent	Fertilised
19/57	Captive	Addled	Present	Absent	Absent	Fertilised
19/100	Wild	No development	Present	Absent	Absent	Fertilised
19/143	Captive	Addled	Present	Absent	Absent	Fertilised
19/147	Wild	No development	Absent	Absent	Absent	Inconclusive†
19/150	Wild	No development	Present	Absent	Absent	Fertilised

*Damaged prior to collection.
†Unsure if germinal disc examined.

Table B11. Egg fates for breeding seasons 2006/07 to 2019/20. Data for the 2019/20 breeding season are based on Hoechst 33342 fluorescent DNA microscopy results, all other seasons are based on necropsy examination alone.

	Egg Fate														
Breeding Season	Hatched*	Died Hatching	Incubation (unspecified)	Died late-incubation	Died mid-incubation	Died early-incubation	Died pre-incubation	Addled	Infertile	Undetermined fertility	Damaged	Soft-shelled	Runt	Unknown†	Total Eggs
2006/07	98	2	3	3	2	8	1	8	18	0	0	0	0	0	143
2007/08	121	4	1	2	4	10	2	2	18	0	0	0	0	15	179
2008/09	96	2	0	0	4	4	8	5	27	0	0	0	0	0	146
2009/10	131	1	0	0	2	9	5	9	15	0	0	0	0	1	173
2010/11	107	5	0	3	3	10	6	4	9	0	2	0	0	1	150
2011/12	81	1	0	2	5	7	1	1	5	0	1	0	0	0	104
2012/13	134	1	0	3	3	4	3	9	15	0	0	0	0	0	172
2013/14	141	1	0	8	3	3	1	11	4	0	0	0	0	0	172
2014/15	170	7	0	6	1	3	8	13	11	0	0	0	0	0	219
2015/16	103	1	0	3	0	3	1	15	10	0	0	0	0	1	137
2016/17	162	1	3	4	5	3	3	12	7	0	1	0	0	5	206
2017/18	215	8	0	2	4	7	4	10	11	0	1	0	1	1	264
2018/19	143	8	0	3	2	5	5	10	8	0	0	1	0	0	185
2019/20	123	8	0	7	8	10	5	0	0	1	4	0	0	0	166

*Includes assisted hatch eggs and those hatched by natal or foster parents as well as artificially incubated eggs.
†No information available, egg disappeared, or egg fostered to the wild and not monitored.

Table B12. Assumed infertility rates of captive- and wild-laid kakī eggs for breeding seasons 2006/07 to 2018/19 based on necropsy examination. Calculations of infertility rates include damaged eggs and eggs with unknown fates where their fertility was known. Infertility calculations were performed with addled eggs either assumed to be unfertilised or fertilised

Breeding Season	Assumed Infertility Rate (addled = excluded)			Assumed Infertility Rate (addled = unfertilised)			Assumed Infertility Rate (addled = fertilised)		
	Captive-laid	Wild-laid	All	Captive-laid	Wild-laid	All	Captive-laid	Wild-laid	All
2006/07	15.7%	11.9%	13.3%	15.7%	19.6%	18.2%	15.7%	10.9%	12.6%
2007/08	19.7%	4.81%	10.6%	19.7%	6.60%	11.6%	19.7%	4.72%	10.5%
2008/09	11.7%	28.1%	19.1%	11.7%	33.3%	21.9%	11.7%	26.1%	18.5%
2009/10	12.3%	6.59%	9.15%	14.7%	13.3%	13.9%	12.0%	6.12%	8.67%
2010/11	7.25%	5.33%	6.25%	8.57%	8.97%	8.78%	7.14%	5.13%	6.08%
2011/12	5.00%	4.65%	4.85%	6.56%	4.65%	5.77%	4.92%	4.65%	4.81%
2012/13	10.7%	8.41%	9.20%	16.7%	12.5%	14.0%	10.0%	8.04%	8.72%
2013/14	1.67%	2.97%	2.48%	6.35%	10.1%	8.72%	1.59%	2.75%	2.33%
2014/15	11.0%	2.26%	5.34%	16.7%	7.80%	11.0%	10.3%	2.13%	5.02%
2015/16	5.13%	9.76%	8.26%	11.9%	21.3%	18.4%	4.76%	8.51%	7.35%
2016/17	4.44%	3.38%	3.63%	6.52%	10.1%	9.27%	4.35%	3.14%	3.41%
2017/18	0.00%	5.53%	4.38%	1.59%	9.62%	8.05%	0.00%	5.29%	4.21%
2018/19	5.41%	4.38%	4.60%	10.3%	9.66%	9.78%	5.13%	4.14%	4.35%
Overall	8.97%	6.58%	7.43%	11.7%	12.1%	11.9%	8.71%	6.19%	7.07%

Table B13. Proportion of hatching failure attributed to fertilisation failure vs. embryo mortality in 2006/07-2018/19 based on necropsy examination alone. Excludes damaged eggs, abnormal eggs (runt and soft-shelled), and eggs with unknown hatching fates. Abbreviations: FF = fertilisation failure, EM = embryo mortality.

	Captive						**Wild**						**All**					
	Addled Eggs						*Addled Eggs*						*Addled Eggs*					
Breeding	*Excluded*		*Unfertilised*		*Fertilised*		*Excluded*		*Unfertilised*		*Fertilised*		*Excluded*		*Unfertilised*		*Fertilised*	
Season	**FF %**	**EM %**	**FF %**	**EM %**	**FF %**	**EM %**	**FF %**	**EM %**	**FF %**	**EM %**	**FF %**	**EM %**	**FF %**	**EM %**	**FF %**	**EM %**	**FF %**	**EM %**
2006/07	66.7	33.3	66.7	33.3	66.7	33.3	40.0	60.0	54.5	45.5	30.3	69.7	48.6	51.4	57.8	42.2	40.0	60.0
2007/08	50.0	50.0	50.0	50.0	50.0	50.0	33.3	66.7	41.2	58.8	29.4	70.6	43.9	56.1	46.5	53.5	41.9	58.1
2008/09	45.0	55.0	45.0	55.0	45.0	55.0	72.0	28.0	76.7	23.3	60.0	40.0	60.0	40.0	64.0	36.0	54.0	46.0
2009/10	47.4	52.6	52.4	47.6	42.9	57.1	46.2	53.8	65.0	35.0	30.0	70.0	46.9	53.1	58.5	41.5	36.6	63.4
2010/11	27.8	72.2	31.6	68.4	26.3	73.7	22.2	77.8	33.3	66.7	19.0	81.0	25.0	75.0	32.5	67.5	22.5	77.5
2011/12	25.0	75.0	30.8	69.2	23.1	76.9	22.2	77.8	22.2	77.8	22.2	77.8	23.8	76.2	27.3	72.7	22.7	77.3
2012/13	60.0	40.0	71.4	28.6	42.9	57.1	47.4	52.6	58.3	41.7	37.5	62.5	51.7	48.3	63.2	36.8	39.5	60.5
2013/14	16.7	83.3	44.4	55.6	11.1	88.9	21.4	78.6	50.0	50.0	13.6	86.4	20.0	80.0	48.4	51.6	12.9	87.1
2014/15	44.4	55.6	56.5	43.5	34.8	65.2	16.7	83.3	42.3	57.7	11.5	88.5	30.6	69.4	49.0	51.0	22.4	77.6
2015/16	40.0	60.0	62.5	37.5	25.0	75.0	61.5	38.5	80.0	20.0	32.0	68.0	55.6	44.4	75.8	24.2	30.3	69.7
2016/17	25.0	75.0	33.3	66.7	22.2	77.8	27.8	72.2	55.2	44.8	17.2	82.8	26.9	73.1	50.0	50.0	18.4	81.6
2017/18	0.00	100	20.0	80.0	0.00	100	34.4	65.6	48.8	51.2	26.8	73.2	30.6	69.4	45.7	54.3	23.9	76.1
2018/19	50.0	50.0	66.7	33.3	33.3	66.7	22.2	77.8	40.0	60.0	17.1	82.9	25.8	74.2	43.9	56.1	19.5	80.5
Overall	42.0	58.0	49.2	50.8	36.8	63.2	36.6	63.4	53.0	47.0	27.1	72.9	38.7	61.3	51.6	48.4	30.6	69.4

Additional eggs examined

Table B14. The results of necropsy and fertility examinations of unhatched eggs of additional species from Aotearoa New Zealand conservation programmes. Abbreviations: ICWT = Isaac Conservation and Wildlife Trust, CS = Cape Sanctuary, ECN = embryonic cell nuclei, TS = trapped sperm, PH = penetration holes, UNK = unknown.

Sample	Facility	Necropsy Examination		Fertility Examination			Fertility
		Stage	Diagnosis	ECN	TS	PH	Status
Kakī x poaka hybrid							
Hybrid 1	Wild	23/24	Died early	–	–	–	Fertilised
Hybrid 2	Wild	23/24	Died early	–	–	–	Fertilised
Hybrid 3	Wild	15/16	Died early	–	–	–	Fertilised
Hybrid 4	Wild	19-21	Died early	–	–	–	Fertilised
Kākāriki karaka (orange-fronted parakeet; *Cyanoramphus malherbi*)							
OFK01	ICWT	22-23	Died early	–	–	–	Fertilised
OFK02	ICWT	–	Potentially infertile	No	Yes	No	UNK*
OFK03	ICWT	–	Potentially infertile	Yes	Yes	No	Fertilised
OFK04	ICWT	–	Potentially infertile	Yes	Yes	No	Fertilised
OFK05	ICWT	–	Potentially infertile	No	No	No	Unfertilised
OFK06	ICWT	–	Potentially infertile	No	No	No	Unfertilised
OFK07	ICWT	–	Potentially infertile	No	No	No	Unfertilised
OFK08	ICWT	–	Potentially infertile	Yes	Yes	No	Fertilised
Tuturuatu (shore plover; *Thinornis novaeseelandiae*)							
SP01	ICWT	<21	Died early	–	–	–	Fertilised
SP02	ICWT	–	Severe infection	–	–	–	–
SP03	CS	–	Potentially infertile	No	No	No	UNK†
SP04	CS	–	Potentially infertile	No	No	No	UNK‡
SP05	CS	–	Potentially infertile	No	No	No	UNK‡
SP06	CS	~35-36	Died mid	–	–	–	Fertilised
SP07	CS	–	Potentially infertile	No	No	Yes	Unfertilised

*Unsure true GD examined.
†Very large number of small, elongated cells – suspect may be bacterial cells.
‡Very little material available to be examined.

Appendix C: Supplementary information for Chapter 4

Appendix C.1. Additional methodological information

Table C1. Summary of life history traits, threat status, and management details for the study species.

	Kakī	**Hihi**
IUCN Red List Status	Critically Endangered (BirdLife International, 2018b).	Vulnerable (BirdLife International, 2022b).
NZTCS Status	Nationally Critical (Robertson *et al.*, 2017).	Nationally Vulnerable (Robertson *et al.*, 2017).
Hatching Failure	28.4% (Heber & Briskie, 2010).	35% (Brekke *et al.*, 2010).
Habitat	Braided rivers and wetlands (Pierce, 2013).	Mature native forests (Castro, 2013).
Primary Diet	Aquatic invertebrates (Pierce, 2013).	Invertebrates, fruit, and nectar (Castro, 2013).
Breeding System	Monogamous solitary nesters, strong pair bonds, high nest site and mate fidelity (Reed *et al.*, 1993; van Heezik *et al.*, 2009; Pierce, 2013).	Complex structure and variable mating system including polygynandry (Castro *et al.*, 1996) and forced copulation by males.
Nest Type	Ground nest consisting of plant material and small stones (Pierce, 2013).	Naturally cavity nesters, readily nest in nestboxes, building a deep woven nest cup with a stick base (Castro *et al.*, 1996).
Mean Clutch Size	3–6 (mean 4) (Pierce, 2013).	2-5 (mean 3) (Castro *et al.*, 1996; Taylor *et al.*, 2005).
Clutches per Season	1–3 (Reed *et al.*, 1993; van Heezik *et al.*, 2005).	1–3 (Castro *et al.*, 1996; Taylor *et al.*, 2005).
Incubation	Biparental; 24–27 days (Pierce, 1982, 2013).	Maternal-only (Castro, 2013); 14–15 days (Ewen *et al.*, 2018).
Breeding Season	July–Jan (egg-laying Sept'–Dec') (Reed *et al.*, 1993; van Heezik *et al.*, 2005; Pierce, 2013).	Sept'–March (Castro, 2013).
Threats	Have suffered massive declines due to predation, habitat loss, and human disturbance (Pierce, 1986, 2013). Reached a bottleneck of 23 birds in 1981 (Maloney & Murray, 2001).	Reduced to single population on Te Hauturu-o-Toi/Little Barrier Island by predation by introduced mammals (Taylor *et al.*, 2005). Continued threats from predation, disease, and inbreeding depression (Ewen *et al.*, 2007; Brekke *et al.*, 2010).
Current Management Interventions	Predator control, captive-breeding, egg-pulling, artificial incubation, forced re-clutching, headstarting, genetic integrity management (BirdLife International, 2018b).	Translocation, predator control, artificial nestbox provision, supplementary feeding, nestbox cleaning for parasite/pest control (BirdLife International, 2017).
Current Population	Free-living population in Te Manahuna/Mackenzie Basin; captive-breeding pairs held at the DOC Kakī Recovery Centre, Twizel and the ICWT.	Remnant population on Te Hauturu-o-Toi, translocated populations on Tititiri Matangi Island, Shakespear Open Sanctuary, Sanctuary Mountain Maungatautari, Rotokare Scenic Reserve, Bushy Park Tarapuruhi, Kapati Island, and Zealandia.
Study Population	As above.	Translocated population on Tiritiri Matangi Island.

Weather station information

Table C2. Details of weather stations selected for each population. Latitude and longitude are given in decimal degrees (DD). The closest weather stations with data available for the study period were selected. As kakī nest locations in Te Manahuna were spread over a large area the chosen weather station was selected as an approximate midpoint of the sites.

