Drivers of female reproductive failure in the hihi, *Notiomystis cincta*

The University Of Sheffield.

Fay Morland

School of Biosciences

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GENERAL SUMMARY

Reproductive failure is costly for individuals, representing a waste of energetic investment and fitness potential. This is particularly true for females, who typically invest more heavily in reproduction than males, producing larger and more energetically costly gametes than males. Despite this, reproductive failure is commonplace across taxa. This thesis addresses the proximate and ultimate drivers of female reproductive failure in a threatened bird, the hihi (*Notiomystis cincta*).

The hihi is endemic to New Zealand and has undergone multiple genetic bottlenecks due to the impacts of invasive predators and habitat loss. On average, 28% of eggs fail to hatch in my focal study population. To investigate the underlying causes of this hatching failure, I first developed novel methods for the DNA extraction and genotyping of embryos which failed very early during development, from eggs that were previously assumed to be unfertilised by macroscopic assessment (Chapter 2). I then investigated the prevalence of fertilisation failure and embryo mortality as causes of hatching failure using data and samples from a 10 year period, specifically identifying the prevalence of previously overlooked early embryo mortality and testing for associations between fertilisation failure and population demographics (Chapter 3). Next, I examined how individual females invest in reproductive traits across their lifespans and whether early-life telomere length – an indicator of early-life developmental conditions – can predict investment strategies and reproductive senescence (Chapter 4). Finally, I look at how nesting site habitat characteristics influence the reproductive success of female hihi, and the extent to which these habitat characteristics are shaped by habitat restoration (Chapter 5).

The work I present here shows that the primary cause of hatching failure in hihi is early embryo mortality, and that this mortality is subject to biases in paternity and offspring sex. I also found that fertilisation failure contributes significantly to hatching failure and is more frequent in years with a smaller population size and more male-skewed adult sex ratio. Female reproductive life-history strategy is predicted by their early-life telomere length, with shorter telomeres indicating a “faster” life-history strategy of larger clutch sizes and higher hatching success but faster senescence. However, early-life telomere length does not predict lifetime reproductive success or lifespan. Finally, I found that the microclimate of the nest site affects the incubation behaviour of female hihi, but despite this behavioural plasticity, warmer nest microclimates lead to lower hatching success and fledging numbers. The microclimate of a nest habitat, as well as other habitat characteristics, are significantly shaped by reforestation planting techniques, suggesting an important role for habitat restoration in shaping suitable nest sites.

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DECLARATION & AUTHOR CONTRIBUTIONS

I, the author, confirm that the Thesis is my own work. I am aware of the University's Guidance on the Use of Unfair Means. This work has not been previously been presented for an award at this, or any other, university.

Chapter 2: I co-designed the study and methods, carried out the fieldwork, lab work, and data analysis, and wrote the manuscript. Nicola Hemmings and Patricia Brekke contributed to the conception of the study and provided comments which improved the manuscript. Selina Patel, under the supervision of Anna Santure at the University of Auckland co-developed (with me) the methods for DNA extraction and contributed to the lab work required for the study. Mhairi McCready and Leani Oosthuizen contributed to the fieldwork required for this study.

Chapter 3: I co-designed the study, carried out the fieldwork, lab work, and data analysis, and wrote the manuscript. Nicola Hemmings and Patricia Brekke contributed to the conception of the study and provided comments which improved the manuscript. Selina Patel, under the supervision of Anna Santure at the University of Auckland, contributed to the DNA extraction and genotyping. Mhairi McCready, Leani Oosthuizen, and past volunteers, students and Department of Conservation staff also contributed to the field work required for this study. Elizabeth Parlato carried out the population modelling for estimations of population size and adult sex ratio.

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CHAPTER 1. GENERAL INTRODUCTION

Reproductive success is the keystone to Darwinian fitness and the basis for natural selection. Failure to reproduce therefore incurs high fitness costs for individuals, and selection is expected to act strongly against it. Despite this, reproductive failure is surprisingly prevalent across taxa. For example, a review of 70 mammals species found that 20% of ova fail to result in offspring that develop beyond the neonatal stage (Stockley, 2003), and on average 10% of birds eggs fail to hatch across all species (Koenig, 1982). Rates of reproductive failure are typically highest in small and/or threatened populations, elevating extinction risk and posing important problems for conservation management (Assersohn et al., 2021; see Appendix 1).

The costs of reproductive failure are higher for the sex that invests most in gamete production and parental care, which is most often females. A meta-analysis of 656 species across six taxa found that energy used by females for gamete production is three times that used for basal metabolism, and females invest 3.5 orders of magnitude more energy in gamete biomass production than males (Hayward and Gillooly, 2011). Gamete production is particularly costly in species where the females produce large, yolky eggs, such as birds (Carey, 1996). For example, egg production causes a 22-27% increase in resting metabolic rate in zebra finches (*Taeniopygia guttata*) and great tits (*Parus major*) (Nilsson and Råberg, 2001; Vézina and Williams, 2005), and experimentally increased investment in egg production leads to lower survival in wild great tits *Parus major* (Visser and Lessells, 2001). The failure of a bird's egg to hatch therefore represents a substantial waste of invested resources and fitness potential. Females also tend to provide a higher proportion of parental care (Clutton-Brock, 2007; Trivers, 1972), with female-only care being common across taxa (Reynolds et al., 2002).

Despite the fact that reproductive failure is usually more costly for females, female-specific reproductive problems (such as female infertility) have received considerably less attention in the scientific literature compared to male-specific issues (Assersohn et al., 2021; Galimov et al., 2021). Studying reproductive failure from the female's perspective is therefore crucial for both our understanding of the evolutionary processes influencing individual fitness, but also to inform conservation management strategies for species that are threatened with extinction. This thesis aims to enhance our understanding of how and why females vary in their ability to reproduce, by investigating the genetic, demographic, ecological and behavioural drivers of female reproductive failure in a threatened bird species.

CAUSES OF REPRODUCTIVE FAILURE IN BIRDS

Reproductive failure can occur at any stage in the female's reproductive cycle: from egg production, sperm acquisition, and fusion of the gametes, through to successful development of the offspring inside and outside of the egg/mother's body, and finally to the survival of offspring to reproduce themselves. In this thesis I consider multiple aspects of reproductive failure, but since considerable research has been carried out on the latter phases (i.e., fledging and recruitment), my primary focus is on the lesser-studied early phases: fertilisation failure of eggs and hatching failure of embryos. In birds, unfertilised ova are laid as fully formed eggs in the same way that fertilised ova are, so both types of failure are manifested in the same way: the failure of eggs to hatch.

EXTRINSIC FACTORS CAUSING HATCHING FAILURE

In birds, hatching failure can be caused by a range of environmental factors, including climate, habitat and social interactions. A seminal study by Koenig (1982) on the factors affecting egg hatchability found that that hatching failure decreased with increasing latitude (distance from the equator), and more recently, studies have shown that other environmental effects, such as high temperatures (Aldredge et al., 2012; Lundblad and Conway, 2021a), low rainfall (Wilcoxen et al., 2011) and chemical pollutants such as insecticides (Burnett et al., 2013; Helander et al., 2002) negatively impact hatching success. Another important cause of hatching failure is microbial infection, which reduces egg viability (Cook et al., 2005, 2003) and is likely to cause embryo mortality. For example, Pinowski *et al.* (1994) found that approximately 70% of unhatched eggs in a wild population of passerines were infected with microorganisms.

Many external factors that affect hatching success originate in the habitat that the female breeds in. For example, a hotter nest microclimate has been associated with decreased hatching success in burrowing owls (*Athene cunicularia*) (Lundblad and Conway, 2021a), and a study on a colony of lesser black-backed gulls (*Larus fuscus*) found that the nest microclimate was influenced by the surrounding vegetation, with nest sites surrounded by more vegetation being favoured by females and having higher hatching success (Kim and Monaghan, 2005). The nest microclimate has also been demonstrated to affect other traits that contribute to reproductive success, with warmer nests leading to increased clutch size (Wiebe, 2001) but reduced fledging success (Mueller et al., 2019). The relationship between the microclimate and reproductive success is evidently complicated, and is likely mediated by

incubation behaviour. Incubating parent birds are known to adjust their behaviour according to environmental conditions (Maziarz, 2019; Sharpe et al., 2021), which may provide a potential behavioural adaptation to increasing atmospheric temperatures and be of particular importance for species vulnerable to environmental change. The habitat of a breeding bird not only has the potential to provide a microclimatic buffer from weather and atmospheric temperatures, but also provides the energy (via food sources) needed for high levels of investment by the female in reproduction. Egg production requires a range of micronutrients alongside high energy reserves (Assersohn et al., 2021), which birds must obtain from their surrounding habitat. Supplementary feeding to ensure sufficient food is available is a common technique in the conservation of bird species, and the importance of the nutrition and energy provided by supplementary feeding has been demonstrated by increased clutch size (Castro et al., 2003; Houston et al., 2007), increased fledging numbers (Doerr et al., 2017), and reduced costs of ectoparasitism in nestlings (Ewen et al., 2009). However, the importance of naturally available food sources for reproductive success has received less research focus.

Social environment can also be important for reproductive outcomes. Koenig (1982) found that social system significantly impacts hatching success, with polygynous and cooperative breeders exhibiting higher hatching failure than monogamous species. Koenig speculated that this could be due to lower fertilisation probability in species with more social interactions, due to increased competition for mates and hence a higher probability of interference during mating and egg laying. Population demographics more generally may also impact an individual's fertility and reproduction. For example, there is some evidence that male-biased sex ratios (which are common in birds, particularly in small populations (Dale, 2001; Gerlach and Le Maitre, 2001; Nadal et al., 1996)) can heighten inter-individual aggression (Kempenaers, 1994; Kvarnemo et al., 1995; Weir et al., 2011), and reduce female survival (Gilmartin and Eberhardt, 1995), reproductive rates (Le Galliard et al., 2005), and juvenile survival (Porter et al., 2004). However, there is a lack of studies addressing how adult ratios influence reproductive outcomes in wild populations with naturally occurring male-biased sex ratios.

INTRINSIC FACTORS CAUSING HATCHING FAILURE

Characteristics of the parents, egg or embryo itself can also lead to hatching failure. For example, low-quality or old male gametes may lead to reduced fertilisation success or early embryo survival (Chenoweth, 2007; Parinaud et al., 1993; Saacke et al., 1994; White et al., 2008). The effects of female gamete quality and other maternal effects on hatching success are less well studied, although there is some evidence that egg quality (McDaniel et al., 1979) and eggshell characteristics (Narushin and

Romanov, 2002) can influence hatchability. Ecological stress also appears to trigger changes in egg quality, which can in turn lead to poorer reproductive outcomes (Saino et al., 2005). However, findings on the effects of egg characteristics on hatchability are at times contradictory, with some studies finding that smaller eggs are less likely to hatch (Potti and Merino, 1996; Serrano et al., 2005; Wiebe and Bortolotti, 1995), whilst others find no relationship between egg size and hatchability (Clifford and Anderson, 2002; Robertson and Cooke, 1993; Smith et al., 1995).

Egg quality, as well as other female fertility traits, may vary considerably between individual females and within individual females across their lifespan. Reproductive senescence, or an increase in the rate of reproductive failure with age, is pervasive across taxa (Lemaître and Gaillard, 2017; Vágási et al., 2021), and individuals vary in their rates of reproductive senescence due to factors such as prior reproductive investment (Nussey et al., 2006; Reed et al., 2008a) or conditions in early life (Balbontín and Møller, 2015). One key individual-based trait, for which there is ample evidence of a link with ageing and reproduction, is telomere length. Telomeres are repetitive sequences of DNA which are situated on the end of chromosomes, acting as a "protective cap" from deterioration, damage, and fusion with adjacent chromosomes. Telomeres are commonly known to shorten with cell replication and once telomeres on chromosomes within a cell reach a critical length, the cell can no longer replicate, and cell death is initiated. Therefore telomeres are linked directly to cellular ageing and have also been implicated in whole organism aging and lifespan in many studies across taxa (e.g. Cawthon et al., 2003; Cherdsukjai et al., 2020; Froy et al., 2021; Haussmann and Vleck, 2002; Heidinger et al., 2012; Muñoz-Lorente et al., 2019; Sánchez-Montes et al., 2020; Seeker et al., 2018; Sohn and Subramani, 2014), including birds (Bichet et al., 2020; Tricola et al., 2018; Vedder et al., 2021). The telomeres of birds have been demonstrated to shorten with stress (Boonekamp et al., 2014a; Herborn et al., 2014; Kotrschal et al., 2007) and reproductive effort (Bauch et al., 2013; Sudyka, 2019; Sudyka et al., 2014). There is evidence that telomere length can predict lifetime reproductive success, but only via effects on lifespan, on which lifetime reproductive success is highly dependent (Eastwood et al., 2019; Heidinger et al., 2021).