Population	**Station Name**	**Agent Number**	**Network Number**	**Latitude** (DD)	**Longitude** (DD)	**Height** (m)
Hihi	Tiri Tiri Lighthouse	1401	A64691	-36.606	174.898	76
Kakī (Te Manahuna)	Lake Tekapo, Air Safaris	4970	H40041	-44.002	170.441	762
Kakī (ICWT)	Christchurch Aero	4843	H32451	-43.493	172.537	37

Table C3. Summary of the number and percentage of amplicon sequence variants (ASVs) which could not be classified at each level ("Unclassified"). For the Greengenes assignments only the number that could be assigned at each level but were not annotated in the database ("Unannotated") is also shown.

Reference Database	Total ASVs		Phylum	Class	Order	Family	Genus	Species
QIIME2 Pipeline								
- All sequences								
Silva v138.1 99%	7648	Unclassified	151 (1.97%)	159 (2.08%)	184 (2.41%)	236 (3.09%)	787 (10.29%)	4931 (64.47%)
Greengenes v13.8 99%	7648	Unclassified	668 (8.73%)	703 (9.19%)	739 (9.66%)	841 (11.00%)	1449 (18.95%)	2117 (27.68%)
		Unannotated	5 (0.07%)	67 (0.88%)	224 (2.93%)	1055 (13.79%)	2324 (30.39%)	4529 (59.22%)
		Unclassified + Unannotated	673 (8.80%)	770 (10.07%)	963 (12.59%)	1896 (24.79%)	3773 (49.33%)	6646 (86.90%)
- Sequences (250-256 nts)								
Silva v138.1 99%	6969	Unclassified	48 (0.69%)	52 (0.75%)	75 (1.08%)	127 (1.82%)	677 (9.71%)	4525 (64.93%)
Greengenes v13.8 99%	6969	Unclassified	41 (0.59%)	62 (0.89%)	96 (1.38%)	196 (2.81%)	796 (11.42%)	1460 (20.95%)
		Unannotated	5 (0.07%)	66 (0.95%)	222 (3.19%)	1052 (15.10%)	2312 (33.18%)	4513 (64.76%)
		Unclassified + Unannotated	46 (0.66%)	128 (1.84%)	318 (4.56%)	1248 (17.91%)	3108 (44.60%)	5973 (85.71%)
DADA2 Pipeline								
- Naïve Bayesian Classifier								
Silva v138.1 99%	7031	Unclassified	23 (0.33%)	53 (0.75%)	168 (2.39%)	811 (11.53%)	1997 (28.40%)	NA
Silva v138.1 99% (with species)	7031	Unclassified	26 (0.37%)	60 (0.85%)	176 (2.50%)	767 (10.91%)	1801 (25.62%)	5408 (76.92%)
Greengenes v13.8 97%	7031	Unclassified	26 (0.37%)	46 (0.65%)	83 (1.18%)	183 (2.60%)	614 (8.73%)	816 (11.61%)
		Unannotated	0 (0.00%)	50 (0.71%)	193 (2.74%)	985 (14.01%)	2412 (34.31%)	5204 (74.02%)
		Unclassified + Unannotated	26 (0.37%)	96 (1.37%)	276 (3.92%)	1168 (16.61%)	3026 (43.04%)	6020 (85.62%)
- IDTAXA (DECIPHER)								
Silva v138	7031	Unclassified	589 (8.38%)	647 (9.20%)	1135 (16.14%)	1719 (24.45%)	3454 (49.13%)	NA

Table C4. Potential contaminants identified using the *decontam* package version 1.16.0 (Davis *et al.*, 2018) applied to all samples (excluding mock communities). Taxonomic assignments are according to the naïve Bayesian classifier with the Silva v138.1 99% database. Where there were differences between this assignment and assignments using other taxonomic reference databases these have been indicated.

Potential Contaminant	Notes	All	Hihi only	Kakī only
Proteobacteria; Gammaproteobacteria; Pseudomonadales; Marinobacteraceae; *Marinobacter*	Genus found in seawater and salt lakes	0.1	0.1	NA
Actinobacteriota; Actinobacteria; Corynebacteriales; Corynebacteriaceae; *Corynebacterium*[a, b]	Genus widely distributed in animal microbiota	0.1	0.1	0.1
Proteobacteria; Alphaproteobacteria; Rhizobiales; Xanthobacteraceae; *Afipia*[a, b, c]	–	0.1	0.1	0.1
Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; *Sphingomonas*[b, d, e]	Genus widely distributed in nature	0.1	0.1	0.2
Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; *Pseudomonas*[a, b, c, d, f]; *stutzeri*	Genus widespread; species isolated from human spinal fluid, straw, manure, soil, and canal water	0.1	0.2	0.1
Proteobacteria; Alphaproteobacteria; Rhizobiales; Beijerinckiaceae; *Methylobacterium-Methylorubrum*[a, b, e]; *jeotgali*[g]	Genus found in soil and water; this species isolated from fermented seafood	0.2	0.2	0.1
Proteobacteria; Gammaproteobacteria; Burkholderiales; Comamonadaceae; *Paucibacter;*	–	0.2	0.2	0.1
Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; *Sphingomonas*[b, d, e]	Genus widely distributed in nature	0.2	0.2	0.1
Proteobacteria; Gammaproteobacteria; Pseudomonadales; Marinobacteraceae; *Marinobacter*	Genus found in seawater and salt lakes	0.2	0.3	0.2
Proteobacteria; Gammaproteobacteria; Burkholderiales; Comamonadaceae; *Comamonas*[a, b, e]		0.3	0.4	0.2
Proteobacteria; Gammaproteobacteria; Pseudomonadales; Halomonadaceae; *Halomonas*	Genus found in saline environments	0.3	0.4	0.2
Proteobacteria; Gammaproteobacteria; Pseudomonadales; Halomonadaceae; *Halomonas*	Genus found in saline environments	0.3	0.5	0.2
Actinobacteriota; Actinobacteria; Corynebacteriales; Corynebacteriaceae; *Corynebacterium*[a, b]; *tuberculostearicum*	Genus widely distributed in animal microbiota; species part of normal human skin flora	0.4	0.4	0.5
Firmicutes; Bacilli; Bacillales; Bacillaceae; *Anoxybacillus*	Genus found in geothermal springs, manure, and milk processing plants	0.4	0.5	0.2
Firmicutes; Bacilli; Bacillales; Bacillaceae; *Bacillus*[b, f]; *halodurans*	Genus found in salty and alkaline conditions; species isolated from soil	0.4	0.5	0.3
Proteobacteria; Gammaproteobacteria; Pseudomonadales; Halomonadaceae; *Halomonas*	Genus found in saline environments	0.5	NA	0.4
Proteobacteria; Gammaproteobacteria; Enterobacterales; Pseudoalteromonadaceae; *Pseudoalteromonas*	Genus of marine bacteria	0.5	NA	0.5
Actinobacteriota; Actinobacteria; Micrococcales; Micrococcaceae; *Micrococcus*[b, c]	Genus found in human skin, animal and dairy products, and beer	0.5	0.5	0.5

Superscript numbers indicate genera or species that have been previously reported as contaminants in a number of studies: [a] reported by Glassing et al. (2016); [b] reported by Salter et al. (2014); [c] reported by Tanner et al. (1998); [d] reported by Laurence et al. (2014); [e] reported by Barton et al. (2006); [f] reported by Grahn et al. (2003); [g] reported by Iulia et al. (2013).

DADA2 pipeline

Raw sequences were also processed in R using the *dada2* package version 1.24.0 and following the DADA2 Pipeline Tutorial (1.16) (Callahan *et al.*, 2016). Based on inspection of the read quality profiles, the forward and reverse reads were trimmed to 240 and 160 bp respectively using filterAndTrim (maxN = 0, maxEE = c(2,2), truncQ = 2). Visualisation of the estimated error rates indicated that the estimated error rates used for the DADA2 algorithm were a good fit to the observed rates. Singletons were removed as part of the default DADA2 quality control settings, and after merging I removed non-target-length sequences outside of the range 250-256 nucleotides. I then removed chimeras, which were identified as 6.7% of the input sequences and accounted for around 0.3% of the merged sequence reads. After all processing an average of 86.47% of reads remained in 7031 ASVs.

Taxonomy was assigned to each ASV using assignTaxonomy, which implements the RDP Naïve Bayesian Classifier algorithm (Wang *et al.*, 2007) based on both the Greengenes v13.8 97% (McDonald *et al.*, 2012) and Silva v138.1 99% (Quast *et al.*, 2013; Yilmaz *et al.*, 2014) reference databases formatted for use with DADA2 (Callahan, 2016; Callahan *et al.*, 2016; McLaren & Callahan, 2021). As with the QIIME2 pipeline, I trialled both Greengenes and Silva for taxonomic classification of the data but decided to proceed with the Silva assignment (**Table C3**). I also tested assigning taxonomy using the IDTAXA method available via the *DECIPHER* Bioconductor package version 2.24.0 (Wright, 2016) due to suggestion of improved classification performance compared to the naïve Bayesian classifier method (Murali *et al.*, 2018). I used the Silva Small Subunit (SSU) v138 training set for classification. Taxonomy was unable to be assigned to a much greater proportion of ASVs when using the IDTAXA method compared to the other methods I trialled. This is perhaps to be expected as this method is recognised as being more conservative than naïve Bayesian classifier methods, with putatively fewer over-classification errors. Although taxonomy assignment using the IDTAXA method may be more accurate, it has not yet been widely applied within the 16S rRNA microbiome literature, greatly limiting comparison between this study and other similar studies. For this reason, the results of the IDTAXA method were not used for any downstream analysis.

While reference sequences were not available for the mock community there were expected to be 25 ASVs in each mock community post-analysis. For the three mock community samples included here, (MC-3) was inferred to have 25 ASVs present while two (MC-1 and MC-2) were inferred to have 22 ASVs. 16 ASVs were consistently identified across the three mock community samples, and the overall composition was similar which gave us confidence to proceed with downstream analysis.

The ASVs were filtered to retain only features annotated to at least phylum level, and removed any non-target sequences assigned to Archaea, chloroplasts, and mitochondria, leaving us with 6625 ASVs. Any Eukaryota had already been removed when the reference databases were reformatted for DADA2 compatibility. I used the decontam package version 1.16.0 (Davis *et al.*, 2018) to detect any contaminants, applying the “prevalence” method which compares sequences in negative controls to those in samples. I found identical results as when decontam was applied to the data imported from the QIIME2 pipeline (see main text), with the same potential contaminants identified for all samples and for the kakī and hihi subsets. As above, 15 potential contaminants were removed before moving forward based on their biologically unexpected presence in the samples and their previous identification as contaminants in 16S rRNA gene sequencing. After all quality control and filtering a total of 6610 ASVs remained, but as 28 of these were present only in mock community and negative control samples the final dataset with these removed consisted of 6582 ASVs.

Appendix C.2. Additional results

Table C5. Summary of the most abundant phyla, families and genera across all eggshell samples and hihi and kakī eggshell samples individually. Mean relative abundances and coverage across samples (in brackets) are given as percentage values. Values in square brackets were not in the top 5 most abundant taxa at that taxonomic level in that subset.

	All eggshell samples	Hihi eggshell samples	Kakī eggshell samples
Phylum			
Proteobacteria	87.97% (100.00%)	87.15% (100.00%)	89.37% (100.00%)
Actinobacteriota	5.70% (97.99%)	5.39% (96.82%)	6.25% (100.00%)
Firmicutes	4.17% (94.78%)	5.10% (92.36%)	2.59% (98.91%)
Bacteroidota	1.01% (73.90%)	1.16% (59.87%)	0.78% (97.83%)
Cyanobacteria	0.28% (26.91%)	0.33% (11.46%)	0.21% (53.26%)
Family			
Pseudomonadaceae	33.97% (98.80%)	14.72% (98.09%)	66.82% (100.00%)
Burkholderiaceae	18.23% (100.00%)	25.56% (100.00%)	5.73% (100.00%)
Halomonadaceae	15.57% (100.00%)	22.84% (100.00%)	3.15% (100.00%)
Pseudoalteromonadaceae	8.30% (100.00%)	12.11% (100.00%)	[1.79%] (100.00%)
Oxalobacteraceae	2.49% (75.90%)	[2.12%] (63.06%)	3.12% (97.83%)
Staphylococcaceae	[1.80%] (75.90%)	2.30% (66.24%)	[0.95%] (92.39%)
Xanthomonadaceae	[1.48%] (69.08%)	[0.69%] (54.14%)	2.83% (94.57%)
Genus			
Pseudomonas	33.88% (98.80%)	14.63% (98.09%)	66.74% (100.00%)
Ralstonia	18.07% (100.00%)	25.32% (100.00%)	5.71% (100.00%)
Halomonas	15.51% (100.00%)	22.76% (100.00%)	3.15% (100.00%)
Pseudoalteromonas	8.30% (100.00%)	12.11% (100.00%)	[1.79%] (100.00%)
Janthinobacterium	2.16% (69.88%)	[1.87%] (56.69%)	2.64% (92.39%)
Staphylococcus	[1.79%] (75.10%)	2.29% (64.97%)	[0.94%] (92.39%)
Stenotrophomonas	[1.44%] (66.27%)	[0.63%] (49.68%)	2.82% (94.57%)

Table C6. Summary of the ASVs identified as being significantly differentially abundant between kakī and hihi eggshell samples. Benjamini-Hochberg (Benjamini & Hochberg, 1995) adjusted p-values (p-adj) are reported for results derived from DESeq2 and the W statistic is reported for ANCOM results. Only statistically significant values are shown.