Telomere shortening is one of several ways in which genetic factors may influence hatching success. Other genetic factors which may impact the embryo directly or indirectly (via the parents) include: genetic incompatibility between parents, which can lead to reduced reproductive success due to increased embryo mortality (Ihle et al., 2015; Pryke and Griffith, 2009a) and decreased maternal investment (Pryke and Griffith, 2009b); embryonic chromosomal abnormalities, which can result in

mortality (Forstmeier and Ellegren, 2010); and inbreeding, the effects of which are well-documented for hatching failure and particularly important in small, threatened populations (Kardos et al., 2016). For example, genetic similarity between parents (Bensch et al., 1994; Cordero et al., 2004; N. L. Hemmings et al., 2012a; Spottiswoode and Møller, 2004) and maternal inbreeding coefficient (Cordero et al., 2004; Keller, 1998) have both been associated with increased hatching failure.

THE INCIDENCE OF FERTILISATION FAILURE VERSUS EMBRYO DEATH IN WILD BIRDS

While hatching failure is ubiquitous across all birds (Koenig, 1982), its prevalence is particularly high in threatened species, reaching as high as 77% in small wild populations (Ferreira et al., 2005; Jamieson and Ryan, 2000). A reduction in population size can be damaging for genetic diversity and overall population viability (Soulé, 1987); population bottlenecks result in lowered heterozygosity, higher levels of inbreeding, and hence increased vulnerability and extinction risk (Clegg et al., 2002; Menken, 1987; Mills and Smouse, 1994). Genetic bottleneck events can also increase the prevalence of hatching failure, with hatching failure rates increasing with the severity of the genetic bottleneck that a population has gone through (Briskie and Mackintosh, 2004; Heber and Briskie, 2010). Hatching failure may limit population growth (White et al., 2015) and has been shown to be prevalent in translocated populations (Jamieson and Ryan, 2000), posing a major problem for the conservation of rare species where conservation efforts are often focused on reintroductions (Clout and Craig, 1995). Understanding the underlying causes of hatching failure is therefore critical for the conservation of threatened bird species.

However, although correlations between hatching failure and the various factors discussed above have been confirmed, the precise mechanisms by which these factors lead to hatching failure are often overlooked. Hatching failure can be due to fertilisation failure or embryo mortality, two discrete problems which likely have different underlying ultimate causes. Fertilisation failure is expected to be driven solely by parental effects, arising from the failure of sperm to reach and fertilise the egg (Adkins-Regan, 2015), which in turn may be due to parental incompatibility (Ihle et al., 2015), low male gamete quality (White et al., 2008), or female hormonal disorders and diseases (Assersohn et al., 2021). Embryo mortality on the other hand, can result from environmental factors disrupting embryonic development (Aldredge et al., 2012; Koenig, 1982; Lundblad and Conway, 2021a), poor individual embryo quality (e.g., due to inbreeding depression; (Bensch et al., 1994; Brekke et al., 2010; Hemmings et al., 2012a), or parental effects such as low maternal investment (Ihle et al., 2017; Narushin and Romanov, 2002). Few studies make the distinction between fertilisation failure and embryo mortality when investigating

hatching failure, meaning that many conclusions drawn about the causes of hatching failure are inaccurate. One example of this is the investigation of the fertility insurance hypothesis (Gibson and Jewell, 1982), which states that females engage in extra-pair copulations in order to avoid infertile eggs. Several studies have investigated this hypothesis in birds (e.g., Ihle et al., 2013; Krokene et al., 1998), whilst incorrectly assuming that unhatched eggs were unfertilised. Recent studies have found that true fertilisation failure is relatively rare in wild birds (Hemmings et al., 2012; Hemmings and Evans, 2020; Savage et al., 2022), highlighting that the importance of fertilisation failure as a cause of hatching failure may have been overestimated (Assersohn et al., 2021; Savage et al., 2022).

METHODS FOR INVESTIGATING THE CAUSES OF HATCHING FAILURE

To identify the underlying cause of hatching failure, we must first accurately determine the reproductive stage at which failure occurs. As highlighted previously, unhatched eggs may result from either fertilisation failure or the death of the embryo (at any stage of development) prior to hatching, but the majority of studies on hatching failure to date fail to distinguish between these two primary causes (e.g. Cordero et al., 2004; Spottiswoode and Møller, 2004), or do so with substantial error (e.g. Brekke et al., 2010; Jamieson and Ryan, 2000; see Assersohn et al., 2021/Appendix 1). There is a practical reason for this: until relatively recently, the distinction between fertilisation failure and early embryo mortality was technically difficult, due to a lack of macroscopic signs of development in the early stages of embryo development (particularly in unhatched eggs that have been left in the nest for some time; Birkhead et al., 2008). Distinguishing between fertilisation failure and embryo mortality, and thereby including the "invisible fraction" (Grafen, 1988) of individuals that fail to survive beyond the early stages of development, is particularly important in research concerning threatened and endangered species, where the underlying causes and appropriate conservation actions may differ dramatically depending on whether reproductive failure is driven primarily by parental fertility issues (i.e., gamete production/transfer problems) or embryo mortality. In this thesis, I used recently developed methods that allow early embryo mortality to be accurately discriminated from fertilisation failure (Birkhead et al., 2008; Assersohn et al., 2021), enabling separate and specific examination of these two proximate causes of hatching failure. These methods involve the isolation and examination of the perivitelline layer which surrounds the yolk, for evidence of sperm penetration, and the germinal disc, where the egg nucleus is located, for evidence of embryonic development. Isolating these components within unhatched eggs, staining them with a fluorescent DNA-binding dye, and examining them microscopically for signs of fertilisation and embryonic development (sperm and embryonic cells) allows accurate determination of fertilisation and early embryo development.

BACKGROUND ON STUDY SYSTEM

The work presented in this thesis was carried out in conjunction with the Zoological Society of London and the Hihi Conservation Project on a population of hihi on Tiritiri Mātangi Island in New Zealand. The hihi is an endemic New Zealand passerine that underwent a near-extinction event in the late 19th century due to the introduction of invasive predators and habitat destruction by European colonisers. The hihi population on Tiritiri Mātangi was established by two translocations in 1995 and 1996 from a source population on Little Barrier Island, and has since grown substantially from eighteen original individuals (twelve males, six females) that started the 1996 breeding season (Armstrong et al., 2002). The Tiritiri Mātangi population has been relatively successful, growing by 34% (on average) each year, and has even been harvested to supply new reintroductions, but there is a high rate of hatching failure (28%) and a risk of inbreeding depression due to the historical genetic bottleneck and small population (Jamieson and Ryan, 2000). The severe reduction in overall population that the only remaining natural population (the remnant population) of hihi have high levels of inbreeding (F_{ROH} = 0.15 as estimated through runs of homozygosity; Duntsch et al., 2021), and this is exacerbated in the Tiritiri Mātangi population by the fact that the reintroduction of the species on the island created a second population bottleneck event. This population is therefore highly inbred (f = 0.08 ± 0.009 as estimated through microsatellite analysis; Brekke et al., 2010).

Detailed breeding data has been recorded for the Tiritiri Mātangi hihi population since 2001, including identities of parents, brood size, hatching and fledging rates. The population also has a long-term pedigree, although extra-pair paternity is common, so genetic relationships are reconstructed with parentage and sibship analysis. Individuals are identifiable by unique colour bands and numbered rings, and nest boxes are monitored daily during the breeding season. Nestlings are ringed and weighed and in addition, blood samples are taken in order to determine genetic paternity and contribute to the genetic pedigree through microsatellite analysis. The result being that the mother, social father and genetic father are known for all nestlings. The clutch size, number of hatched chicks, and the number of fledglings are recorded for every clutch as part of the standard monitoring. There are two constant effort transect surveys of the hihi population on Tiritiri Mātangi carried out every year, one pre-breeding survey and one post-breeding survey. The long-term data and ongoing monitoring provide a powerful framework to comprehensively investigate questions regarding variation in female reproductive success.

Hihi are socially monogamous within a given breeding season but highly promiscuous, with a high occurrence of extra-pair paternity (Ewen et al., 1999). 89% of broods have extra-pair paternity and the average extra-pair paternity within a brood is 68%, but can range up to 100% (Brekke et al., 2013). Extra-pair copulations occur frequently during the breeding season (Castro et al., 1996a; Ewen et al., 2004). The behaviour involved in extra-pair copulations in this species is different to that of within-pair copulations and unique to hihi. Extra-pair copulations involve multiple males chasing a female, who will show evasive and sometimes aggressive avoidant behaviour (Castro et al., 1996a; Low, 2005), and concludes in the successful male pinning the female on her back on the ground in a face-to-face forced copulation (Castro et al., 1996a; Ewen et al., 2004). During the breeding season, from September to February, hihi lay on average 2 clutches of 2 to 5 eggs, and the proportion of these eggs that fail to hatch is high (28%).

AIMS AND OBJECTIVES

This thesis aims to investigate the causes of female reproductive failure using the hihi as a model threatened species with high inbreeding levels and intensive conservation management. Reproductive failure is considered on multiple scales, from fine-scale variation in reproductive failure within individuals, to population and ecosystem level effects on reproductive failure. The research presented addresses key gaps in the literature by i) developing new methods for the genetic sampling and genotyping of individuals who fail at the early stages of embryonic development; ii) examining the factors affecting true fertilisation failure and early embryo mortality; iii) investigating the effect of early-life telomere length on within-individual variation in multiple reproductive traits across the lifespan; and iv) considering the long-term implications of habitat restoration on reproductive success. In Chapter 2, I present novel field and lab methods for sampling and extracting DNA from embryos that die very early in development, and test these methods on eggs that have experienced post-mortem incubation. I also test whether the DNA obtained can be used for microsatellite genotyping, sex-typing and paternity analysis. In Chapter 3, I investigate the relative importance of fertilisation failure and embryo mortality as causes of hatching failure in hihi over a 10-year study period. I examine whether embryo mortality is subject to sex and paternity biases, and whether dead embryos have higher inbreeding coefficients compared to hatched individuals. I also explore long-term patterns of embryo mortality and fertilisation failure in relation to the demographic effects of sex ratio and population size.

In Chapter 4, I investigate the lifelong effect of a female's early-life telomere length on lifespan, lifetime reproductive success, life-history strategy, and reproductive ageing of multiple reproductive traits (clutch size, hatching success and fledging success). In Chapter 5, I assess the role of a historic planting regime in shaping current availability of natural food sources and habitat microclimate at the nest sites of hihi. I also explore the importance of the microclimate and the abundance and diversity of natural food availability for female incubation behaviour and reproductive success.

During my PhD programme, I also co-wrote (as joint first author) a review paper on the causes of hatching failure in birds, which is now published (Assersohn et al., 2021). Although not directly included as a chapter in my thesis, the content of this review paper is relevant to much of the work I present here, and I have therefore included a copy of it as an Appendix (Appendix 1).

CHAPTER 2.