ASV	DESeq2 p-adj	ANCOM W
Greater abundance in hihi eggshell samples compared to kakī eggshell samples		
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Burkholderiaceae; g__*Ralstonia*	–	72
p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacterales; f__Pseudoalteromonadaceae; g__*Pseudoalteromonas*	2.13e-22	78
p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Halomonadaceae; g__*Halomonas*	2.17e-12	78
p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Halomonadaceae; g__*Halomonas*	5.41e-07	78
p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Halomonadaceae; g__*Halomonas*	2.35e-12	78
p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Halomonadaceae; g__*Halomonas*	0.0441	69
p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Halomonadaceae; g__*Halomonas*	–	68
p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Halomonadaceae; g__*Halomonas*	0.0363	67
p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__*Pseudomonas*	–	69
Greater abundance in kakī eggshell samples compared to hihi eggshell samples		
p__Actinobacteriota; c__Actinobacteria; o__Corynebacteriales; f__Nocardiaceae; g__*Rhodococcus*	1.36e-29	75
p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Microbacteriaceae	2.03e-76	73
p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Microbacteriaceae; g__*Leucobacter*; s__*Leucobacter_sp.*	2.88e-13	63
p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Microbacteriaceae; g__*Microbacterium*	2.79e-16	77
p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Microbacteriaceae; g__*Microbacterium*	0.0358	–
p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Microbacteriaceae; g__*Pseudoclavibacter*	1.95e-26	73
p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Micrococcaceae	1.36e-09	–
p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Micrococcaceae	0.0013	–
p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Micrococcaceae; g__*Paeniglutamicibacter*	4.91e-24	73
p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Micrococcaceae; g__*Paeniglutamicibacter*; s__*Arthrobacter_sp*.	2.84e-55	75
p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Sanguibacteraceae; g__*Sanguibacter-Flavimobilis*	1.87e-07	74
p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__*Bacillus*	1.29e-47	–
p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__*Bacillus*	0.0255	–
p__Firmicutes; c__Bacilli; o__Exiguobacterales; f__Exiguobacteraceae; g__*Exiguobacterium*	1.54e-102	63
p__Proteobacteria; c__Alphaproteobacteria; o__Acetobacterales; f__Acetobacteraceae; g__*Roseomonas*; s__*Acetobacteraceae_bacterium*	1.20e-41	72
p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Devosiaceae; g__*Devosia*	1.25e-39	72
p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Rhizobiaceae; g__*Pseudochrobactrum*	2.00e-33	73
p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Rhizobiaceae; g__*Pseudochrobactrum*	6.22e-17	72
p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__*Sphingomonas*	0.0484	–

p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__*Sphingomonas*; s__*Sphingomonas_faeni*	–	59
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Alcaligenaceae; g__*Verticiella*; s__*metagenome*	3.56e-10	62
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Comamonadaceae	1.27e-40	74
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Oxalobacteraceae; g__*Herbaspirillum*	2.89e-09	75
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Oxalobacteraceae; g__*Janthinobacterium*	1.18e-17	72
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Oxalobacteraceae; g__*Massilia*	0.0134	–
p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__*Pseudomonas*	8.24e-45	79
p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__*Pseudomonas*	2.22e-16	73
p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__*Pseudomonas*	6.62e-28	68
p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__*Pseudomonas*	1.36e-09	65
p__Proteobacteria; c__Gammaproteobacteria; o__Xanthomonadales; f__Xanthomonadaceae; g__*Stenotrophomonas*; s__*Stenotrophomonas_rhizophila*	3.94e-26	68
Total significantly differentially abundant ASVs	*35*	*32*

Table C7. Summary of the genera identified as being significantly differentially abundant between kakī and hihi eggshell samples. Benjamini-Hochberg (Benjamini & Hochberg, 1995) adjusted p-values (p-adj) are reported for results derived from DESeq2 and the W statistic is reported for ANCOM results. Only statistically significant values are shown. One genus (*Ralstonia*) was identified by DESeq2 as being more abundant in kakī samples, but identified by ANCOM as being more abundant in hihi samples and is marked with an asterisk (*).

Genus	DESeq2 p-adj	ANCOM W
Greater abundance in hihi eggshell samples compared to kakī eggshell samples		
p__Proteobacteria;c__Gammaproteobacteria; o__Enterobacterales; f__Pseudoalteromonadaceae; g__*Pseudoalteromonas*	5.12E-04	112
p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Halomonadaceae; g__*Halomonas*	4.70e-06	112
*p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Burkholderiaceae; g__*Ralstonia*	–	113
Greater abundance in kakī eggshell samples compared to hihi eggshell samples		
p__Acidobacteriota; c__Acidobacteriae; o__Acidobacteriales; f__uncultured; g__uncultured	0.0172	–
p__Acidobacteriota; c__Acidobacteriae; o__Bryobacterales; f__Bryobacteraceae; g__*Bryobacter*	0.0020	–
p__Acidobacteriota; c__Blastocatellia; o__Blastocatellales; f__Blastocatellaceae; __	0.0155	–
p__Acidobacteriota; c__Vicinamibacteria; o__Vicinamibacterales; f__uncultured; g__uncultured	0.0042	–
p__Actinobacteriota; c__Acidimicrobiia; o__IMCC26256; f__IMCC26256; g__IMCC26256	0.0400	–
p__Actinobacteriota; c__Acidimicrobiia; o__Microtrichales; f__uncultured; g__uncultured	6.04e-04	–
p__Actinobacteriota; c__Actinobacteria; o__Corynebacteriales; f__Corynebacteriaceae; g__*Corynebacterium*	1.12e-05	–
p__Actinobacteriota; c__Actinobacteria; o__Corynebacteriales; f__Mycobacteriaceae; g__*Mycobacterium*	0.0047	–
p__Actinobacteriota; c__Actinobacteria; o__Corynebacteriales; f__Nocardiaceae; g__*Rhodococcus*	5.47e-16	108
p__Actinobacteriota; c__Actinobacteria; o__Frankiales; f__Acidothermaceae; g__*Acidothermus*	2.66e-07	–
p__Actinobacteriota; c__Actinobacteria; o__Frankiales; f__Geodermatophilaceae; g__*Blastococcus*	0.0299	–
p__Actinobacteriota; c__Actinobacteria; o__Frankiales; f__Geodermatophilaceae; g__*Geodermatophilus*	0.0475	–
p__Actinobacteriota; c__Actinobacteria; o__Frankiales; f__Geodermatophilaceae; g__*Klenkia*	0.0042	–
p__Actinobacteriota; c__Actinobacteria; o__Frankiales; f__Geodermatophilaceae; g__*Modestobacter*;	0.0206	–
p__Actinobacteriota; c__Actinobacteria; o__Frankiales; f__Sporichthyaceae; g__*Longivirga*	0.0039	–
p__Actinobacteriota; c__Actinobacteria; o__Kineosporiales; f__Kineosporiaceae; __	7.58e-06	–
p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Intrasporangiaceae; __	2.87e-04	–
p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Microbacteriaceae; g__*Leucobacter*	5.33e-23	–
p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Microbacteriaceae; g__*Microbacterium*	1.11e-21	111
p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Microbacteriaceae; g__*Pseudoclavibacter*	1.11e-33	109
p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Microbacteriaceae; __	1.06e-23	105
p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Micrococcaceae; g__*Paeniglutamicibacter*	5.22e-30	111
p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Micrococcaceae; __	6.93e-07	–
p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Sanguibacteraceae; g__*Sanguibacter-Flavimobilis*	3.29e-11	111
p__Actinobacteriota; c__Actinobacteria; o__PeM15; f__PeM15; g__*PeM15*	0.0145	–

p__Actinobacteriota; c__Actinobacteria; o__Propionibacteriales; f__Propionibacteriaceae; g__*Cutibacterium*	0.0449	–
p__Actinobacteriota; c__Actinobacteria; o__Pseudonocardiales; f__Pseudonocardiaceae; g__*Actinopolyspora*	0.0032	–
p__Actinobacteriota; c__Thermoleophilia; o__Gaiellales; f__Gaiellaceae; g__*Gaiella*	0.0020	–
p__Actinobacteriota; c__Thermoleophilia; o__Gaiellales; f__uncultured; g__uncultured	0.0204	–
p__Actinobacteriota; c__Thermoleophilia; o__Solirubrobacterales; f__Solirubrobacteraceae; g__*Conexibacter*	0.0104	–
p__Actinobacteriota; c__Thermoleophilia; o__Solirubrobacterales; f__Solirubrobacteraceae; g__uncultured	0.0247	–
p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__*Prevotella_7*	0.0111	–
p__Bacteroidota; c__Bacteroidia; o__Chitinophagales; f__Chitinophagaceae; g__*Ferruginibacter*	0.0104	–
p__Bacteroidota; c__Bacteroidia; o__Chitinophagales; f__Chitinophagaceae; g__*Sediminibacterium*	0.0475	–
p__Bacteroidota; c__Bacteroidia; o__Chitinophagales; f__Chitinophagaceae; g__*Segetibacter*	6.64e-09	–
p__Bacteroidota; c__Bacteroidia; o__Chitinophagales; f__Chitinophagaceae; g__uncultured	0.0042	–
p__Bacteroidota; c__Bacteroidia; o__Chitinophagales; f__Saprospiraceae; g__uncultured	0.0148	–
p__Bacteroidota; c__Bacteroidia; o__Cytophagales; f__Hymenobacteraceae; g__*Hymenobacter*	2.18e-06	99
p__Bacteroidota; c__Bacteroidia; o__Cytophagales; f__Spirosomaceae; g__*Arcicella*	0.0302	–
p__Bacteroidota; c__Bacteroidia; o__Cytophagales; f__Spirosomaceae; g__*Dyadobacter*	0.0042	–
p__Bacteroidota; c__Bacteroidia; o__Cytophagales; f__Spirosomaceae; g__*Fibrella*	0.0206	–
p__Bacteroidota; c__Bacteroidia; o__Flavobacteriales; f__Flavobacteriaceae; g__*Flavobacterium*	0.0015	–
p__Bacteroidota; c__Bacteroidia; o__Flavobacteriales; f__Weeksellaceae; g__*Chryseobacterium*	1.15e-04	–
p__Bdellovibrionota; c__Bdellovibrionia; o__Bdellovibrionales; f__Bdellovibrionaceae; g__*Bdellovibrio*	0.0171	–
p__Chloroflexi; c__KD4-96; o__KD4-96; f__KD4-96; g__KD4-96	1.20e-07	–
p__Cyanobacteria; c__Cyanobacteriia; o__Cyanobacteriales; f__Chroococcidiopsaceae; g__*Crinalium_SAG_22.89*	0.0089	–
p__Cyanobacteria; c__Cyanobacteriia; o__Cyanobacteriales; f__Phormidiaceae; g__*Tychonema_CCAP_1459-11B*	0.0003	–
p__Cyanobacteria; c__Cyanobacteriia; o__Synechococcales; f__Cyanobiaceae; g__*Cyanobium_PCC-6307*	0.0206	–
p__Deinococcota; c__Deinococci; o__Deinococcales; f__Deinococcaceae; g__*Deinococcus*	2.31e-06	–
p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__*Bacillus*	5.23e-05	–
p__Firmicutes; c__Bacilli; o__Bacillales; f__Planococcaceae; g__*Sporosarcina*	0.0375	–
p__Firmicutes; c__Bacilli; o__Bacillales; f__Planococcaceae; __	0.0107	–
p__Firmicutes; c__Bacilli; o__Bacillales; __; __	0.0162	–
p__Firmicutes; c__Bacilli; o__Exiguobacterales; f__Exiguobacteraceae; g__*Exiguobacterium*	1.12e-05	–
p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Listeriaceae; g__*Brochothrix*	0.0145	–
p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Streptococcaceae; g__*Streptococcus*	0.0047	–
p__Firmicutes; c__Bacilli; o__Mycoplasmatales; f__Mycoplasmataceae; g__*Ureaplasma*	0.0268	–
p__Firmicutes; c__Bacilli; o__Staphylococcales; f__Staphylococcaceae; g__*Staphylococcus*	0.0080	–
p__Firmicutes; c__Clostridia; o__Clostridiales; f__Caloramatoraceae; g__*Fonticella*	0.0400	–
p__Firmicutes; c__Clostridia; o__Clostridiales; f__Clostridiaceae; g__*Clostridium_sensu_stricto_1*	7.68e-05	–
p__Firmicutes; c__Clostridia; o__Peptostreptococcales-Tissierellales; f__Family_XI; g__*Anaerococcus*	0.0196	–

p__Firmicutes; c__Clostridia; o__Peptostreptococcales-Tissierellales; f__Family_XI; g__*Finegoldia*	0.0449	–
p__Firmicutes; c__Clostridia; o__Peptostreptococcales-Tissierellales; f__Family_XI; g__*Peptoniphilus*	0.0020	–
p__Fusobacteriota; c__Fusobacteriia; o__Fusobacteriales; f__Leptotrichiaceae; g__*Leptotrichia*	0.0145	–
p__Gemmatimonadota; c__Gemmatimonadetes; o__Gemmatimonadales; f__Gemmatimonadaceae; g__*Gemmatimonas*	0.0072	–
p__Gemmatimonadota; c__Gemmatimonadetes; o__Gemmatimonadales; f__Gemmatimonadaceae; g__uncultured	9.60e-04	–
p__Myxococcota; c__Myxococcia; o__Myxococcales; f__Myxococcaceae; g__*P3OB-42*	0.0156	–
p__Myxococcota; c__Polyangia; o__Haliangiales; f__Haliangiaceae; g__*Haliangium*	0.0475	–
p__Planctomycetota; c__Planctomycetes; o__Gemmatales; f__Gemmataceae; g__uncultured	0.0203	–
p__Planctomycetota; c__Planctomycetes; o__Isosphaerales; f__Isosphaeraceae; g__*Aquisphaera*	0.0173	–
p__Planctomycetota; c__Planctomycetes; o__Isosphaerales; f__Isosphaeraceae; g__uncultured	0.0344	–
p__Planctomycetota; c__Planctomycetes; o__Isosphaerales; f__Isosphaeraceae; __	0.0162	–
p__Planctomycetota; c__Planctomycetes; o__Pirellulales; f__Pirellulaceae; g__uncultured	0.0042	–
p__Proteobacteria; c__Alphaproteobacteria; o__Acetobacterales; f__Acetobacteraceae; g__*Roseomonas*	1.82e-23	108
p__Proteobacteria; c__Alphaproteobacteria; o__Caulobacterales; f__Caulobacteraceae; g__*Brevundimonas*	0.0302	–
p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Beijerinckiaceae; g__*alphaI_cluster*	0.0085	–
p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Beijerinckiaceae; g__*Methylocystis*	0.0206	–
p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Beijerinckiaceae; g__*Rhodoblastus*	7.00e-05	–
p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Beijerinckiaceae; g__*Roseiarcus*	0.0068	–
p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Devosiaceae; g__*Devosia*	6.38e-37	109
p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Rhizobiaceae; g__*Pseudochrobactrum*	1.63e-32	109
p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Rhizobiales_Incertae_Sedis; g__uncultured	0.0250	–
p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Rhodomicrobiaceae; g__*Rhodomicrobium*	0.0268	–
p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Xanthobacteraceae; __	0.0466	–
p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae; g__*Rhodobacter*	0.0475	–
p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__*Polymorphobacter*	3.17e-07	–
p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__*Rhizorhapis*	0.0013	–
p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__*Sphingomonas*	0.0020	–
p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__*Sphingorhabdus*	0.0012	–
p__Proteobacteria; c__Alphaproteobacteria; o__Tistrellales; f__Geminicoccaceae; g__*Candidatus_Alysiosphaera*	0.0210	–
p__Proteobacteria; c__Gammaproteobacteria; __; __; __	0.0366	–
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Alcaligenaceae; g__*Verticiella*	0.0054	–
*p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Burkholderiaceae; g__*Ralstonia*	1.59e-36	–
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Comamonadaceae; g__*Rhodoferax*	0.0020	–
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Comamonadaceae; __	5.56e-34	105
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Methylophilaceae; g__*Methylotenera*	0.0475	–
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Neisseriaceae; g__*Neisseria*	0.0449	–