OBTAINING AND ANALYSING GENETIC SAMPLES FROM WILD BIRD EMBRYOS THAT DIE EARLY IN DEVELOPMENT – A FIELD AND LAB GUIDE

ABSTRACT

Early embryo mortality has recently been proven to be a significant component of reproductive failure in populations of threatened birds. Due to the difficulty in identifying eggs that have suffered early embryo mortality, this cause of reproductive failure has historically been underestimated and overlooked. Here we describe methods for isolating embryonic material from unhatched wild bird eggs, and efficiently extracting DNA from those samples. We test these methods on unhatched eggs collected from a threatened wild bird population, which were left in the nest until the end of the incubation period to avoid disturbance, and therefore underwent post-mortem incubation. We demonstrate that it is possible to achieve DNA yields sufficient for a wide range of molecular techniques, including microsatellite genotyping for parentage analysis and sex-typing. Aspects of the sampling procedure are shown to affect downstream DNA yields and microsatellite amplification rates, and species-specific microsatellite markers have higher amplification success rates than cross-species markers. We make recommendations for each stage of the process and suggestions for potential protocol improvements and modifications.

INTRODUCTION

Long-term, individual based field studies of wild animals are recognised as a valuable asset to research (Taig-Johnston et al., 2017). Observing populations over multiple years with individual level detail provides unique insight into processes such as demography, aging, selection and climate change, and contributes to various fields, from conservation (Margalida, 2017) to ecology and evolution (Clutton-Brock and Sheldon, 2010). Often, long-term studies involve exhaustive genetic sampling of all individuals to allow pedigree construction, facilitating our understanding of topics such as life-history, quantitative trait variation, adaptation and inbreeding depression (Pemberton, 2008) . However, long-term studies often suffer from a phenomenon known as the 'invisible fraction' (Grafen, 1988); a subset of individuals that are unaccounted for because they die before they are sampled. Depending on the size of the invisible fraction, their exclusion from studies could have significant implications for our understanding of demographic and evolutionary processes.

In wild bird populations, there is evidence that the invisible fraction may be substantial (Hemmings and Evans, 2020). Birds provide an ideal system for identifying individuals that die early, since each embryo is contained within a hard-shelled egg, outside of the mother's body, which can be easily examined and sampled. Despite this, early embryo mortality, defined here as the death of an embryo before it is visible to the naked eye, is often unaccounted for in avian population monitoring. This is either because non-developed eggs are assumed to be unfertilised (Birkhead et al., 2008) or because they are not sampled for molecular analysis. For example, studies which use molecular techniques such as sex determination or parentage analysis to study differential embryonic mortality between the sexes (Brekke et al., 2010; DuRant et al., 2016; Eiby et al., 2008; English et al., 2014), or between within-pair and extra-pair offspring (Whittingham and Dunn, 2001), often miss the invisible fraction of early failed embryos in their assessment of primary sex ratio.

The inclusion of early-dead embryos in genetic analyses of wild bird populations is hampered by potential technical challenges, the most significant of these being DNA degradation. In most long-term studies or monitoring programmes, unhatched eggs remain in the nest until the end of the incubation period, to reduce the risk of mistakenly removing a viable egg. This is particularly important for species of conservation concern. However, the microclimate inside a bird's nest during incubation provides the ideal environment for post-mortem DNA degradation. Although nest temperatures during incubation vary depending on species, habitat and nest characteristics (e.g. 15-26°C in northern flickers (Wiebe, 2001), 35°C in ostrich (Swart et al., 1987), and 28-52°C in horned lark (Hartman and Oring, 2003)), in general, nests are kept warm by incubating parents to enable embryonic development. This warm environment is likely to accelerate DNA degradation in deceased embryos by facilitating autolysis (Williams et al., 2015). Despite this, DNA samples taken from deceased late-stage embryos, which have been incubated post-mortem, have been successfully used for genetic sex determination (Cichoń et al., 2005). Genetic analysis of early embryos has also been demonstrated using samples taken from live embryos at 1-4 days of development (Strausberger and Ashley, 2001), and embryos that died at an early stage of development and were then incubated for up to 2 days post-death (Kato et al., 2017). However, the successful extraction and molecular analysis of DNA from embryos in unhatched eggs that have died early and undergone a significant period of post-mortem incubation, as is often required by restricted data sampling regimes of wild/protected species, has not been reported. Methods outlining tissue sampling for DNA extraction of fully incubated, early-failed embryos will prove useful for researchers hoping to sample unhatched eggs from wild bird populations, since the ability to use

such samples will provide access to a demographic group that has thus far been missing from genetic and demographic studies of wild populations.

Here we describe methods for (i) collecting and storing unhatched eggs in the field for subsequent embryonic DNA extraction; (ii) obtaining cell samples from embryos that died very early in unhatched eggs and underwent the full incubation period, and (iii) extracting DNA from those early embryo samples. We assess the quantity of DNA obtained from early-stage embryos using these methods and its suitability for microsatellite genotyping, sex determination and paternity analysis, compared to blood samples taken from live individuals from the same species.

PROTOCOL RECOMMENDATIONS AND SPECIAL CONSIDERATIONS FOR EARLY EMBRYO SAMPLES

SAMPLE COLLECTION AND STORAGE

When collecting unhatched eggs for analysis from wild nests, it is vital to avoid jeopardising the success of the breeding attempt. This is of particular importance when working with species of conservation concern. We recommend candling eggs no earlier than 4 days after the onset of incubation to identify non-developing eggs before removal. In protected species, undeveloped eggs may need to be left in the nest until after any other eggs have hatched (or a few days beyond the incubation period, if no eggs hatch). In either instance, to limit DNA degradation after collection, we recommend unhatched eggs are stored at 1-5 °C as soon as possible. Samples can be placed in a cool box with ice packs when in the field and afterwards transferred to a standard refrigerator where they should remain until processing.

SAMPLE PROCESSING AND CELL ISOLATION

To assess an undeveloped egg for signs of fertilisation and embryonic cells, first open the egg into phosphate buffered saline (PBS) to locate and isolate the germinal disc and the section of perivitelline layer surrounding it, following the methods of Birkhead et al (2008). Unhatched, undeveloped eggs are usually found in two distinct states when collected from the field: either intact, with the yolk and albumen remaining separate inside the shell, or addled (yolk broken down and mixed with albumin, often degraded to some degree). This distinction has been used in the past to falsely categorise eggs as unfertilised when they are intact, or as failed embryos when they are addled (e.g. Cook et al., 2005). However, intact or separated eggs may be fertilised and contain embryonic cells (Assersohn et al., 2021; Birkhead et al., 2008).

Embryonic cells can be found either in the germinal disc or attached to the overlying perivitelline layer. When the egg is intact, locating and isolating the germinal disc, appearing as a white ring or spot, is straightforward. However, when the contents of failed eggs collected from the field are heavily degraded or "addled", searching the sample for pieces of perivitelline layer provides the best opportunity of locating the germinal disc and embryonic cells. When examining pieces of perivitelline layer, clean off any excess yolk with a hair loop (see Birkhead et al. 2008) or by agitating in PBS solution. However, take care not to clean the perivitelline layer so thoroughly as to potentially dislodge any embryonic cells which may be attached. The germinal disc and perivitelline layer should be stained with the fluorescent DNA dye Hoechst 33342 (Invitrogen, UK) and examined with a fluorescence microscope to search for embryonic cells, which will appear as clustered fluorescent-blue cells (Figure 1). For a more detailed explanation of the equipment and methodology used to locate and isolate the germinal disc and perivitelline layer, please see Appendix 1 (Assersohn et al. 2021) and the open access protocols and videos referred to in therein ("Practical resources for identifying the causes of hatching failure in birds"). If embryonic cells have been located on the germinal disc or the perivitelline layer, transfer the sample from the microscope slide into absolute ethanol using a pipette, to be stored for later DNA extraction. Once the majority has been transferred, pipette ethanol directly onto the slide and agitate, before pipetting back up and into the sample tube, to ensure any remaining sample is captured.

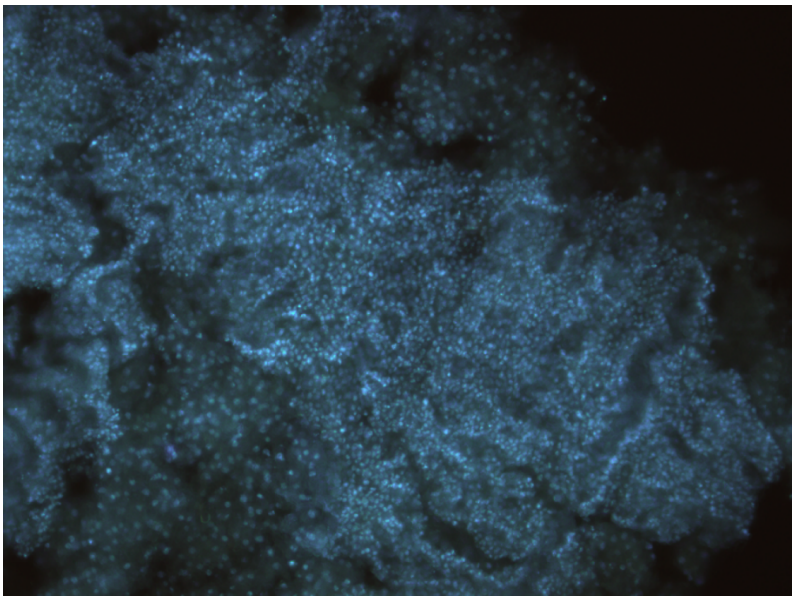

Figure 1 Hihi (Notiomystis cincta) embryonic cells stained with Hoechst fluorescent dye, as viewed at a magnificantion of x20.

DNA EXTRACTION

Some samples from embryos that die at a very early stage of development contain a very small number of cells; therefore, it is necessary to take special steps to maximise the amount of tissue retained for the DNA extraction. DNA can be extracted from the samples using the Qiagen DNeasy Blood and Tissue kit spin-column protocol for tissue samples (Purification of Total DNA from Animal Tissues (Spin-Column Protocol)), with the following modifications. Instead of starting step 1 of the protocol by placing tissue is a separate microcentrifuge tube, the ethanol-preserved perivitelline layer and germinal disc samples should be centrifuged in their original sample tubes at maximum speed (13,000 RPM) for 1 minute. All ethanol should be removed using a pipette, taking care not to disturb the tissue pellet at the bottom. After ethanol removal, 180ul Buffer ATL and 40ul Proteinase K should be added directly to the sample tubes. Sample tubes should then be incubated on a rocking platform at 56°C for one hour. Only after this step should the samples be transferred into new 1.5 ml Eppendorf tubes, followed by incubation in a rotating oven at 56°C overnight or until the samples are completely lysed. The method of lysis in the original sample tube was adopted to ensure that no cells (e.g., those potentially attached to the walls of the sample tube), were missed on tissue removal. Following this modification, the Qiagen Blood and Tissue kit protocol can be followed without further modifications from steps 3 to 7.

CASE STUDY: NOTIOMYSTIS CINCTA

METHODS

STUDY SYSTEM

The eggs we used to test our methods were collected from a managed population of hihi, *Notiomystis cincta,* which nest in nest boxes on Tiritiri Mātangi Island, Hauraki Gulf. Unhatched eggs with no sign of embryonic development were collected from this population across two breeding seasons spanning 2019-2021, screened for signs of embryonic development, and sampled for DNA. The hihi is an IUCN listed vulnerable species and exists only in predator-free reserves in the North Island of New Zealand. As is typical for many protected bird species, there are limitations to the amount of nest interference that is permitted during the hihi incubation period. Hihi have a 14-day incubation period, and eggs were collected on day 13, to limit disturbance to natural incubation behaviour and comply with permits. Before collection, eggs were candled at the nest box with a long torch to ensure there was no embryonic development visible. A long torch was used because it allows eggs to be candled in situ, avoiding the need to handle or remove eggs from the nest. In the field, undeveloped eggs were stored in small, sealable sample bags and placed inside plastic centrifuge tubes. The sample bags fitted closely

into the tube and buffered the egg from movement and impact during transportation to the field station/lab. The centrifuge tubes containing eggs were placed into a cool box with ice blocks for transportation from the field site to a fridge, in order to minimise tissue and DNA degradation as much as possible. The samples were then examined within 19 days of collection using the microscopic methods described in the Protocol Recommendations (section ii). Of 167 eggs collected, 147 (88%) were found to contain embryonic cells, samples of which were taken for DNA extraction using the methods described in the Protocol Recommendations (section iii). Of these, 101 samples were from the first season (2019-20) and 46 samples were from the second season (2020-21). If embryonic development was visible macroscopically or via a stereomicroscope, the embryo's developmental stage was classified using the Hamburger-Hamilton (HH) embryonic development staging series (Hamburger and Hamilton, 1951).

The DNA yields of these samples are compared to the DNA yields from 347 fledgling blood samples. The blood samples were taken from nestlings at day 21 and extracted in 2022 using the standard Qiagen DNeasy Blood and Tissue kit spin-column protocol for tissue samples (Purification of Total DNA from Animal Tissues (Spin-Column Protocol)). The microsatellite amplifications of these samples are compared to the microsatellite sequencing results using nestling blood samples from 2012 – 2019.

MICROSATELLITE ANALYSIS

DNA samples from embryos that died early in development and remained in the nest for the full incubation period were genotyped using 20 microsatellite markers, 2 sex-typing markers (Z002a and Z037b; Dawson, 2007; Dawson et al., 2015), 15 species-specific markers, and 3 markers developed for other passerines (Brekke et al., 2009, two primers listed in this paper were not used: Nci86C10 and MSLP4).

Each 6.75µL PCR contained 2µl of 20ng/µl of genomic DNA, 3µl of Qiagen PCR Kit Mix and 1.75µl of 1 of 4 multiplex primer mixes. Each multiplex primer mix contained 4-6 primers at 0.2 - 0.6µM concentration. Primers were grouped into multiplex mixes based on annealing temperature and allele size range (Brekke et al., 2009). The PCR followed a thermal cycle of 9°C for 15 minutes, followed by 30 cycles of 94°C for 30 s, annealing temperature of 56°C or 64°C (depending on primer mix, see Brekke et al., 2009) for 30 s, 72°C for 90 seconds, and a final step of 72 °C for 10 minutes. Microsatellites were assigned using an Applied Biosystems 3130 Genetic Analyser and Geneious software with the Microsatellite plugin.

RESULTS

DNA QUANTITY

Most samples (139 out of 145) yielded DNA following extraction using the methods described in the Protocol Recommendations. DNA quantity was assessed with a Qubit fluorometer. The mean total DNA quantity obtained per egg from the samples was 2,122ng (n=144), but values for individual samples ranged from 6.6ng to 12,400ng. The yield of DNA obtained varied between embryo stages and sample type (Table 1). For comparison, the expected yield of DNA from bird blood when using the Qiagen DNeasy Blood and Tissue kit is 9,000 – 40,000ng ("DNeasy Blood & Tissue Kits," Accessed: 19-09-2022). However, the mean yield of DNA using 20µl of blood was less than that obtained for some embryo samples types, possibly because of the conservative extraction technique used for embryos. As expected, the quantity of DNA obtained was higher for embryos that died at a later stage of development, i.e., those that didn't reveal development on candling but an embryo was obvious via macroscopic inspection on opening/dissection (approx. HH stage 8 onwards). For very early embryonic samples (≤ HH stage 7), where no development was visible on macroscopic examination, samples of perivitelline layer with the germinal disc attached provided the highest yields of DNA on average, compared to embryonic cells alone.

We did not find a relationship between processing latency (i.e., the time between egg collection and dissection/storage of sample in ethanol) and DNA quantity or functionality in downstream genetic analysis. The maximum latency between removal from the nest and processing for any of our samples was 19 days (range = 2-19 days).

Table 1 - The mean yield of DNA from germinal disc, perivitelline layer (PVL), and embryos of different developmental stages (HH Stage refers to Hamburger-Hamilton developmental stages (Hamburger and Hamilton, 1951).

HH Stage of Embryo	Sample Type (sample size)	Mean Yield of DNA (ng)
≤ 7	Germinal Disc (n = 34)	707
≤ 7	PVL (n = 20)	638
≤ 7	Germinal Disc + PVL (n = 17)	1,934
≥ 8	Germinal Disc/Embryo (n = 24)	3,688
≥ 8	Germinal Disc + PVL (n = 9)	6,170
Nestling	20µl whole blood (n = 357)	2,648

MICROSATELLITE ANALYSIS

Of all samples processed, only 21 out of 145 samples amplified across the entire microsatellite panel. However, the majority (134 samples) showed at least some amplification. Mean microsatellite amplification rates were similar for pre- and post-HH stage 8 embryos (Table 2) and very poor amplification rates were found only for perivitelline layer samples categorised as having very few embryonic cells found during microscopic examination (average success rate = 10%, n = 6). These results suggest that prioritising samples in which the embryo or germinal disc can be found, and discounting samples for which very few embryonic cells are found during microscopic examination, will prove the most efficient approach if resources and/or time are limited.

The average microsatellite success rate was 69% across samples, representing a higher failure rate in microsatellite amplification than blood samples taken from fledglings from the same population (Figure 2). Early embryo DNA showed the highest rates of amplification failure with cross-species microsatellite primers: Dpu16 (46%), Tgu-Gga (41%) and BMC04 (41%); the top three highest failing microsatellite markers were cross-species markers. This suggests that genotyping efforts for early embryonic samples may be more productive when using species-specific microsatellite markers. The length of the target

allele was not associated with microsatellite failure rate (Figure 3), suggesting that DNA degradation may not be the cause of the amplification failures.

Table 2 - The percentage of microsatellite markers (out of 40) that successfully amplified in embryo samples of different developmental stages and the most common sample types.

HH Stage of Embryo	Sample Type (sample size)	Percent of Markers Amplified
≤ 7	Germinal Disc (n = 41)	72.6%
≤ 7	Perivitelline layer (n = 26)	61.8%
≤ 7	Germinal Disc + Perivitelline layer (n = 22)	65.2%
≥ 8	Germinal Disc/Embryo (n = 33)	71.7%
≥ 8	Germinal Disc + Perivitelline layer (n = 12)	66%

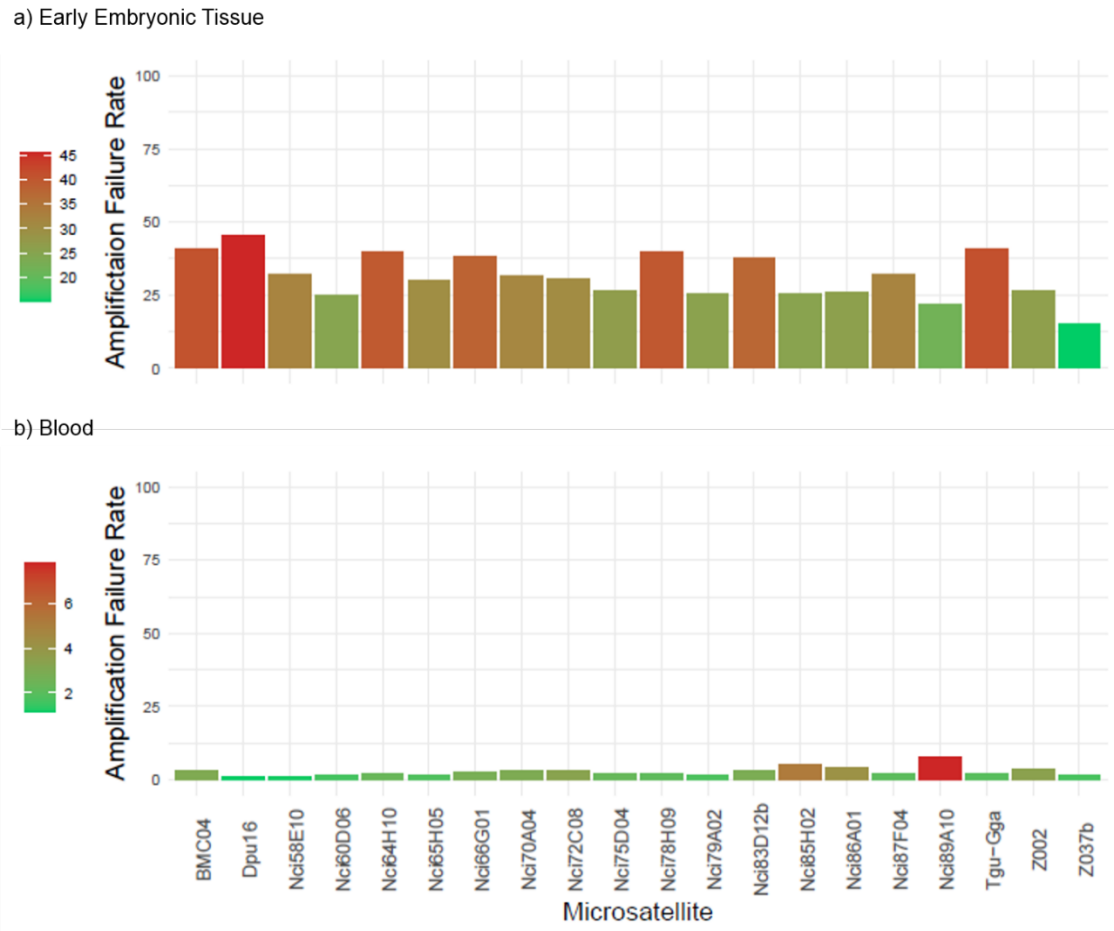

Figure 2. The microsatellite amplification failure rates for DNA obtained from a) cell samples taken from embryos that died early and b) blood samples taken from nestlings. The green to red colour gradient indicates the markers which failed the least (green) to markers which failed the most (red), relative to the failure rates for each sample type.

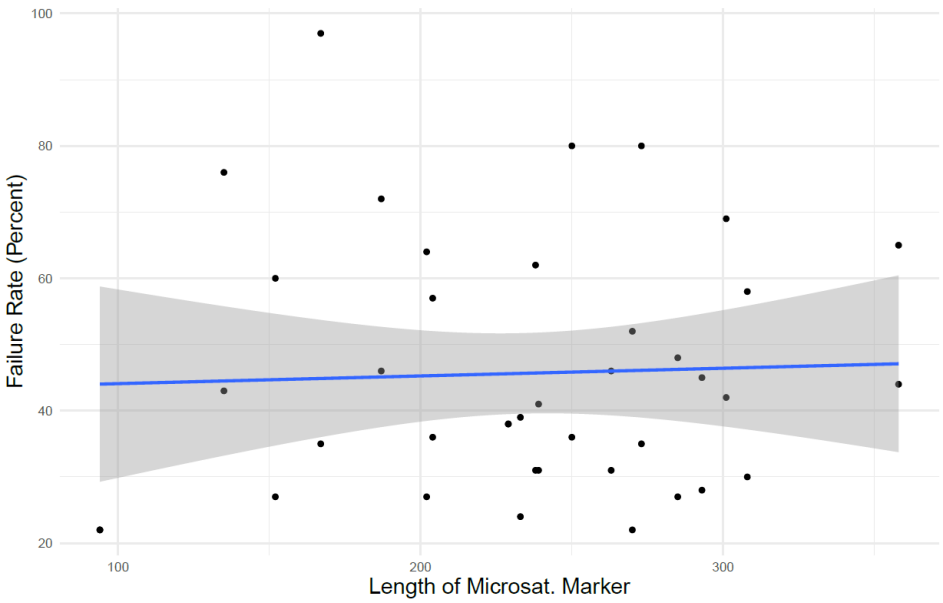

Figure 3. The rate of failure of microsatellite amplification performed on DNA from degraded, deceased early bird embryos. The failure rate had no association with the length of the microsatellite marker.

It is recommended to consider downstream requirements when sampling embryos in the early stages of development (≥ 7 in the Hamburger and Hamilton Embryonic Staging Series). DNA extracted from the germinal disc/embryonic material provided the highest success rate in microsatellite analysis; however, extracting DNA from the perivitelline layer and germinal disc together provided the highest yields of DNA.

PATERNITY ANALYSIS & SEX TYPING

Paternity assignments and duplicate checks were performed in Colony, including information on candidate maternal and paternal genotypes and maternal siblings (samples from the same nest). The parameters used were full-likelihood analysis method and high likelihood precision. There were no cases of DNA contamination from the mother or (social) father detected as duplicates by Colony. When maternal ID was excluded from the information provided to Colony, fewer mothers were correctly inferred. The majority of samples (84%) could be sexed from the 2 sex-typing markers used in microsatellite analysis.