p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Neisseriaceae; g__uncultured	0.0370	–
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Nitrosomonadaceae; g__*Ellin6067*	0.0072	–
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Oxalobacteraceae; g__*Herbaspirillum*	2.45e-14	109
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Oxalobacteraceae; g__*Janthinobacterium*	5.59e-29	105
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Oxalobacteraceae; g__*Massilia*	0.0017	–
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__SC-I-84; g__SC-I-84	0.0042	–
p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacterales; f__Pasteurellaceae; __	0.0475	–
p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Moraxellaceae; g__*Acinetobacter*	0.0054	–
p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__*Azotobacter*	4.92e-04	–
p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__*Pseudomonas*	2.70e-124	109
p__Proteobacteria; c__Gammaproteobacteria; o__Xanthomonadales; f__Xanthomonadaceae; g__*Stenotrophomonas*	7.39e-33	107
p__Verrucomicrobiota; c__Verrucomicrobiae; o__Chthoniobacterales; f__Chthoniobacteraceae; g__*Candidatus_Udaeobacter*	0.0027	–
p__Verrucomicrobiota; c__Verrucomicrobiae; o__Chthoniobacterales; f__Chthoniobacteraceae; g__*Chthoniobacter*	0.01629	–
Total significantly differentially abundant genera	112	18

Table C8. Summary of the families identified as being significantly differentially abundant between kakī and hihi eggshell samples. Benjamini-Hochberg (Benjamini & Hochberg, 1995) adjusted p-values (p-adj) are reported for results derived from DESeq2 and the W statistic is reported for ANCOM results. Only statistically significant values are shown. One family (Burkholderiaceae) was identified by DESeq2 as being more abundant in kakī samples, but identified by ANCOM as being more abundant in hihi samples and is marked with an asterisk (*).

Family	DESeq2 p-adj	ANCOM W
Greater abundance in hihi eggshell samples compared to kakī eggshell samples		
*p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Burkholderiaceae	–	87
p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacterales; f__Pseudoalteromonadaceae	0.0002	86
p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Halomonadaceae	5.08e-07	86
Greater abundance in kakī eggshell samples compared to hihi eggshell samples		
p__Acidobacteriota; c__Acidobacteriae; o__Acidobacteriales; f__uncultured	0.0083	–
p__Acidobacteriota; c__Acidobacteriae; o__Bryobacterales; f__Bryobacteraceae	0.0004	–
p__Acidobacteriota; c__Acidobacteriae; o__Solibacterales; f__Solibacteraceae	0.0412	–
p__Acidobacteriota; c__Vicinamibacteria; o__Vicinamibacterales; f__uncultured	0.0011	–
p__Actinobacteriota; c__Acidimicrobiia; o__IMCC26256; f__IMCC26256	0.0217	–
p__Actinobacteriota; c__Acidimicrobiia; o__Microtrichales; f__Iamiaceae	0.0412	–
p__Actinobacteriota; c__Acidimicrobiia; o__Microtrichales; f__Microtrichaceae	0.0136	–
p__Actinobacteriota; c__Acidimicrobiia; o__Microtrichales; f__uncultured	9.78E-05	–
p__Actinobacteriota; c__Actinobacteria; o__Corynebacteriales; f__Corynebacteriaceae	0.0008	–
p__Actinobacteriota; c__Actinobacteria; o__Corynebacteriales; f__Mycobacteriaceae	0.0022	–
p__Actinobacteriota; c__Actinobacteria; o__Corynebacteriales; f__Nocardiaceae	3.16E-17	81
p__Actinobacteriota; c__Actinobacteria; o__Frankiales; f__Acidothermaceae	5.60E-09	–
p__Actinobacteriota; c__Actinobacteria; o__Frankiales; f__Geodermatophilaceae	0.0061	–
p__Actinobacteriota; c__Actinobacteria; o__Frankiales; f__Nakamurellaceae	0.0317	–
p__Actinobacteriota; c__Actinobacteria; o__Frankiales; f__Sporichthyaceae	0.0001	–
p__Actinobacteriota; c__Actinobacteria; o__Frankiales; f__uncultured	0.0412	–
p__Actinobacteriota; c__Actinobacteria; o__Kineosporiales; f__Kineosporiaceae	0.0118	–
p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Intrasporangiaceae	3.34E-06	–
p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Microbacteriaceae	1.03E-26	82
p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Micrococcaceae	2.20E-28	81
p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Sanguibacteraceae	4.49E-12	87
p__Actinobacteriota; c__Actinobacteria; o__PeM15; f__PeM15	0.0061	–
p__Actinobacteriota; c__Actinobacteria; o__Propionibacteriales; f__Propionibacteriaceae	0.0196	–
p__Actinobacteriota; c__Coriobacteriia; o__OPB41; f__OPB41	0.0363	–
p__Actinobacteriota; c__Thermoleophilia; o__Gaiellales; f__Gaiellaceae	0.0005	–

p__Actinobacteriota; c__Thermoleophilia; o__Gaiellales; f__uncultured	0.0118	–
p__Actinobacteriota; c__Thermoleophilia; o__Solirubrobacterales; f__Solirubrobacteraceae	0.0011	–
p__Bacteroidota; c__Bacteroidia; o__Chitinophagales; f__Chitinophagaceae	0.0412	–
p__Bacteroidota; c__Bacteroidia; o__Chitinophagales; f__Saprospiraceae	5.32E-05	–
p__Bacteroidota; c__Bacteroidia; o__Cytophagales; f__Hymenobacteraceae	2.55E-06	–
p__Bacteroidota; c__Bacteroidia; o__Cytophagales; f__Microscillaceae	0.0089	–
p__Bacteroidota; c__Bacteroidia; o__Flavobacteriales; f__Flavobacteriaceae	7.74E-05	–
p__Bacteroidota; c__Bacteroidia; o__Flavobacteriales; f__Weeksellaceae	0.0002	–
p__Bacteroidota; c__Bacteroidia; o__Sphingobacteriales; f__Sphingobacteriaceae	0.0001	–
p__Bdellovibrionota; c__Bdellovibrionia; o__Bdellovibrionales; f__Bdellovibrionaceae	0.0013	–
p__Bdellovibrionota; c__Oligoflexia; o__0319-6G20; f__0319-6G20	0.0375	–
p__Chloroflexi; c__KD4-96; o__KD4-96; f__KD4-96	1.99E-09	–
p__Chloroflexi; c__Ktedonobacteria; o__C0119; f__C0119	0.0486	–
p__Cyanobacteria; c__Cyanobacteriia; o__Cyanobacteriales; f__Nostocaceae	0.0027	–
p__Cyanobacteria; c__Cyanobacteriia; o__Cyanobacteriales; f__Phormidiaceae	1.34E-06	–
p__Cyanobacteria; c__Cyanobacteriia; o__Leptolyngbyales; f__Leptolyngbyaceae	0.0170	–
p__Cyanobacteria; c__Cyanobacteriia; o__Pseudanabaenales; f__Pseudanabaenaceae	0.0202	–
p__Cyanobacteria; c__Cyanobacteriia; o__Synechococcales; f__Cyanobiaceae	0.0071	–
p__Deinococcota; c__Deinococci; o__Deinococcales; f__Deinococcaceae	1.25E-06	–
p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae	2.25E-05	–
p__Firmicutes; c__Bacilli; o__Bacillales; f__Planococcaceae	9.22E-06	–
p__Firmicutes; c__Bacilli; o__Exiguobacterales; f__Exiguobacteraceae	2.16E-06	–
p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Aerococcaceae	0.0222	–
p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Listeriaceae	0.0076	–
p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Streptococcaceae	0.0022	–
p__Firmicutes; c__Bacilli; o__Mycoplasmatales; f__Mycoplasmataceae	0.0168	–
p__Firmicutes; c__Bacilli; o__Staphylococcales; f__Staphylococcaceae	0.0042	–
p__Firmicutes; c__Clostridia; o__Clostridiales; f__Caloramatoraceae	0.0267	–
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__Hungateiclostridiaceae	0.0058	–
p__Firmicutes; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae	0.0143	–
p__Firmicutes; c__Clostridia; o__Peptostreptococcales-Tissierellales; f__Family_XI	0.0058	–
p__Firmicutes; c__Clostridia; o__Peptostreptococcales-Tissierellales; f__Peptostreptococcaceae	0.0217	–
p__Fusobacteriota; c__Fusobacteriia; o__Fusobacteriales; f__Leptotrichiaceae	0.0071	–
p__Gemmatimonadota; c__Gemmatimonadetes; o__Gemmatimonadales; f__Gemmatimonadaceae	0.0001	–
p__Myxococcota; c__Myxococcia; o__Myxococcales; f__Myxococcaceae	0.0007	–
p__Myxococcota; c__Polyangia; o__Haliangiales; f__Haliangiaceae	0.0294	–

p__Planctomycetota; c__Planctomycetes; o__Isosphaerales; f__Isosphaeraceae	0.0486	–
p__Planctomycetota; c__Planctomycetes; o__Pirellulales; f__Pirellulaceae	0.0016	–
p__Proteobacteria; c__Alphaproteobacteria; o__Acetobacterales; f__Acetobacteraceae	6.55E-15	81
p__Proteobacteria; c__Alphaproteobacteria; o__Caulobacterales; f__Caulobacteraceae	0.0202	–
p__Proteobacteria; c__Alphaproteobacteria; o__Caulobacterales; f__Hyphomonadaceae	0.0195	–
p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Beijerinckiaceae	0.0005	–
p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Devosiaceae	2.94E-42	83
p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Rhizobiaceae	5.77E-33	82
p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Rhizobiales_Incertae_Sedis	0.0154	–
p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Rhodomicrobiaceae	0.0155	–
p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Xanthobacteraceae	0.0170	–
p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae	0.0006	–
p__Proteobacteria; c__Alphaproteobacteria; o__Tistrellales; f__Geminicoccaceae	0.0202	–
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Alcaligenaceae	0.0042	–
*p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Burkholderiaceae	1.12E-40	–
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Comamonadaceae	2.94E-42	78
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Neisseriaceae	0.0134	–
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Nitrosomonadaceae	3.03E-05	–
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Oxalobacteraceae	6.11E-37	78
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__Rhodocyclaceae	0.0089	–
p__Proteobacteria; c__Gammaproteobacteria; o__Burkholderiales; f__SC-I-84	0.0013	–
p__Proteobacteria; c__Gammaproteobacteria; o__Diplorickettsiales; f__Diplorickettsiaceae	0.0217	–
p__Proteobacteria; c__Gammaproteobacteria; o__Legionellales; f__Legionellaceae	0.0240	–
p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Moraxellaceae	9.44E-05	–
p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae	2.06E-132	82
p__Proteobacteria; c__Gammaproteobacteria; o__Xanthomonadales; f__Rhodanobacteraceae	0.0007	–
p__Proteobacteria; c__Gammaproteobacteria; o__Xanthomonadales; f__Xanthomonadaceae	3.50E-37	81
p__Verrucomicrobiota; c__Verrucomicrobiae; o__Opitutales; f__Opitutaceae	0.0317	–
p__Verrucomicrobiota; c__Verrucomicrobiae; o__Pedosphaerales; f__Pedosphaeraceae	0.0125	–
Total significantly differentially abundant families	92	14

Table C9. Summary of the phyla identified as being significantly differentially abundant between kakī and hihi eggshell samples. Benjamini-Hochberg (Benjamini & Hochberg, 1995) adjusted p-values (p-adj) are reported for results derived from DESeq2 and the W statistic is reported for ANCOM results. Only statistically significant values are shown.

Phylum	DESeq2 p-adj	ANCOM W
Greater abundance in hihi eggshell samples compared to kakī eggshell samples		
Firmicutes	0.0045	11
Total significantly differentially abundant phyla	1	1

Table C10. Results of a multivariate Adonis test for kakī samples generated using *adonis* in QIIME2 to test for any interaction between eggs being wild- or captive-laid, female ID, and clutch number. Significant p-values are highlighted in bold.