Table 3 – A summary of the results of the paternity analysis of embryos that failed early in development using microsatellite markers and Colony. Colony was run with either maternal sibship included and maternal ID excluded, or with maternal sibship and maternal ID included.

	Maternal ID Excluded	Maternal ID Included
Maternity Correctly Inferred	44%	62%
Parentage Probability	0.44	0.79

CONCLUSIONS & LIMITATIONS

The results of our study suggest that sampling tissue for DNA extraction and downstream molecular analyses, from bird embryos that die early in development and undergo post-mortem incubation in wild nests, is possible and highly applicable to a wide range of wild population studies across multiple fields. With particular care given to sample collection, microscopic fertility examination techniques, and DNA extraction protocols (Figure 4), it is possible to obtain high yields of genomic DNA, even with very

early embryos (≤ HH stage 7). We were able to isolate DNA of relatively high molecular yield despite the small quantities of often degraded embryonic tissue available, and we found this DNA to be suitable for several downstream molecular techniques including microsatellite genotyping, which we demonstrated for sex typing and parentage assignment.

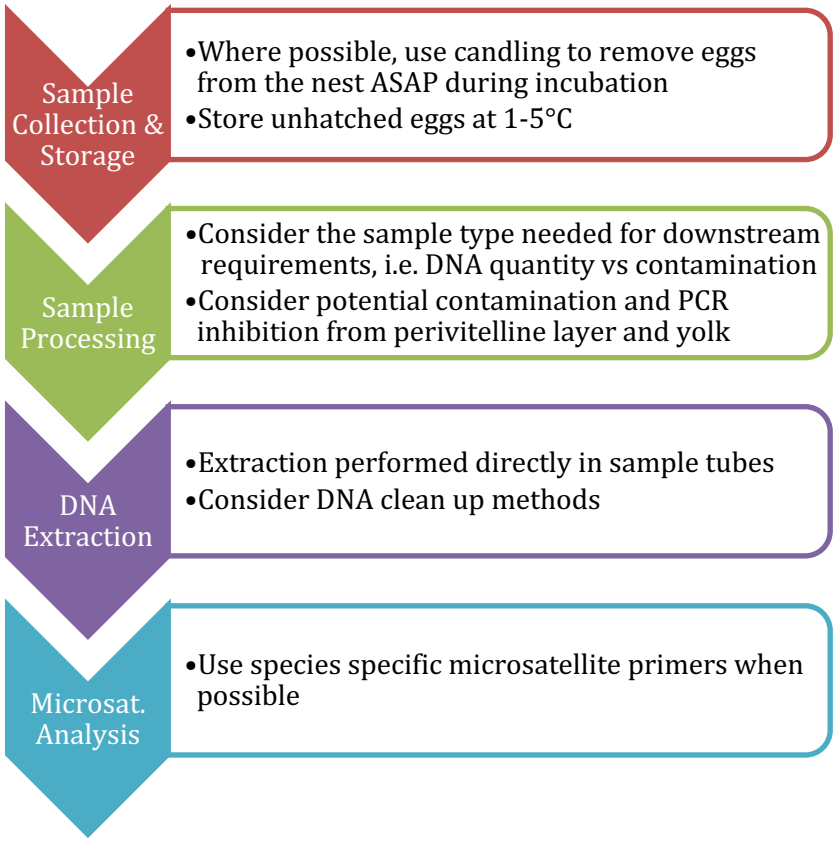

Figure 4. A summary of the important steps recommended during sample collection, storage, processing, DNA extraction and microsatellite analysis.

In our study, the mean amount of DNA obtained from early-dead embryos in our study was 2,122ng, which is ample for a number of different molecular techniques. For example, a minimum of 10ng of DNA per is recommended per reaction for multiplex microsatellite analysis (Brekke et al., 2010; Narina et al., 2011; Neff et al., 2000), 2.5ng of DNA per sample is required for RAD-seq (Etter et al., 2011), 20-1000ng for Illumina Sequencing ("Sample Requirements," Accessed: 05-09-2022), depending on the precise method, and a maximum of 200ng is required for Sanger sequencing ("Sample Requirements," Accessed: 05-09-2022). In addition, only 6 out of 145 samples extracted in this study yielded no DNA at all, which is a higher success rate than a previous attempt to extract DNA from bird faeces, where the extraction success rate was 34-80% depending on the method used (Pons et al., 2007).

An advantage of microsatellite analysis is that it can be applied to degraded DNA due to the short sequence of microsatellite target regions (Queller et al., 1993). We demonstrate here that microsatellite analysis of DNA samples taken from embryos that died early in development, and were then left in the nest for up to two weeks, has an average microsatellite amplification success rate of 60%. Although this is substantially lower than the average amplification success rate when using blood samples stored in ethanol immediately after sampling (97%; Figure 2), it does at least allow some degree of genotyping and downstream analysis (for example, paternity analysis and sex-typing). It has been demonstrated that short microsatellite markers are more effective for degraded DNA, such as that from museum specimens (Nakahama and Isagi, 2017), but our results suggest that DNA degradation may not be a major issue in early-dead embryo samples, as we see no relationship between the length of the microsatellite marker and the amplification failure rate. Microsatellite amplification failure rates may instead be due to the presence of PCR inhibitors in the egg, including proteins and fats (Acharya et al., 2017; Schrader et al., 2012), and proteases (Schrader et al., 2012), which make up a large component of the egg yolk (Kowalska et al., 2021; Shbailat et al., 2016). 412 proteins have also recently been identified in the chicken perivitelline layer (Brégeon et al., 2022). Samples containing perivitelline layer had the worst rates of microsatellite amplification in this study, which may be a consequence of the large number of proteins present in those samples. An improvement on the methods presented here could involve a DNA purification step, such as ethanol precipitation, silica columns or magnetic bead cleaning.

Our results also showed that cross-species microsatellite primers were more likely to fail on early embryo DNA samples compared to species-specific primers. Microsatellite sequencing with cross-species primers has previously been used successfully on DNA obtained from faecal samples (e.g. Wultsch et al., 2014) and the benefits of using cross-species primers include a reduced time commitment and cost compared to developing and optimising species-specific primers as well as utility across many species (Dawson et al., 2010). However, it is reasonable to expect cross-species primers to show higher rates of failure in this case due to their lack of complete specificity.

Until recently, early embryo mortality has been largely ignored in avian population studies, and individuals that die early in development have for the most part been omitted from molecular studies. As such, our understanding of the evolutionary processes driving population dynamics has been limited. For example, the only previous study (to our knowledge) that has included early embryos in an

assessment of primary sex ratio found that a female-biased secondary sex ratio in Eurasian tree sparrow (*Passer montanus*) was due to differential mortality in very early embryos (≤ HH stage 5), with 97% of early mortalities being males (Kato et al., 2017). Here we have shown that molecular information can be relatively easily obtained from the "invisible fraction" (Grafen, 1988) of bird embryos that die early, and this information is likely to have important implications for ecology, evolution, and conservation. Specifically, genetic data on the invisible fraction will provide vital information on levels of selection and plasticity in wild populations, as well as improving our understanding of life-history trade-offs (e.g., sex allocation), reproductive biology (e.g., levels of extra-pair paternity) and inbreeding depression. Genetic and potentially genomic analysis of embryos that die early in development has the potential to inform these and many other areas of study. We hope that the methods described in this chapter will be widely adopted, allowing a more in-depth exploration of the nature of the invisible fraction and the causes of early embryo mortality.

CHAPTER 3.

MALE-BIASED SEX RATIOS AND SMALL POPULATION SIZES AFFECT PATTERNS OF EMBRYO MORTALITY AND INFERTILITY IN A THREATENED PASSERINE

ABSTRACT

Up to 77% of eggs laid by threatened bird species fail to hatch. However, the mechanisms driving this hatching failure are poorly understood. Many unhatched eggs are misclassified as "infertile", but the true prevalence of and drivers behind fertilisation failure in birds are unknown. Here, using 10 years of data including 4,372 unhatched eggs and methods to accurately discriminate between fertilisation failure and embryonic mortality, we investigate the relative importance of these mechanisms of hatching failure and how they are influenced by population-level factors. We show that the majority of unhatched eggs in a population of a threatened bird, the hihi (*Notiomystis cincta*) fail to hatch due to mortality in the early stages of embryonic development. Fertilisation failure is also a significant cause of hatching failure and is higher in years with smaller populations and more male-biased adult sex ratios, which we propose is due to stress induced by increased levels of male harassment and forced extra-pair copulations. The offspring of extra-pair males are more likely to die during early development, which may also be due to maternal stress resulting from forced extra-pair copulations. Male embryos are more likely to die during early embryo development, but surprisingly this is not linked to inbreeding. Embryo death is not associated with higher inbreeding coefficients in this population, which may indicate the occurrence of genetic purging during the population's history.

INTRODUCTION

Reproductive failure signifies a fitness cost for individuals, particularly females who often invest more heavily than males in reproduction (Clutton-Brock, 2007; Hayward and Gillooly, 2011; Trivers, 1972). In birds, females incur a large physiological cost from the production of eggs (Nilsson and Råberg, 2001), so unhatched eggs represent not only wasted fitness potential but also wasted energy and resources. Average rates of hatching failure across all birds are around 10% (Koenig, 1982) but hatching failure is much more prevalent in threatened bird species, with rates of up to 77% in small genetically isolated populations (Jamieson and Ryan, 2000). Identifying the drivers of hatching failure is therefore an important goal for bird conservation (Assersohn et al., 2021).

Narrowing in on the physiological mechanisms underlying hatching failure requires an accurate distinction between its two potential causes: embryo mortality and fertilisation failure. Fertilisation failure occurs when sperm and ova fail to fuse, which signals a problem that is directly impacting the parents. There are many possible mechanisms of fertilisation failure originating from male, female, or pair incompatibility (female infertility reviewed in: Assersohn et al., 2021). For example, fertilisation failure can be due to male disorders such as low numbers of sperm (Hemmings and Birkhead, 2015) or azoospermia (Lifjeld et al., 2007), or behavioural incompatibility issues leading to copulation failure. Female issues with gamete production or function as a result of female reproductive disorders and diseases may also lead to fertilisation failure (Rosen, 2012; Srinivasan et al., 2014), as well as issues with sperm storage, release, and rejection (Pizzari and Birkhead, 2000). However, the processes driving infertility are not as well understood as those driving embryo mortality, due to the difficulty identifying fertilisation failure, particularly in wild populations. Early embryo death, on the other hand, may result from a range of extrinsic and/or intrinsic factors affecting the embryo directly, or from the indirect effects of poor parental quality. For example, embryo development is affected by a range of climatic variables such as temperature (Aldredge et al., 2012; Eiby et al., 2008; Lourens et al., 2005; Serrano et al., 2005), rainfall (Wilcoxen et al., 2011) and humidity (Bruzual et al., 2000) the latter of which also increases the risk of microbial infection which can lead to embryo mortality (Cook et al., 2005, 2003; Pinowski et al., 1994). Intrinsic factors, such as chromosomal abnormalities (Forstmeier and Ellegren, 2010) or low-quality male (Chenoweth, 2007; Parinaud et al., 1993; Saacke et al., 1994) or female (McDaniel et al., 1979; Narushin and Romanov, 2002) gametes have also been linked to embryo death. In small populations, early embryo mortality is likely to be the result ofinbreeding depression (Kardos et al., 2016). Genetic similarity between parents (Bensch et al., 1994; Cordero et al., 2004; N. L. Hemmings et al., 2012a; Spottiswoode and Møller, 2004), high maternal inbreeding coefficient (Cordero et al., 2004; Keller, 1998), and the severity of the genetic bottleneck a population has been through (Briskie and Mackintosh, 2004) have all been associated with increased rates of hatching failure, although whether this failure is mostly due to fertilisation failure or embryo death is poorly understood.

While the distinction between fertilisation failure and embryo death is obvious when an embryo is visible inside an unhatched egg, in many species the majority of failed eggs contain embryos that have died very early, and therefore show no macroscopic signs of development (Hemmings and Evans, 2020; Savage et al., 2022). These eggs are often assumed to be unfertilised by researchers and conservation practitioners (Assersohn et al., 2021), leading to the overestimation of infertility in bird populations and a persistent “invisible fraction” of individuals that die before sampling in long-term population studies (Hemmings & Evans 2020).

Although several environmental and genetic factors influencing hatching failure have been well studied, the importance of population demographic factors, such as population size and sex ratio, have received less attention. Little is known about the influence of naturally occurring adult sex ratios on reproductive failure (including infertility and embryo mortality rates) in wild populations. Research in experimental systems has revealed that the adult sex ratio can affect individual reproductive success (Galimov et al., 2021; Ward and FitzGerald, 1988) and female fecundity due to male harassment in male biased populations (Le Galliard et al., 2005). Population size and sex ratio have been shown to be linked in some bird species, with smaller populations having more skewed sex ratios, either towards males (Gerlach and Le Maitre, 2001; Nadal et al., 1996) or females (Fry et al., 1987). Small populations are also more susceptible to stochasticity in population demographic factors, such as sex ratio, and the associated extinction risks (Lande et al., 2003). A strongly skewed adult sex ratio in a population may also increase sexual competition between the more numerous sex and bring costs for the rarer sex. For example, female survival and reproductive success have been shown to be directly impacted by aggressive male behaviours in populations with male-skewed adult sex ratios (Gilmartin and Eberhardt, 1995; Porter et al., 2004), potentially due to increased stress exposure. The effect of stress on reproduction is well studied in poultry (reviewed in: Assersohn et al., 2021), and it has been suggested that both acute and chronic stress exposure in birds can trigger an "emergency life-history stage" which focuses on self-preservation and survival, resulting in a lack of reproductive investment and suppression of reproductive behaviours such as chick provisioning (Wingfield et al., 1998). Stress in birds can lead to lower investment in reproduction and parental care (Angelier and Chastel, 2009; Blas, 2015) and heightened corticosterone levels in eggs, which has been demonstrated to lead to reduced hatching success (Khan et al., 2016), impaired development (Eriksen et al., 2003) and lower quality hatchlings (Saino et al., 2005). In addition, stress exposure can reduce circulating levels of reproductive hormones (Henriksen et al., 2011; Novero et al., 1991), which could affect hormonally controlled aspects of fertility and reproduction such as sperm release (Ito et al., 2011).

In wild bird populations, the average adult sex ratio is typically male-skewed, and more heavily so in threatened populations (Donald, 2007a). Mayr (1939) was among the first to highlight stark differences between the sex ratios of offspring (secondary sex ratio) and adults (tertiary sex ratio) in bird populations. Although the average adult sex ratio in birds is male biased, the average secondary sex ratio is balanced between males and females (Donald, 2007a). This would suggest that the primary sex ratio (sex ratio at fertilisation) and pre-hatching mortality also shows no sex bias. However, previous studies have found embryo mortality to be male biased (Brekke et al., 2010; Cichoń et al., 2005; Whittingham and Dunn, 2001). Sex biases in embryo mortality in birds could result from sex-biased inbreeding depression (Brekke et al., 2010; Vega-Trejo et al., 2022) or differences in optimal incubation

temperature between the sexes (Eiby et al., 2008). Previous studies on sex bias in embryo mortality have not included an assessment of embryos that died very early in development (0-3 days incubation), before macroscopic signs of development are visible, so our understanding of primary sex ratios and sex-biased embryo mortality in birds is incomplete. This omission means that other factors that may influence patterns of early embryo mortality, such as paternity bias, have also been overlooked. Extra-pair paternity in the clutch has been proposed to lead to higher overall hatching success (Kempenaers et al., 1999), and higher survival probability of individuals (Charmantier et al., 2004), but whether this proposed benefit of female promiscuity extends to embryo survival remains to be investigated.

This study aims to examine how patterns of hatching failure and infertility vary with population demographic changes in a long-term monitored population of hihi (*Notiomystis cincta*), which is under conservation management on the island reserve Tiritiri Mātangi. The hihi is a threatened, New Zealand endemic passerine bird which, following population decline and subsequent conservation efforts, consists of a remnant population and numerous reintroduced populations (Brekke et al., 2011). The species therefore has a history of multiple genetic bottlenecks and its populations are of small size, with high inbreeding levels (Brekke et al., 2010; Duntsch et al., 2021). Hihi have an extremely promiscuous mating system with females experiencing forced extra-pair copulations, which become more frequent with an increasingly male biased sex ratio (Ewen et al., 2004). Hihi also have consistently high rates of hatching failure (28%). The adult sex ratio in this population has been found to on have no effect on adult female survival or number of fledglings produced, despite the high occurrence of female harassment when the population is male biased (Ewen et al., 2011a). However, the impact on other aspects of female reproduction such as fertility and hatching success, has not been investigated. Peak levels of female harassment by males occur from the day before laying begins to the day before the last egg is laid (Low, 2005), so higher levels of male-male competition and harassment during this time may be more likely to influence earlier stages of the reproductive process. Higher levels of stress (in both females and males) may lead to lower fertilisation rates, and increased disturbance of females by males during the laying period (Low, 2005) may lead to higher embryo mortality rates. Using a long-term dataset spanning 10 years, which incorporates a genetic pedigree, reproductive data, population demographic estimates, and the developmental and fertilisation status of 4,372 unhatched eggs, we assess whether patterns of fertilisation failure and embryo mortality are associated with population size and adult sex ratio. We also examine the evidence for biases in sex, paternity, and inbreeding level, in the thus far unsampled subset of individuals that die very early during embryo development.

METHODS

This study was carried out on a closed, managed population of hihi, *Notiomystis cincta,* on the island of Tiritiri Mātangi in northern New Zealand. At 21 days old, each individual bird is colour ringed for identification purposes and has blood samples taken for microsatellite analysis which allows sexing and paternity analysis for inclusion in a long-term pedigree. Since 1995, two constant effort transect surveys have been carried out each year: one pre-breeding survey in September, one post-breeding survey in February. An integrated population modelling framework (Parlato et al., 2021) was used to estimate the population size and sex ratio using the sighting data obtained from biannual surveys and breeding data.

The population of hihi on Tiritiri Mātangi use nest boxes provided across the island. During the breeding season (September-February) the population is monitored closely to record accurate dates for laying, hatching and fledging. Clutch size, hatching success and fledging success are also recorded for each nesting attempt. Unhatched eggs are collected from nest boxes three days after the last egg in a clutch has hatched (14-day incubation period). Unhatched eggs are opened to inspect the contents; obvious embryos are approximately staged according to Hamburger & Hamilton (1951); see also Hemmings & Birkhead (2015) for passerine staging comparison and estimations of development times) and are categorised here as: "Early Embryo" (Hamburger-Hamilton Stage 1 – 21 / day 0 – day 5), "Mid Embryo" (Hamburger-Hamilton Stage 22 – 36 / day 5 – day 10) and "Late Embryo" (Hamburger-Hamilton stage 37 – hatching / day 11 – day 14). Previously, unhatched eggs without signs of embryonic development have been categorised as unfertilised if the yolk remained intact and was separate from the albumen, and undeveloped eggs which were addled/rotten have been assumed to be fertilised. Between 2010 and 2017, unhatched eggs without signs of embryonic development upon being opened were stored without the shell in 10% formalin. From 2018 to 2022, unhatched eggs were candled for signs of development and frozen in the shell. The formalin-preserved and frozen eggs were later dissected between 2019 - 2022 and examined for accurate evidence of fertilisation and embryonic development using the microscopic techniques described in Assersohn et al., 2021 (see associated open access resources). Following these examinations, unhatched eggs were re-classified as fertilised if there was evidence of embryonic development (embryonic tissue visible under the microscope) and/or sperm penetration of the perivitelline layer surrounding the yolk. Eggs were deemed as unfertilised if there was no evidence of embryonic development or sperm reaching the egg.

Fledglings, nestlings and dead embryos, including early stage embryos extracted from the frozen unhatched eggs detailed above, were sexed via microsatellite analysis, using two sex-typing markers (Z002a and Z037b; Dawson, 2007, Dawson et al., 2015), following methods in Brekke et al., (2015) (see

Chapter 2). Paternity analysis was also performed using microsatellite analysis: DNA samples from embryos that died early in development and remained in the nest for the full incubation period were genotyped using 20 microsatellite markers: 2 sex-typing markers (Z002a and Z037b; 26, 27), 15 species-specific markers, and 3 markers developed for other passerines (Brekke et al., 2009; two primers listed in this paper were not used: Nci86C10 and MSLP4; see Chapter 2). Paternity assignments were performed in Colony (Jones and Wang, 2010), including information on candidate maternal and paternal genotypes and maternal siblings (samples from the same nest). There were no cases of DNA contamination from the mother or (social) father detected as duplicates by Colony. Inbreeding coefficients (F_{PED}) were estimated using a long-term genetically resolved pedigree and the pedigreemm package. Individuals were only included in the analysis involving inbreeding coefficients if two generations of close relatives, i.e. parents and the dam and sire of both parents, were present in the pedigree.

All data analysis was carried out in R (version 1.4.1717). The sex and paternity biases in mortality were compared across the developmental stages using glmmTMB with a binomial distribution. The variation in inbreeding coefficients with sex, paternity and stage of development at which mortality occurred were tested in glmmTMB with tweedie distribution which accounts for zero inflation. The effect of sex ratio and population size on egg infertility rate and hatching rate was tested using glmmTMB with binomial distribution. All models included dam ID and year as random effects.

RESULTS

Fertilisation Failure vs Embryo Mortality

The proportion of hihi eggs that failed to hatch due to fertilisation failure in the study population (16.7% ± 13.3% SD) was significantly lower than previously thought (Figure 3.a; Wilcoxon test paired by year: median difference between assumed vs actual infertile eggs per breeding season = -81.5, p = 0.003). The most common cause of hatching failure was embryo mortality at a very early stage of development (Figure 1.b), prior to stage 8 on the Hamburger and Hamilton (1951) chick developmental series and within the first two days of hihi embryo development, at which stage there are few obvious macroscopic signs of embryonic development, particularly in partially degraded eggs. Early embryo mortality was found to cause on average 56.8% (± 15% SD) of all hatching failure in hihi, which is higher than that caused by mid (9.6% ± 4.9% SD) or late (16.8% ± 5.7% SD) embryo mortality, or by fertilisation failure (16.7% ± 13.3% SD).

Demographic Effects on Fertilisation Failure and Embryo Mortality

The probability of an egg being fertilised was significantly reduced when the size of the population decreased (Figure 1, glmm: estimate = -0.02, df = 2,748, p = <0.001) and the sex ratio of the population became more male biased (Figure 2, glmm: estimate = -2.83, df = 2,748, p = 0.001). However, the overall hatching success of eggs was not significantly affected by population size (glmm: estimate = -0.0018, df= 2,748, p=0.81,) nor the adult sex ratio (glmm: estimate = -0.4, df = 2,748, p=0.59). Population size and sex ratio were tested in separate models because they are significantly correlated (Figure 1c, r = -0.76, df = 9, p= 0.007). Annual population-level hatching success was negatively associated with annual population-level fertilisation failure rates (Figure 3.a, Pearsons correlation: cor = -0.6, df = 8, p = 0.06), although this relationship was marginally non-significant. Individual-level hatching success rates (annual average) were significantly negatively associated with individual-level infertility rates (annual average) (Figure 3.b, Pearsons correlation: cor = -0.23, df = 368, p = <0.001).