		Weighted UniFrac distance			**Unweighted UniFrac distance**			**Jaccard distance**			**Bray-Curtis distance**		
	df	F.model	R^2	Pr(>F)	F.model	R^2	Pr(>F)	F.model	R^2	Pr(>F)	F.model	R^2	Pr(>F)
Wild vs. captive	1	14.2891	0.1096	**0.001**	2.0834	0.0216	**0.002**	1.5939	0.0174	**0.001**	13.1390	0.0927	**0.001**
Female ID	16	2.0570	0.2524	**0.011**	1.4621	0.2428	**0.001**	1.1942	0.2084	**0.001**	2.5312	0.2858	**0.001**
Clutch number	1	14.2045	0.1089	**0.001**	1.8819	0.0195	**0.002**	1.9668	0.0215	**0.001**	19.0586	0.1345	**0.001**
Residuals	69	–	0.5291	–	–	0.7161	–	–	0.7527	–	–	0.4870	–
Total	87	–	1.0000	–	–	1.0000	–	–	1.0000	–	–	1.0000	–

Table C11. Summary of the most abundant phyla, families and genera across eggshell samples from captive- and wild-laid kakī eggs. Mean relative abundances are given as percentage values.

	Captive-laid	Wild-laid
Phylum		
Proteobacteria	84.81%	90.89%
Actinobacteriota	7.378%	5.868%
Firmicutes	4.945%	1.800%
Bacteroidota	1.118%	0.662%
Cyanobacteria	0.456%	0.122%
Family		
Pseudomonadaceae	52.54%	71.58%
Burkholderiaceae	9.605%	4.435%
Halomonadaceae	5.217%	2.463%
Xanthomonadaceae	2.642%	2.898%
Oxalobacteraceae	3.025%	3.154%
Pseudoalteromonadaceae	3.580%	1.195%
Staphylococcaceae	2.097%	0.573%
Sanguibacteraceae	1.219%	1.649%
Genus		
Pseudomonas	52.49%	71.49%
Ralstonia	9.592%	4.422%
Halomonas	5.216%	2.463%
Janthinobacterium	2.440%	2.710%
Stenotrophomonas	2.622%	2.884%
Pseudoalteromonas	3.580%	1.043%
Staphylococcus	2.057%	0.567%
Sanguibacter-Flavimobilis	1.219%	1.649%

Table C12. Findings from previous studies of eggshell microbiome in wild birds. ARISA = automated ribosomal intergenic spacer analysis, LC = Least Concern, NT = Near Threatened, VU = Vulnerable. For simplicity of comparison with this study taxonomic names are matched to those used within the Silva v138.1 database (Quast et al., 2013; Yilmaz et al., 2014).

Reference	Study Species	*IUCN Red List* Classification	Method	Dominant Eggshell Phyla Composition
(Basso *et al.*, 2022)	Brown-headed cowbird (*Molothrus ater*)	LC	16S rRNA gene sequencing	Actinobacteriota, Bacteroidota, Proteobacteria
	Ovenbird (*Seiurus aurocapilla*)	LC		
(Song *et al.*, 2022)	Oriental tit (*Parus minor*)	NA	16S rRNA gene sequencing	Actinobacteriota, Firmicutes, Bacteroidota, Planctomycetota, Proteobacteria
(Ruiz-Castellano *et al.*, 2019)	Spotless starling (*Sturnus unicolor*)	LC	Culture	Actinobacteriota, Firmicutes, Proteobacteria
(Boonyarittichaikij *et al.*, 2018)	Great tit (*Parus major*)	LC	qPCR	Firmicutes, Bacteroidota, Proteobacteria
	Eurasian blue tit (*Cyanistes caeruleus*)	LC		
(Peralta-Sánchez *et al.*, 2018)	17 species	LC (one VU)	Culture and ARISA and 16S rRNA gene sequencing	Actinobacteriota, Firmicutes, Bacteroidota, Proteobacteria
(Geltsch *et al.*, 2018)	Common cuckoo (*Cuculus canorus*)	LC	Culture and 16S rRNA gene sequencing	Actinobacteriota, Firmicutes, Bacteroidota, Proteobacteria
	Great reed-warbler (*Acrocephalus arundinaceus*)	LC		
(Tomás *et al.*, 2018)	Spotless starling (*Sturnus unicolor*)	LC	Culture and ARISA	Firmicutes, Proteobacteria
(Bollinger *et al.*, 2018)	Eastern bluebird (*Sialia sialis)*	LC	Culture	Proteobacteria
(Lee *et al.*, 2017)	Eurasian magpie (*Pica pica*)	LC	qPCR	Firmicutes, Proteobacteria
(Potter *et al.*, 2013, 2014, 2017)	House wren (*Troglodytes aedon*)	LC	Culture and 16S rRNA gene sequencing	Actinobacteriota, Firmicutes, Proteobacteria
	American kestrel (*Falco sparverius*)	LC		
	Purple martin (*Progne subis*)	LC		
(Schmitt *et al.*, 2017a, 2017b)	Tree swallow (*Tachycineta bicolor*)	LC	Culture	Firmicutes, Proteobacteria
(Ruiz-Castellano *et al.*, 2016)	Spotless starling (*Sturnus unicolor*)	LC	Culture	Firmicutes, Proteobacteria
(Grizard *et al.*, 2015)	Red-capped lark (*Calandrella cinerea*)	LC	16S rRNA gene sequencing	Actinobacteriota, Proteobacteria
(Soler *et al.*, 2011a, 2011b, 2012a, 2012b, 2015)	62 species	LC (two NT)	Culture	Firmicutes, Proteobacteria
(Grizard *et al.*, 2014)	Rock dove (*Columba livia*)	LC	16S rRNA gene sequencing	Firmicutes, Proteobacteria
(Lee *et al.*, 2014)	Eurasian magpie (*Pica pica*)	LC	qPCR and 16S rRNA pyrosequencing	Actinobacteriota, Firmicutes, Bacteroidota, Proteobacteria

(Brandl *et al.*, 2014)	Common reed-warbler (*Acrocephalus scirpac*eus)	LC	Culture and ARISA	Firmicutes, Proteobacteria
(Martín-Vivaldi *et al.*, 2014)	Common hoopoe (*Upupa epops*)	LC	Culture	Firmicutes, Proteobacteria
(Peralta-Sánchez *et al.*, 2010, 2014)	Barn swallow (*Hirundo rustica*)	LC	Culture	Firmicutes, Proteobacteria
(Peralta-Sánchez *et al.*, 2012)	24 species	LC (one VU)	Culture	Firmicutes, Proteobacteria
(Wang *et al.*, 2011)	Western bluebird (*Sialia mexicana*)	LC	Culture and 16S rRNA gene sequencing	Actinobacteriota, Firmicutes, Bacteroidota, Proteobacteria
	Tree swallow (*Tachycineta bicolor*)	LC		
	Violet-green swallow (*Tachycineta thalassina*)	LC		
(Ruiz-de-Castañeda *et al.*, 2011b)	European pied flycatcher (*Ficedula hypoleuca*)	LC	Culture	Actinobacteriota, Firmicutes, Proteobacteria
(Martín-Platero *et al.*, 2010)	Common hoopoe (*Upupa epops*)	LC	Culture and 16S rRNA gene sequencing	Firmicutes, Proteobacteria
	Great tit (*Parus major*)	LC		
	Little owl (*Athene noctua*)	LC		
	Spotless starling (*Sturnus unicolor*)	LC		
	Eurasian magpie (*Pica pica*)	LC		
	Great spotted cuckoo (*Clamator glandarius*)	LC		
(Shawkey *et al.*, 2009)	Pearly-eyed thrasher (*Margarops fuscatus*)	LC	16S rRNA gene sequencing	Actinobacteriota, Bacteroidota, Cyanobacteria, Firmicutes, Proteobacteria

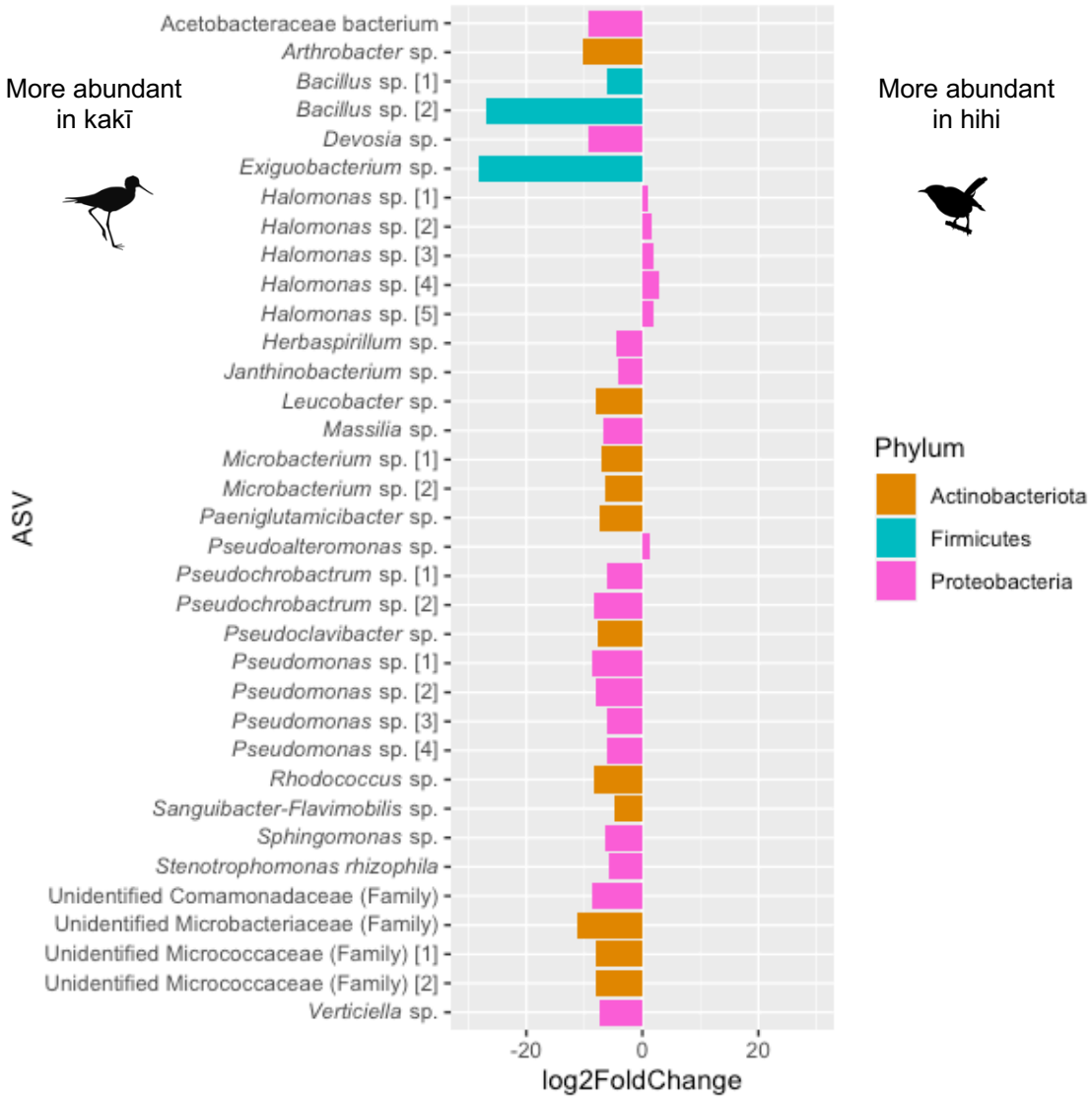


Figure C1. Symmetrical barplot showing the ASVs identified by DESeq2 as being differentially abundant between hihi and kakī eggshells. Negative log2FoldChange values indicate taxa were more abundant in kakī samples, while positive log2FoldChange values indicate taxa were more abundant in hihi samples.

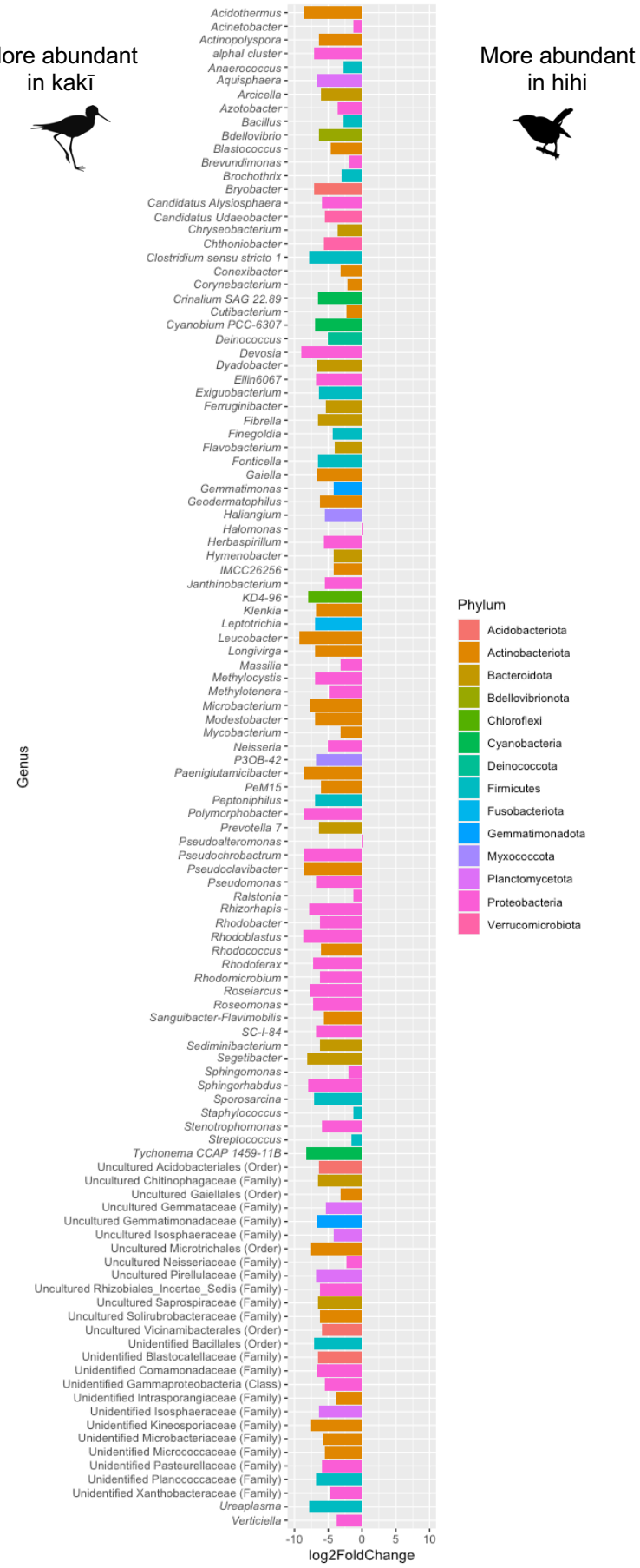


Figure C2. Symmetrical barplot showing the genera identified by DESeq2 as being differentially abundant between hihi and kakī eggshells. Negative log2FoldChange values indicate taxa were more abundant in kakī samples, while positive log2FoldChange values indicate taxa were more abundant in hihi samples.

Figure C3. Symmetrical barplot showing the families identified by DESeq2 as being differentially abundant between hihi and kakī eggshells. Negative log2FoldChange values indicate taxa were more abundant in kakī samples, while positive log2FoldChange values indicate taxa were more abundant in hihi samples.

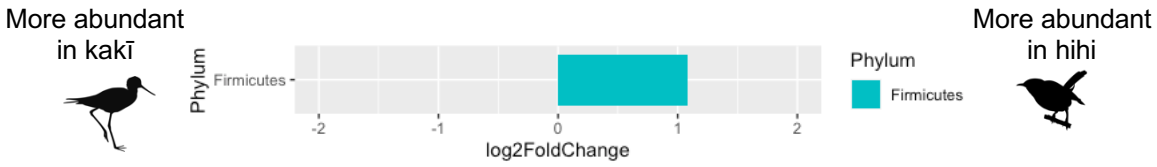


Figure C4. Symmetrical barplot showing the phylum identified by DESeq2 as being differentially abundant between hihi and kakī eggshells. Negative log2FoldChange values indicate taxa were more abundant in kakī samples, while positive log2FoldChange values indicate taxa were more abundant in hihi samples.

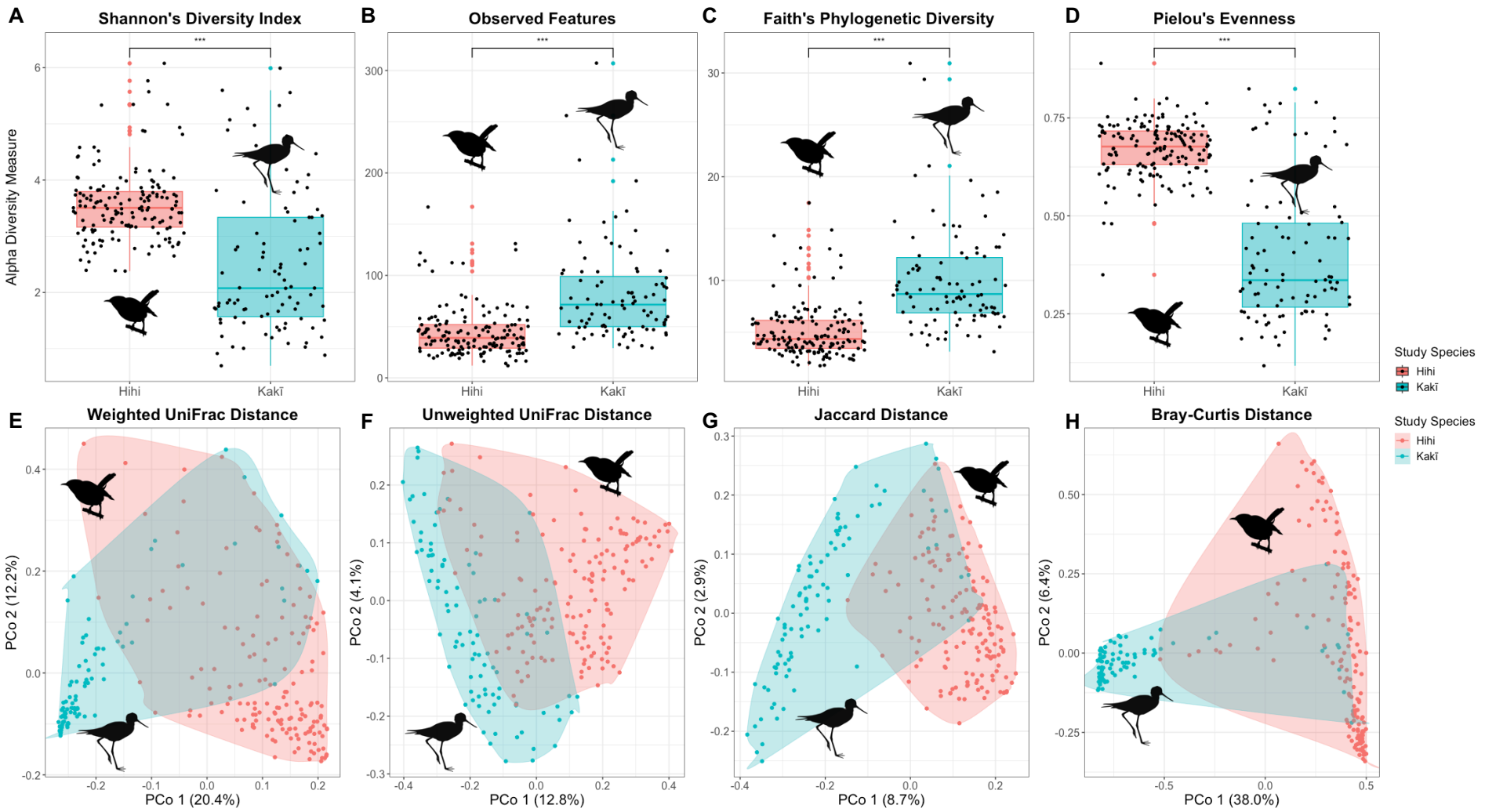


Figure C5. Visualisations of alpha and beta diversity metrics for hihi and kakī eggshells. A–D) Box-and-whisker plots of Shannon's diversity index, observed features, Faith's PD, and Pielou's Evenness, E–H) Principal coordinates analysis (PCoA) based on weighted UniFrac, unweighted UniFrac, Jaccard, and Bray-Curtis distances. Levels of significance are as represented as: ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

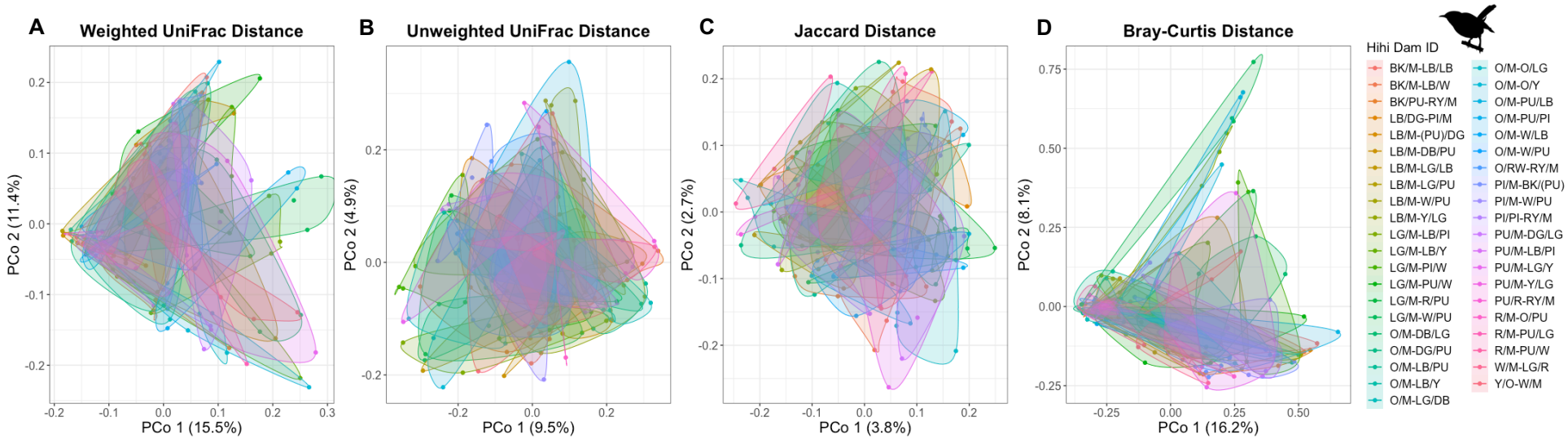


Figure C6. Visualisations of beta diversity metrics of eggshells from hihi eggs laid by different females. PCoA plots based on A) weighted UniFrac, B) unweighted UniFrac, C) Jaccard, and D) Bray-Curtis distances.

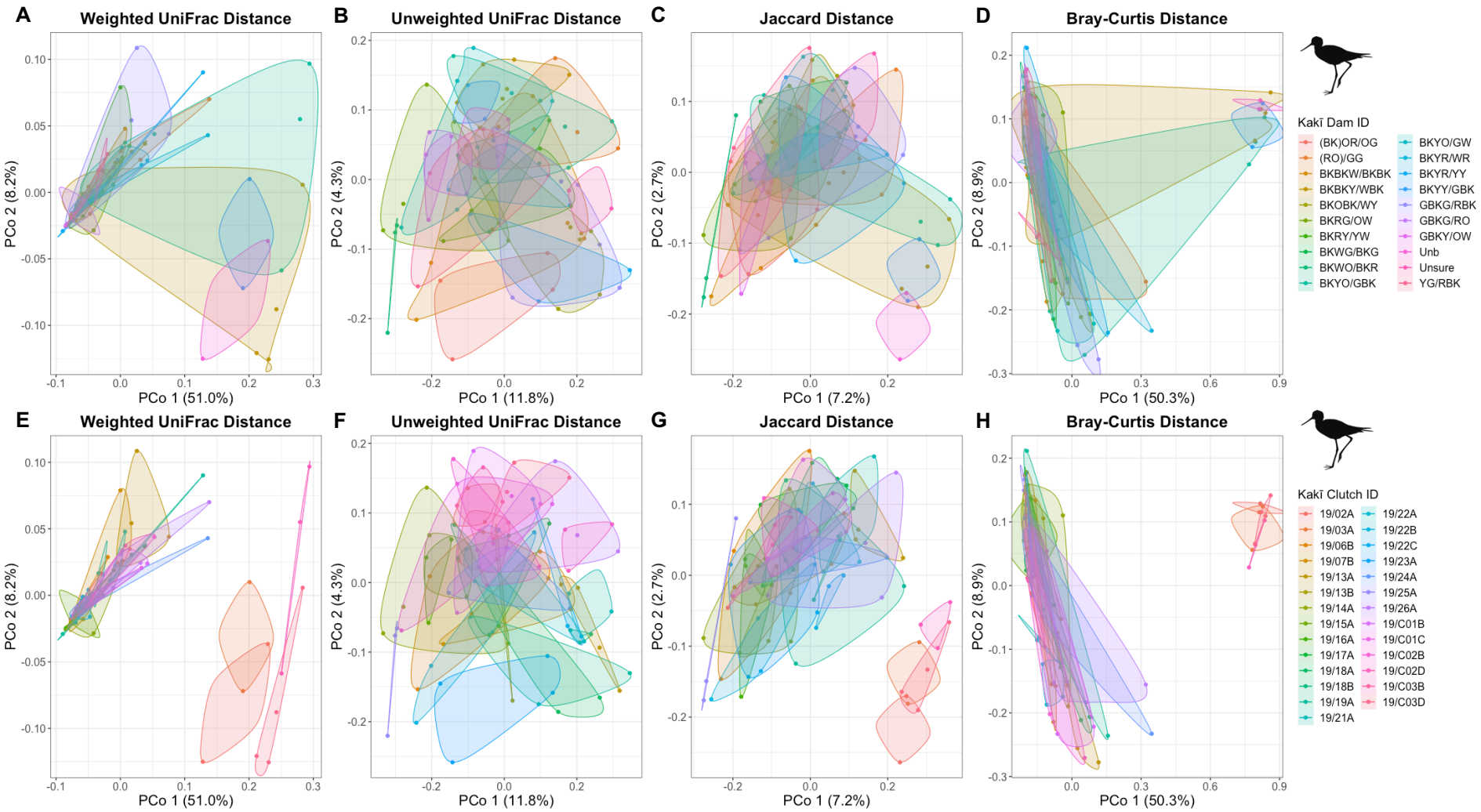


Figure C7. Visualisations of beta diversity metrics of eggshells from kakī eggs laid by different females and the same females. Repeated clutches from the same females are represented by the same number (e.g. 19/18) with a different letter (A–D) as a suffix. PCoA plots based on weighted UniFrac, unweighted UniFrac, Jaccard, and Bray-Curtis distances for kakī eggshell samples with points coloured and encircled based on A–D) dam ID, and E–H) clutch ID.