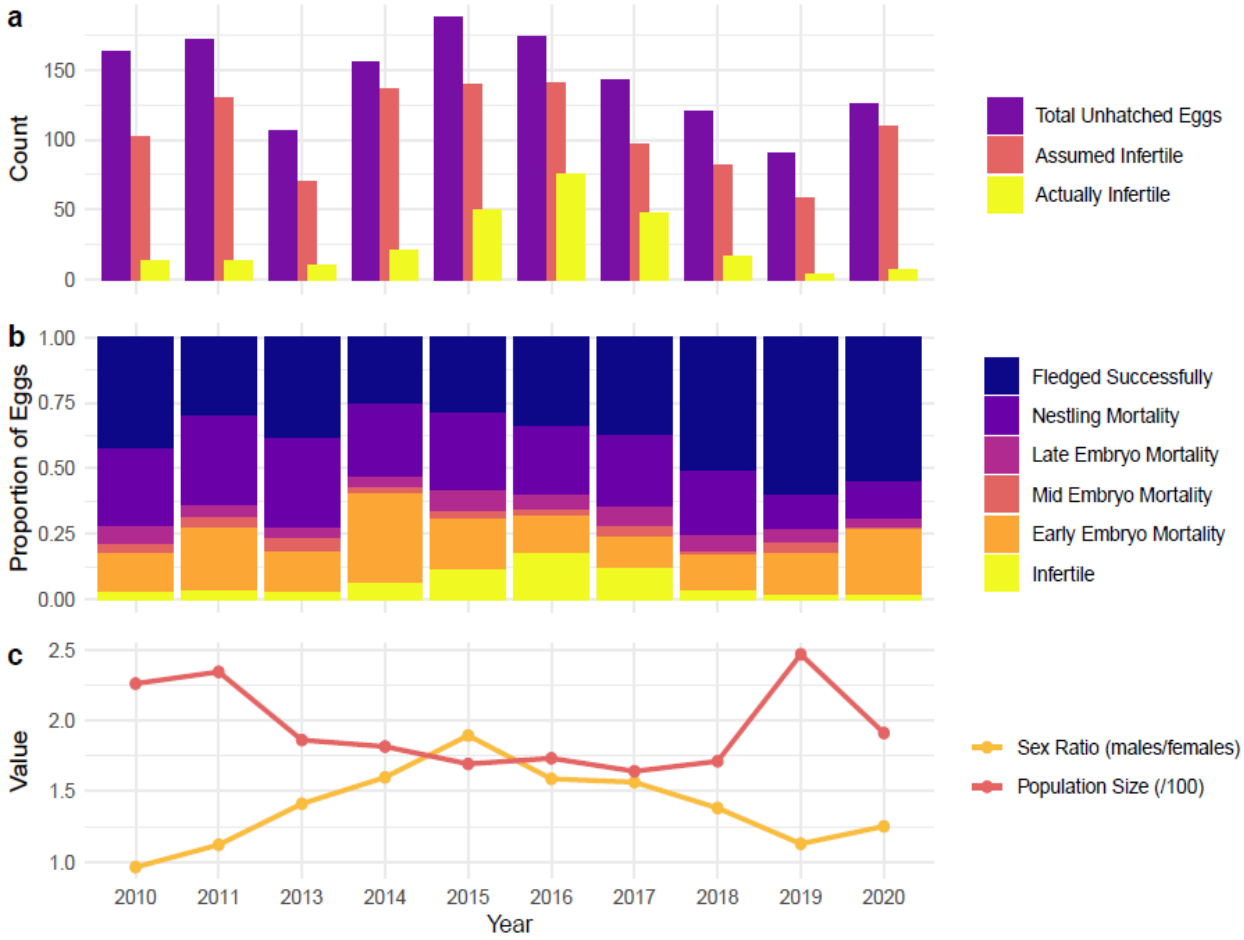

Figure 1. How egg outcomes vary in a population of hihi across 10 years. a) The total number of eggs which failed to hatch after the full incubation period for every year, compared with the proportion of eggs previously assumed to be unfertilised due to having no macroscopic embryonic development, and the proportion determined to be truly unfertilised through microscopic analysis of egg contents. b) The outcome of every egg laid in the population across every year (as proportions of the total number of eggs laid), highlighting early embryo mortality (before macroscopic signs of development) as the main cause of hatching failure in this population. c) The sex ratio and size of the population of hihi on Tiritiri Matangi across 10 years. These two demographic factors are significantly correlated and have significant effects on the infertility rates of hihi eggs in this population.

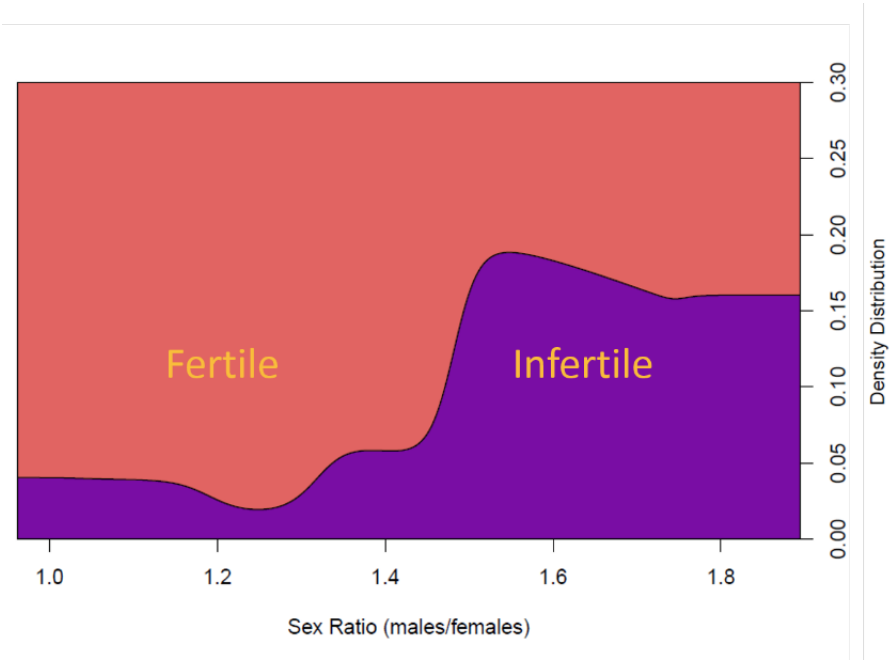

Figure 2. The density distribution of the probability that a hihi egg laid will be unfertilised given the dynamic sex ratio of the population of hihi on Tiritiri Matangi. The sex ratio and size of this population are significantly correlated, making it impossible to separate their respective effects on infertility rate of eggs.

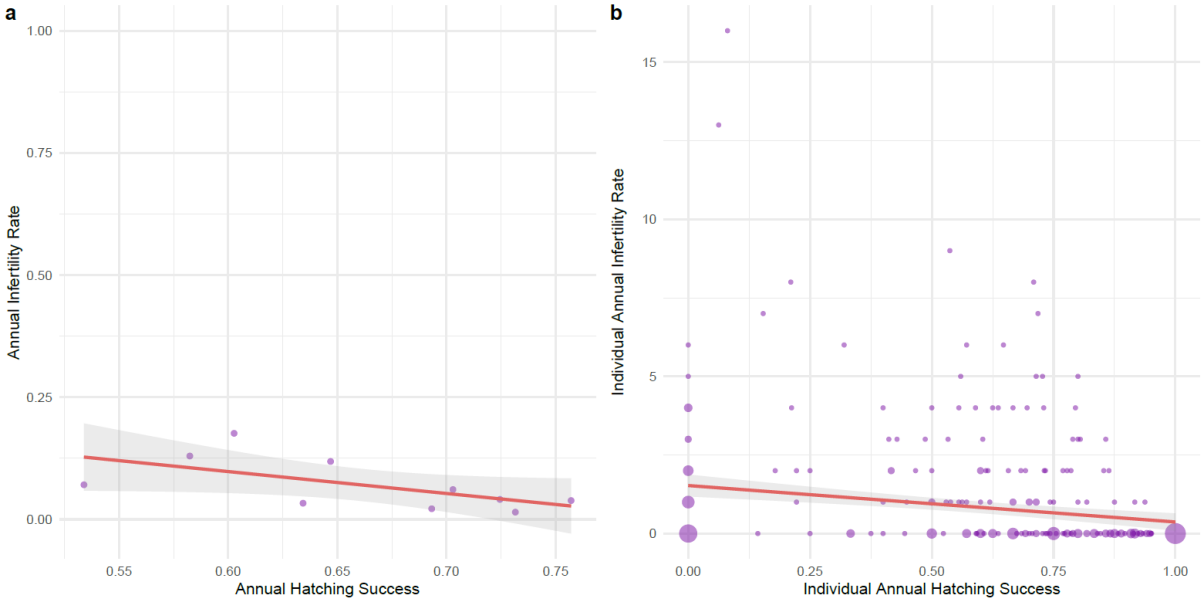

Figure 3. Fertilisation failure is a significant contributor to individual annual hatching success and is negatively associated with hatching success on the population level. a) Hatching success of the whole population is negatively associated with the population level fertilisation failure rate (each point represents 1 year), although this relationship was marginally non-significant. b) Individual level hatching success is significantly negatively associated with increased individual level fertilisation failure rates, measured per female per year.

Patterns in Embryo Mortality: Sex Bias

The average primary sex ratio (pre-hatch males/females = 1.01 ± 0.16 SD) and secondary (nestling) sex ratio (males/females = 1.01 ± 0.15 SD) were found to be practically equal. However, the adult/tertiary sex ratio is consistently male biased in this population (Figure 3.c), with an average sex ratio

(males/females) of 1.38 ± 0.27 SD. Early embryo mortality, the most common cause of hatching failure, was male biased (males/females = 1.4, Figure 5.a), with the number of males dying at late stages of development being significantly lower than those dying during early embryo mortality, but this was not the case for embryos that died at the mid stages of development or those that hatched (Table 1). Overall, there was no significant difference in the sex ratio of embryos that died (males/females = 1.02) or those that hatched (males/females = 1.01) (glmm: estimate = 0.008, df = 430, p = 0.93).

Table 1. Summary table of two glmmTMB models with binomial distribution of the sex bias in hihi embryos according to the embryo outcome including those which fail at early, mid and late stages of embryonic development, and those which hatch successfully. The model included dam and year as random effects.

Parameter	*Estimate*	*Std. Error*	*p*
(Intercept) Early Embryo Mortality	*0.4*	*0.2*	*0.054*
Embryo Outcome			
Mid Embryo Mortality	*-0.47*	*0.29*	*0.098*
Late Embryo Mortality	*-0.5*	*0.25*	***<0.05***
Hatched	*-0.38*	*0.21*	*0.064*

Patterns in Embryo Mortality: Extra-Pair vs Within-Pair Paternity

There was no bias in paternity with the developmental stage reached by individuals (Figure 1.c, Anova: Chisq = 1.85, df = 3, p = 0.6). The rate of extra pair paternity for both hatched individuals and individuals which suffered embryo mortality was 60%.

Patterns in Embryo Mortality: Inbreeding

The average inbreeding coefficient (F_{PED}) across all individuals sampled was 0.023 ± 0.045. A model of the associations between embryo outcome (embryo mortality at early, mid or late stages or successful hatching), paternity, sex, and inbreeding coefficient found that overall, the outcome of an embryo did not significantly predict inbreeding coefficient (Figure 5.b, Anova: Chisq = 7.4, df = 3, p = 0.059,), nor did the sexes significantly differ in their inbreeding coefficients (Figure 5.b, Anova: Chisq = 0.78, df = 1, p = 0.37). Surprisingly, individuals that hatched successfully had significantly higher inbreeding coefficients than individuals that died during early embryo development (Figure 5.b, glmm: estimate = 0.84, df = 2,498, p = 0.04), and individuals fathered by within-pair males had significantly higher inbreeding coefficients than those fathered by extra-pair males (Figure 4, glmm: estimate = 0.25, df = 2,498, p = <0.001)

Table 2. Summary table of a glmmTMB model with Tweedie distribution of the inbreeding coefficient of hihi, which is significantly higher in offspring resulting from within pair parternity (WPP) and individuals that hatch. The model included dam and year as random effects.