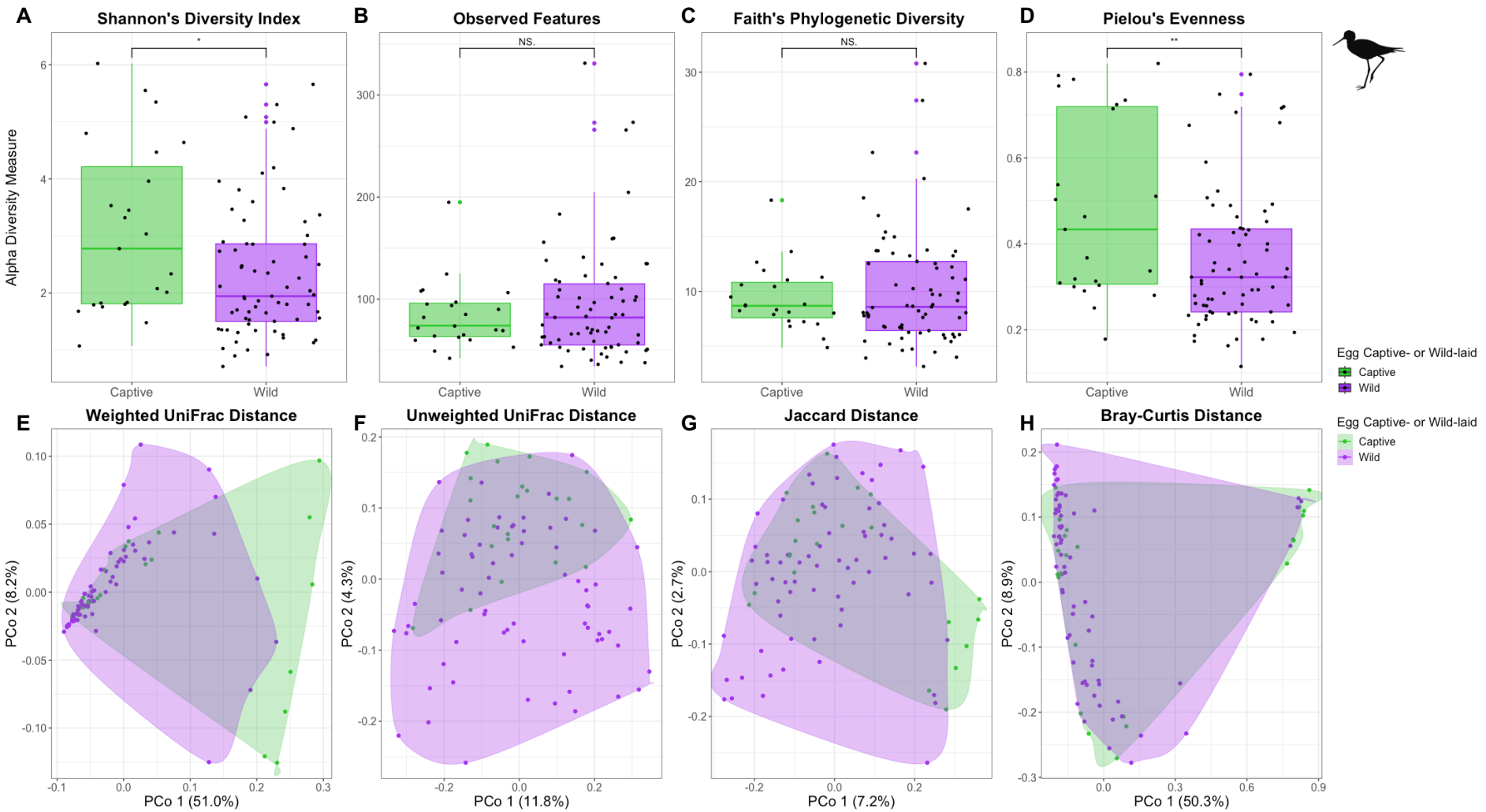


Figure C8. Visualisations of alpha and beta diversity metrics of eggshells from captive- and wild-laid kakī eggs. A–D) Box-and-whisker plots of Shannon's diversity index, observed features, Faith's PD, and Pielou's Evenness, E–H) PCoA plots based on weighted UniFrac, unweighted UniFrac, Jaccard, and Bray-Curtis distances. Levels of significance are as represented as: ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

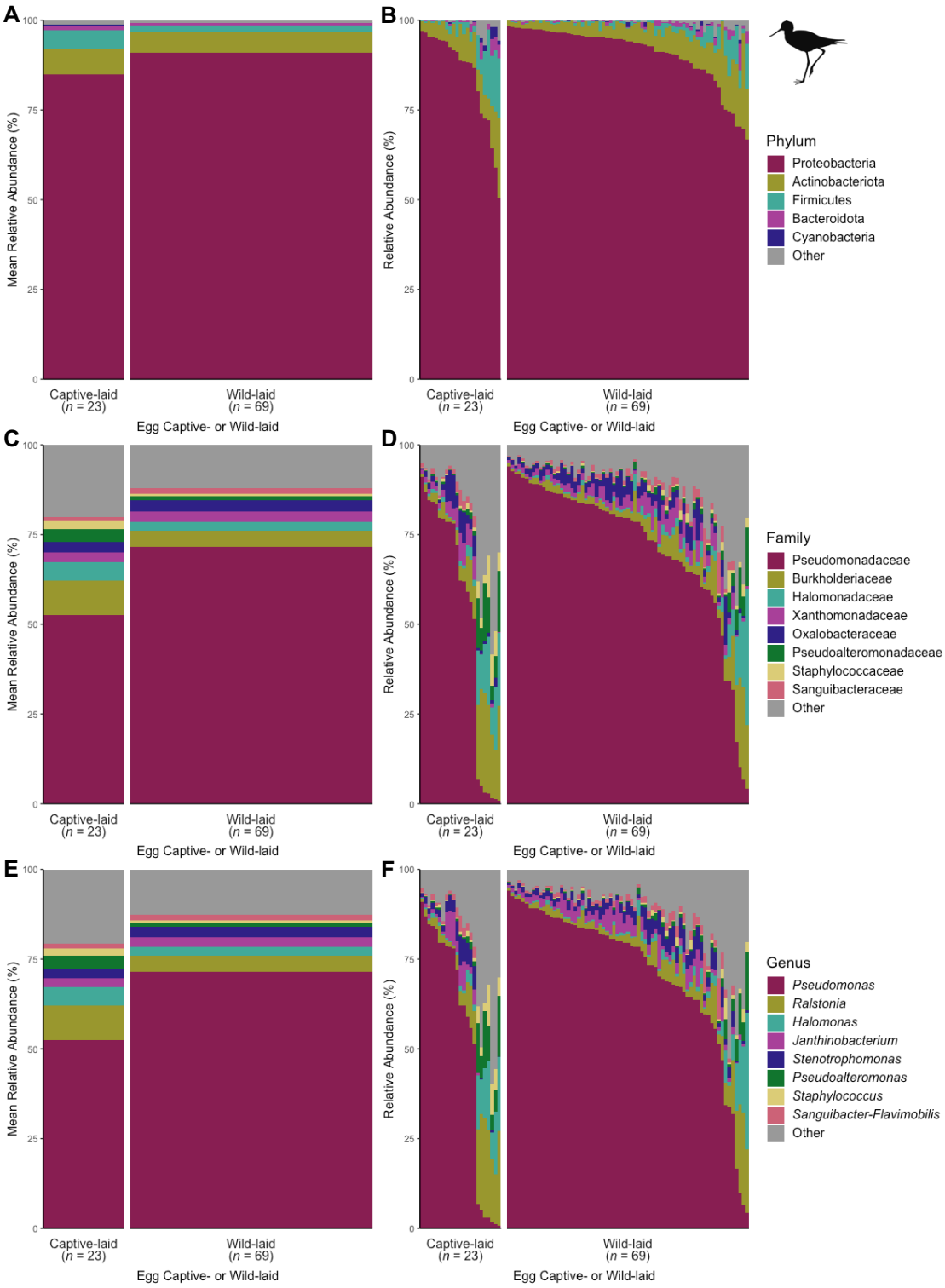


Figure C9. Relative abundances of A–B) phyla, C–D) families, and E–F) genera across eggshell samples from captive- and wild-laid kakī eggs. The left of each pair of figures shows the mean relative abundances across all samples at that taxonomic level, while the right shows the relative abundance in each sample. The top five most abundant phyla and top eight most abundant families and genera are named, with the remaining taxa represented by 'Other'. The width of each panel is representative of the sample size of the groups.

Appendix D: Supplementary information for Chapter 5

Checks for multicollinearity

Table D1. Assessment of correlations between continuous variables using Pearson correlation coefficients. Cut-offs for interpretation were as follows: ±1.0-0.9 = very high correlation; ±0.9-0.7 = high correlation; ±0.7-0.5 = moderate correlation; ±0.5-0.3 = low correlation; ±0.3-0.1 = very low correlation; ±0.1-0.0 = negligible correlation.

Parameter 1	Parameter 2	*r*	df	*p*-value	Correlation interpretation
Hihi					
Dam age	Sire age	0.0396	155	0.6229	negligible
Dam age^2	Sire age^2	0.0032	155	0.9687	negligible
Dam age	Dam total social partners	0.8045	155	**<0.001**	high
Dam age^2	Dam total social partners	0.8085	155	**< 0.001**	high
Dam age	Dam total genetic partners	0.9340	155	**<0.001**	very high
Dam age^2	Dam total genetic partners	0.9168	155	**<0.001**	very high
Sire age	Sire total social partners	0.7397	155	**<0.001**	high
Sire age^2	Sire total social partners	0.8038	155	**<0.001**	high
Sire age	Sire total genetic partners	0.8025	155	**<0.001**	high
Sire age^2	Sire total genetic partners	0.7942	155	**<0.001**	high
Mean daily temp.	Min. daily temp.	0.9698	155	**<0.001**	very high
Mean daily temp.	Max. daily temp.	0.7627	155	**<0.001**	high
Min. daily temp.	Max. daily temp.	0.6821	155	**<0.001**	moderate
Kakī					
Dam age	Sire age	0.6058	86	**<0.001**	moderate
Dam age^2	sire age^2	0.4797	86	**<0.001**	low
Dam age	Dam total social partners	0.7712	86	**<0.001**	high
Dam age^2	Dam total social partners	0.8048	86	**<0.001**	high
Sire age	Sire total social partners	0.6099	90	**<0.001**	moderate
Sire age^2	Sire total social partners	0.6082	90	**<0.001**	moderate
Egg weight	Est. days incubation	-0.2683	87	**0.011**	very low
Mean daily temp.	Min. daily temp.	0.7247	90	**<0.001**	moderate
Mean daily temp.	Max. daily temp.	0.7636	90	**<0.001**	high
Min. daily temp.	Max. daily temp.	0.1612	90	0.1250	very low
Est. days incubation	Relative abundance *Pseudomonas*	-0.4724	90	**<0.001**	low

Table D2. Assessment of correlations between categorical variables using pairwise chi-squared tests of independence and Cramér's V. Interpretation of Cramér's V values were as follows: 0.1–0.3 = weak association; 0.3–0.5 = moderate association; and >0.5 = strong association.

Parameter 1	Parameter 2	*Cramér's V*	df	*p*-value	Correlation interpretation
Hihi					
Dam experienced vs. naive	Sire experienced vs. naive	0.3532	1	**<0.001**	moderate
Kakī					
Dam experienced vs. naive	Sire experienced vs. naive	0.7034	1	**<0.001**	strong
Nest location	Dam ID	0.9738	247	**<0.001**	strong
Egg captive- vs. wild-laid	Dam captive- vs. wild-laid	0.3974	1	**<0.001**	moderate
Egg captive- vs. wild-laid	Sire captive- vs. wild-laid	0.4116	1	**<0.001**	moderate
Dam captive- vs. wild-laid	Sire captive- vs. wild-laid	0.4453	1	**<0.001**	moderate

Table D3. Assessment of correlations between categorical and continuous variables using point biserial correlation coefficients. Cut-offs for interpretation were as follows: ±1.0-0.9 = very high correlation; ±0.9-0.7 = high correlation; ±0.7-0.5 = moderate correlation; ±0.5-0.3 = low correlation; ±0.3-0.1 = very low correlation; ±0.1-0.0 = negligible correlation.

Parameter 1	Parameter 2	r_{pb}	df	*p*-value	Correlation interpretation
Hihi					
Dam age	Dam experienced vs. naive	0.7556	155	**<0.001**	high
Dam age^2	Dam experienced vs. naive	0.6079	155	**<0.001**	moderate
Sire age	Sire experienced vs. naive	0.6631	155	**<0.001**	moderate
Sire age^2	Sire experienced vs. naive	0.5128	155	**<0.001**	moderate
Kakī					
Dam age	Dam experienced vs. naive	0.5070	86	**<0.001**	moderate
Dam age^2	Dam experienced vs. naive	0.3700	86	**<0.001**	low
Sire age	Sire experienced vs. naive	0.7089	90	**<0.001**	high
Sire age^2	Sire experienced vs. naive	0.6019	90	**<0.001**	moderate

Relative abundances of taxa

Table D4. Summary of the most abundant phyla, families and genera across hatched and unhatched eggshell samples from hihi and kakī. Mean relative abundances are given as percentage values.

	Hihi eggshell samples		**Kakī eggshell samples**	
	Hatched	Unhatched	Hatched	Unhatched
Phylum				
Proteobacteria	86.42%	89.74%	88.65%	92.13%
Firmicutes	6.095%	3.537%	2.829%	1.653%
Actinobacteriota	5.239%	5.115%	6.547%	5.085%
Bacteroidota	0.953%	0.665%	0.884%	0.362%
Cyanobacteria	0.544%	0.011%	0.208%	0.193%
Family				
Burkholderiaceae	25.30%	25.27%	5.590%	6.254%
Halomonadaceae	22.70%	20.31%	3.174%	3.067%
Pseudomonadaceae	15.44%	16.87%	65.90%	70.34%
Pseudoalteromonadaceae	11.58%	11.79%	1.707%	2.116%
Orbaceae	0.000%	2.907%	0.000%	0.000%
Staphylococcaceae	2.785%	1.304%	1.052%	0.577%
Moraxellaceae	1.981%	1.798%	0.708%	0.555%
Oxalobacteraceae	2.292%	2.537%	3.143%	3.041%
Xanthomonadaceae	0.742%	0.573%	2.865%	2.715%
Sanguibacteraceae	0.272%	0.000%	1.575%	1.414%
Micrococcaceae	0.900%	1.627%	1.167%	1.525%
Genus				
Ralstonia	25.03%	24.88%	5.575%	6.247%
Halomonas	22.70%	20.31%	3.173%	3.067%
Pseudomonas	15.32%	16.83%	65.83%	70.23%
Pseudoalteromonas	11.58%	11.79%	1.707%	2.116%
Orbus	0.000%	2.907%	0.000%	0.0005
Staphylococcus	2.778%	1.304%	1.034%	0.5775
Janthinobacterium	2.067%	2.309%	2.639%	2.6565
Acinetobacter	1.637%	1.085%	0.537%	0.430%
Stenotrophomonas	0.688%	0.521%	2.846%	2.711%
Sanguibacter-Flavimobilis	0.272%	0.000%	1.575%	1.414%
Pseudochrobactrum	0.070%	0.050%	1.138%	0.823%

Model selection

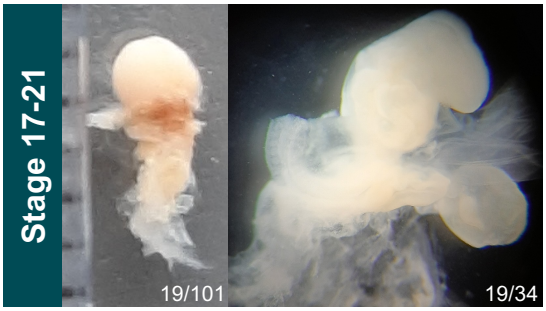
Table D5. Model selection to assess the effects of microbiome diversity on hatching success in hihi and kakī. Dam ID was included as a random term in all models. All continuous variables were scaled and centred. *K* = number of parameters in the model, AIC = Akaike's information criterion, AIC_c = corrected AIC for small sample sizes, Δi = difference in the AIC_c from that of the strongest model, RL = relative likelihood, *wi* = Akaike weight. Shading highlights models within $\Delta AIC_c \leq 2$ of the best model which were included in model averaging.

no.	Model	*K*	AIC	AIC_c	Δi	RL	w_i
Hihi binary hatching success (*n* = 106)							
1	Shannon's diversity index + dam age^2	4	104.651	105.047	0.000	1.000	0.205
2	Shannon's diversity index + dam age	4	104.722	105.118	0.072	0.965	0.198
3	Shannon's diversity index × dam age^2	5	105.488	106.088	1.041	0.594	0.122
4	Shannon's diversity index × dam age	5	105.518	106.118	1.071	0.585	0.120
5	Shannon's diversity index	3	107.915	108.151	3.104	0.212	0.043
6	dam age	3	108.237	108.472	3.425	0.180	0.037
7	dam age^2	3	108.410	108.645	3.598	0.165	0.034
8	Shannon's diversity index × mean daily rainfall + dam age	6	108.059	108.908	3.861	0.145	0.030
9	Shannon's diversity index × mean daily temp. + dam age^2	6	108.097	108.945	3.898	0.142	0.029
10	Shannon's diversity index × mean daily rainfall + dam age^2	6	108.124	108.972	3.926	0.140	0.029
11	Shannon's diversity index × mean daily temp. + dam age	6	108.152	109.000	3.953	0.139	0.028
12	Pielou's evenness	3	109.463	109.698	4.651	0.098	0.020
13	Relative abundance of *Staphylococcus*	3	109.671	109.906	4.860	0.088	0.018
14	Relative abundance of *Enterococcus*	3	110.155	110.390	5.343	0.069	0.014
15	Null	2	110.353	110.469	5.423	0.066	0.014
16	Observed features	3	110.686	110.921	5.874	0.053	0.011
17	Faith's PD	3	111.280	111.516	6.469	0.039	0.008
18	Mean daily rainfall	3	111.382	111.617	6.570	0.037	0.008
19	Mean daily temp.	3	111.812	112.047	7.000	0.030	0.006
20	Sire age	3	111.819	112.054	7.007	0.030	0.006
21	Relative abundance of *Pseudomonas*	3	112.181	112.417	7.370	0.025	0.005
22	Sire age^2	3	112.246	112.481	7.434	0.024	0.005
23	Cutch size	3	112.320	112.555	7.508	0.023	0.005
24	Relative abundance of Enterobacteriaceae	3	112.352	112.588	7.541	0.023	0.005
Hihi proportional hatching success per clutch (*n* = 157)							
1	Dam age	3	304.689	304.845	0.000	1.000	0.227
2	Dam age^2	3	304.954	305.110	0.265	0.876	0.199
3	Null	2	306.461	306.538	1.693	0.429	0.098
4	Mean daily rainfall	3	307.652	307.809	2.963	0.227	0.052
5	Sire age	3	308.220	308.376	3.531	0.171	0.039
6	Clutch size	3	308.355	308.511	3.666	0.160	0.036
7	Relative abundance of Enterobacteriaceae	3	308.381	308.538	3.693	0.158	0.036
8	Relative abundance of *Enterococcus*	3	308.411	308.568	3.723	0.155	0.035
9	Mean daily temp.	3	308.434	308.591	3.746	0.154	0.035
10	Sire age^2	3	308.438	308.595	3.749	0.153	0.035
11	Observed features	3	308.438	308.595	3.750	0.153	0.035
12	Faith's PD	3	308.440	308.597	3.752	0.153	0.035
13	Shannon's diversity index	3	308.441	308.598	3.753	0.153	0.035
14	Relative abundance of *Pseudomonas*	3	308.456	308.613	3.767	0.152	0.035
15	Pielou's evenness	3	308.460	308.617	3.771	0.152	0.034
16	Relative abundance of *Staphylococcus*	3	308.460	308.617	3.772	0.152	0.034
Kakī binary hatching success (*n* = 85)							
1	Est. days of incubation	3	84.334	84.630	0.000	1.000	0.342
2	Est. days of incubation + egg captive- or wild-laid	4	84.389	84.889	0.259	0.878	0.300
3	Est. days of incubation × egg captive- or wild-laid	5	85.658	86.418	1.788	0.409	0.140
4	Est. days of incubation × relative abundance of *Pseudomonas*	5	88.114	88.874	4.243	0.120	0.041
5	Egg captive- or wild-laid	3	88.646	88.943	4.313	0.116	0.040
6	Observed features	3	89.846	90.142	5.512	0.064	0.022
7	Relative abundance of *Enterococcus*	3	90.568	90.864	6.234	0.044	0.015
8	Relative abundance of *Staphylococcus*	3	90.761	91.057	6.427	0.040	0.014
9	Clutch number	3	90.934	91.230	6.600	0.037	0.013
10	Faith's PD	3	91.230	91.526	6.896	0.032	0.011
11	Null	2	91.410	91.556	6.926	0.031	0.011
12	Shannon's diversity index	3	91.677	91.974	7.344	0.025	0.009
13	Egg weight	3	92.053	92.349	7.719	0.021	0.007
14	Mean daily rainfall	3	92.310	92.606	7.976	0.019	0.006
15	Pielou's evenness	3	92.503	92.799	8.169	0.017	0.006
16	Relative abundance of *Pseudomonas*	3	92.808	93.104	8.474	0.014	0.005
17	Relative abundance of Enterobacteriaceae	3	92.986	93.282	8.652	0.013	0.005
18	Mean daily temp.	3	93.221	93.517	8.887	0.012	0.004
19	Clutch size	3	93.244	93.541	8.911	0.012	0.004
20	Dam age	3	93.358	93.654	9.024	0.011	0.004
21	Dam age^2	3	93.362	93.658	9.028	0.011	0.004
Kakī proportional hatching success per clutch (*n* = 85)							
1	Clutch number + mean daily temp.	4	170.636	171.136	0.000	1.000	0.198

2	Mean daily temp.	3	171.172	171.468	0.332	0.847	0.167
3	Clutch number	3	171.952	172.248	1.112	0.574	0.113
4	Est. days of incubation	3	172.297	172.593	1.457	0.483	0.095
5	Clutch number × mean daily temp	5	172.553	173.312	2.176	0.337	0.067
6	Egg captive- or wild-laid	3	173.875	174.171	3.035	0.219	0.043
7	Null	2	174.087	174.233	3.097	0.213	0.042
8	Mean daily rainfall	3	174.090	174.386	3.250	0.197	0.039
9	Observed features	3	174.645	174.941	3.805	0.149	0.029
10	Clutch size	3	174.876	175.173	4.036	0.133	0.026
11	Relative abundance of Enterobacteriaceae	3	175.121	175.417	4.281	0.118	0.023
12	Shannon's diversity index	3	175.206	175.503	4.367	0.113	0.022
13	Faith's PD	3	175.366	175.662	4.526	0.104	0.021
14	Pielou's evenness	3	175.570	175.867	4.730	0.094	0.019
15	Relative abundance of *Enterococcus*	3	175.759	176.056	4.920	0.085	0.017
16	Dam age^2	3	175.767	176.064	4.927	0.085	0.017
17	Egg weight	3	175.847	176.144	5.008	0.082	0.016
18	Dam age	3	175.919	176.215	5.079	0.079	0.016
19	Relative abundance of *Pseudomonas*	3	175.987	176.283	5.147	0.076	0.015
20	Relative abundance of *Staphylococcus*	3	176.065	176.361	5.225	0.073	0.014

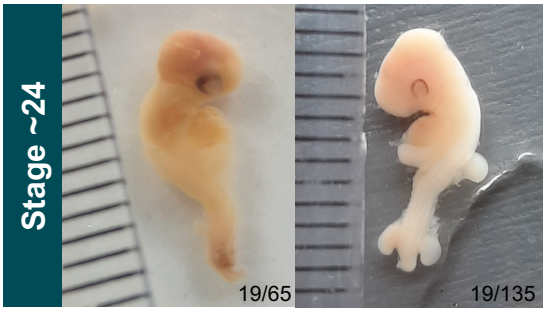
Appendix E: Kakī embryo staging guide

Developed based on previous work by Hamburger & Hamilton (1951), Hemmings & Birkhead (2016), Brown (2017), and Araújo *et al.* (2019).



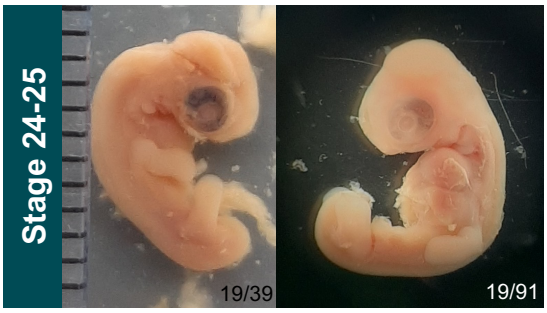
Total length: 4-5mm | **Est. age**: ~2-3 days

Key features:
- Lateral body-folds extend around circumference of body
- Wing- and leg-buds lifted off body
- Leg buds bigger than wing buds
- Optic cup completely formed, eyes unpigmented/faint gray hue



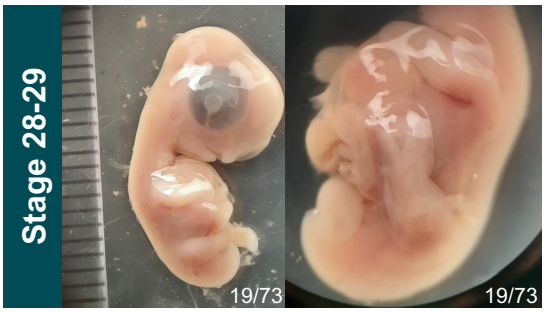
Total length: 10-13mm | **Est. age:** ~4-5 days

Key features:
- Wing- and leg-buds longer than wide
- Toe-plate in leg-bud distinct; toes not yet demarcated
- Eye pigmentation = distinct



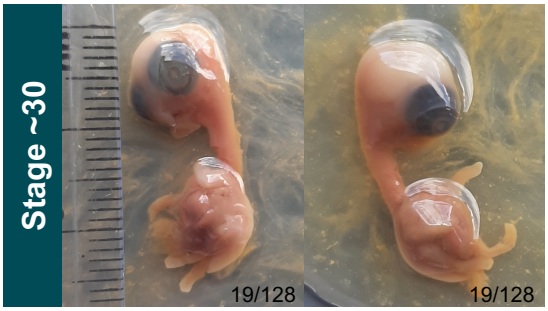
Total length: 10-12mm | **Est. age:** ~4-5 days

Key features:
- Elbow and knee-joints distinct
- Digital plate in wing distinct; digits not yet demarcated
- Eye pigmentation = distinct



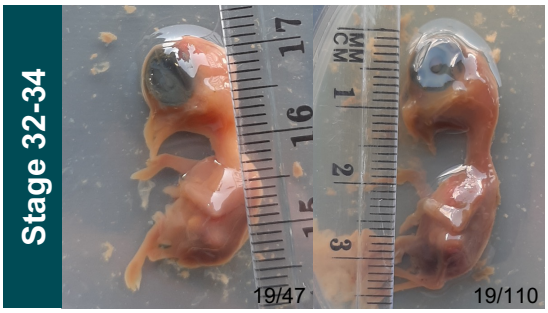
Total length: 20mm | **Est. age:** 6-7 days

Key features:
- Grooves between 1st, 2nd, and 3rd digits indicated
- Grooves between toes are distinct on surfaces of toe-plate
- A distinct outgrowth of the beak is visible in profile



Total length: 24mm | **Est. age:** 7-8 days

Key features:
- Major segments of wing/leg demarcated
- Wing/leg bent in elbow/knee joint
- Web contours between first two digits and all toes are slightly curved concave lines
- Feather germs in two dorsal rows either side of spinal cord at brachial level; three rows at leg level; none on thigh
- Egg tooth distinct, slightly protruding



Total length: 28-35mm | **Est. age:** ~8-12 days

Key features:
- Differential growth of 2nd digit and 3rd toe
- Contours of webs between digits and toes are concave and arched
- Feather-germs on tail, scapula, neck, wing's posterior edge
- Feather-germs on thigh protruding conspicuously

Total length: 55mm | **Est. age:** ~19 days

Key features:
- Eyelids almost fully developed, opening between lids much reduced
- Claws on toes flattened laterally and curved ventrally; dorsal tips opaque; tip of claw on wing opaque
- Feather germs and feathers becoming advanced over whole body

Total length: 55-64mm | **Est. age:** 20-23 days

Key features:
- Few changes from previous stages
- Eyelids completely closed
- Continued growth and maturation of feathers and cornification of nail