Parameter	*Estimate*	*Std. Error*	*p*
(Intercept)	*-5.47*	*0.4*	***<0.001***
Paternity			
WPP	*0.25*	*0.058*	***<0.001***
Embryo Outcome			
Mid Embryo Mortality	*0.68*	*0.4*	*0.093*
Late Embryo Mortality	*0.71*	*0.38*	*0.062*
Hatched	*0.85*	*0.37*	***<0.05***
Sex			
Male	*-0.047*	*0.053*	*0.37*

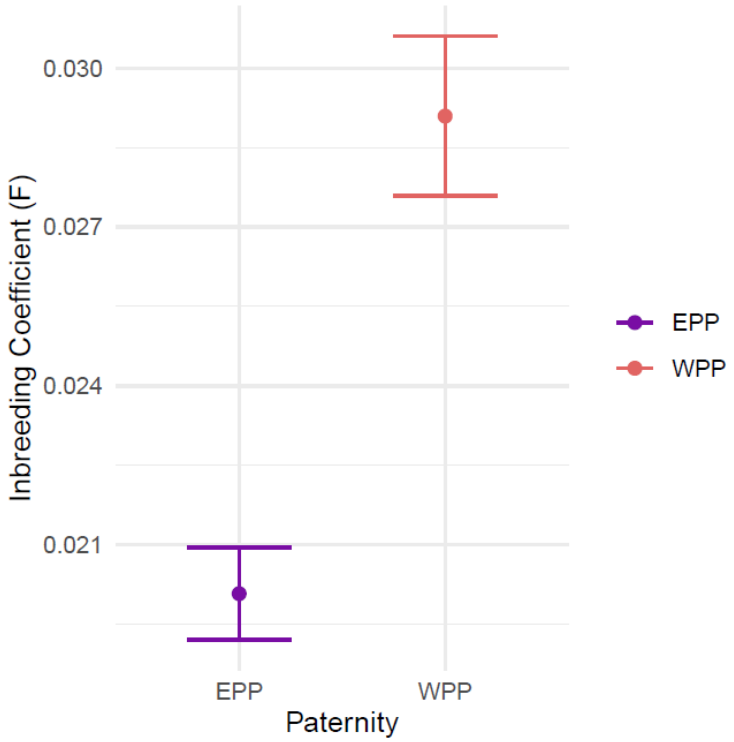

Figure 4. Mean (point) and standard errors (bars) of the inbreeding coefficients of extra-pair (n = 2,029) and within-pair (1,250) hihi offspring from the years 2010 -2020 (excluding 2012), including failed embryos and hatched individuals.

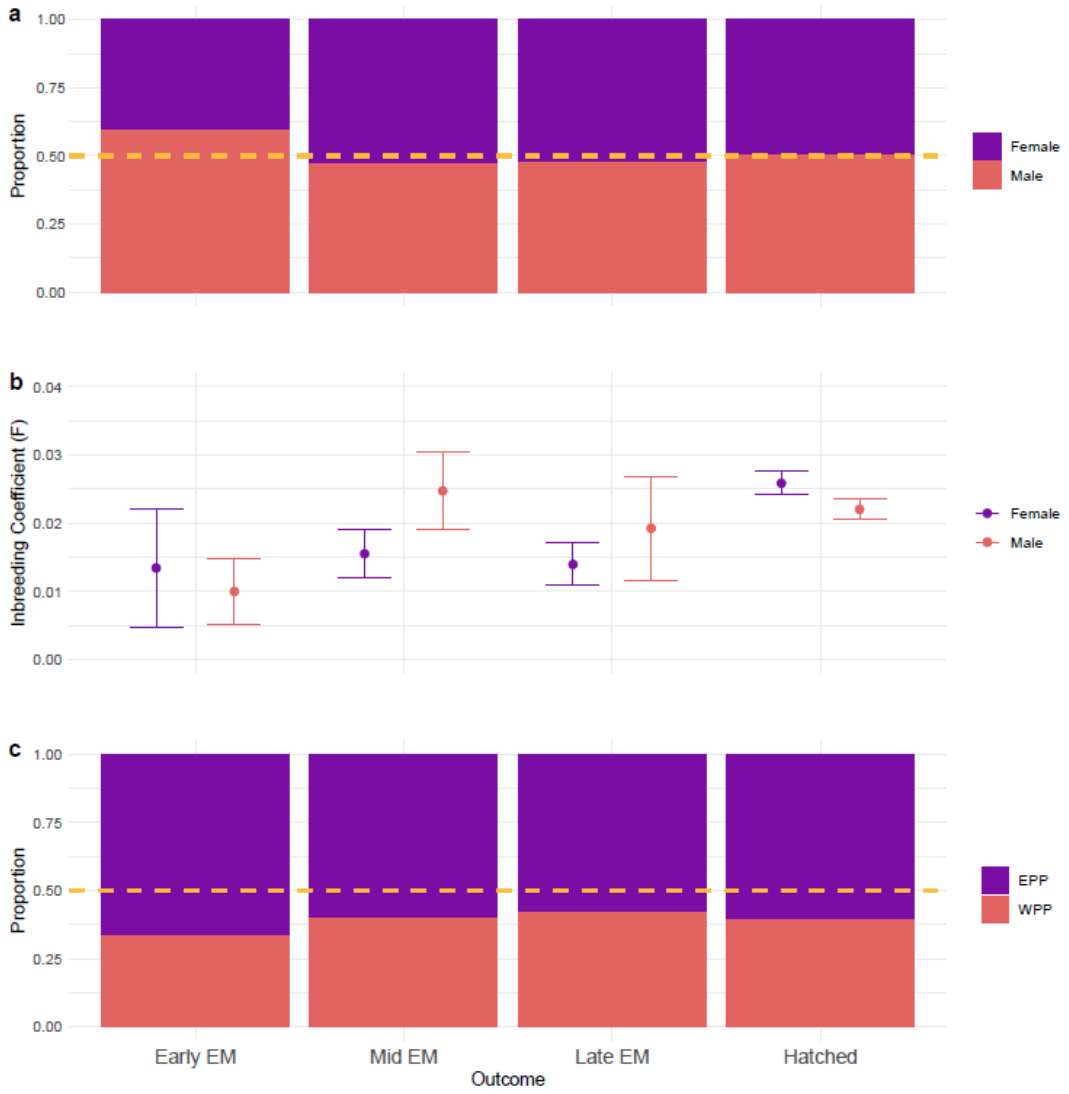

*Figure 5. The biases in mortality at different stages of embryonic development: early embryo mortality (Early EM), mid embryo mortality (Mid EM), late embryo mortality (Late EM) compared to individuals that hatched. a) The proportion of males and females that died at the different stages of development compared to those that hatched; b) the inbreeding coefficients of males and females that died at different stages of development compared to those that hatched; c) the proportion of extra-pair paternity (EPP) and within-pair paternity (WPP) of individuals that died at different stages of development compared to those which hatched. Plotted sample sizes are: a) Sex: Early EM = 114, Mid EM = 216, Late EM = 103, Hatched = 2,370; b) Inbreeding*Sex: Early EM = 11, Mid EM = 74, Late EM = 164, Hatched = 1,618); c) Paternity: Early EM = 105, Mid EM = 108, Late EM = 218, Hatched = 2,356; d) Inbreeding*Paternity: Early EM = 105, Mid EM = 109, Late EM = 220, Hatched = 2,835*

DISCUSSION

Here we show that, in a reintroduced population of an inbred, threatened, endemic New Zealand passerine, the hihi (*Notiomystis cincta),* the main cause of hatching failure (57% of unhatched eggs) is early embryo mortality, and this important source of early-stage losses from the population is subject to both sex and paternity bias. Egg infertility rates in this population of hihi are significantly lower than was previously assumed based upon macroscopic examination of egg contents. This finding supports that of previous studies on a number of different bird species, which have found infertility to be less of a concern than was previously thought (Hemmings et al., 2012; Savage et al., 2022), and adds to the

body of evidence suggesting that research and conservation efforts should be focused on reducing embryo mortality rates in threatened species.

Despite the relatively low incidence of fertilisation failure, we found that infertility rates vary between years according to population size and sex ratio. Population size and sex ratio are interlinked: in years when the population size is smaller, the adult sex ratio is more skewed (towards males), reflecting a common pattern in birds, particularly those with threatened (Donald, 2007a) and/or small, isolated populations (Dale, 2001). When the sex ratio in this population of hihi is more male-biased, infertility rates are higher. We propose that these elevated rates of fertilisation failure may be explained by physiological stress, resulting from extremely high levels of male-male competition and female harassment under male-biased sex ratios (Castro et al., 1996b; Ewen et al., 2011a, 2004).

Sex biases in small populations are proposed to be driven by lower survival or recruitment of the rarer sex (Dale, 2001; Donald, 2007a). However, the exact mechanisms behind these differential mortality rates remains unknown (Donald, 2007a; Morrison et al., 2016). The origin of the consistent male bias in the adult sex ratio of hihi is elusive, as the primary and secondary sex ratios in this population are practically equal. A reasonable explanation is that post-hatching, post-fledging, or adult mortality is higher in females than males, although this remains to be confirmed and would be an interesting avenue for further study. Previous studies have shown that female birds have lower survival rates than males in some species (Sillett and Holmes, 2002; Woolfenden et al., 2001) and others have demonstrated that this is a direct cause of male-biased sex ratios (Githiru and Lens, 2006; Liker and Székely, 2005; Gerlach and Le Maitre, 2001).

Increased rates of fertilisation failure in years when the sex ratio is extremely male-biased is perhaps unexpected, since with more males per female, there are more opportunities for copulations, sperm transfer, and therefore fertilisation. However, hihi have an extremely promiscuous mating system, notable for its high levels of female harassment by males and high rates of extra-pair paternity (on average 62% (data from this study) ranging up to 100%; Brekke et al., 2013). Extra-pair copulations frequently (Low, 2005), if not always (Ewen et al., 2004), take the form of "forced face-to-face copulations", and this unique behaviour involves multiple males chasing females, pinning her down on her back to the ground and pecking her, sometimes culminating in cloacal contact between one chasing male and the target female (Castro et al., 1996b; Ewen et al., 2004; Low, 2005). The copulations are described as 'forced' because of the female's reaction to the pursuits, which involves evasive flying behaviour, hiding in ground vegetation or leaf litter, and attempting to fly away or kick the male when pinned (Castro et al., 1996b; Low, 2005). Due to the female resistance, it is reasonable to assume that

these forced copulations are stressful and physiologically costly for females, particularly if they occur at a high frequency, which may be the case when the sex ratio of the population is more male-biased, a pattern that has been demonstrated in other species (Howe et al., 2017; Liker et al., 2013; Lodé et al., 2005; Weir et al., 2011). During the breeding season, the frequency of forced copulation attempts averages four per hour (Castro et al., 1996b), but can be as high as 16 per hour (Ewen et al., 2004), and it has been observed that male harassment increases in heavily male biased years (Ewen et al., 2004). In other species, aggressive male behaviour in male-biased populations can negatively affect female survival and reproductive output (Gilmartin and Eberhardt, 1995; Porter et al., 2004). An increase in male harassment in male-biased populations can lead to altered female behaviour such as selecting non-preferred habitats with higher predation risk (Darden and Croft, 2008) and reducing foraging (Magurran and Seghers, 1994), which may have consequences for fertility and egg production, in addition to reduced survival, fecundity and recruitment (Le Galliard et al., 2005).

One potential mechanism underlying higher fertilisation failure rates in years with a more male-biased sex ratio is stress-related disruption to female reproductive hormones. An experimental study on Japanese quail *(Coturnix japonica)* showed that higher maternal corticosterone levels lead to lower fertility rates of eggs, although how fertilisation success was determined was not described (Schmidt et al., 2009). There is evidence that sperm release from sperm storage tubules in birds is triggered by the reproductive hormone progesterone (Ito et al., 2011), levels of which has been shown to decrease in heat-stressed hens (Novero et al., 1991). It is also possible that females are more likely to eject sperm transferred during forced copulations. In chickens (*Gallus gallus domesticus),* for example, females are more likely to reject sperm from non-preferred males with whom they are coerced to mate (Pizzari and Birkhead, 2000). However, if this was the case in hihi, we may not expect the high rates of extra-pair paternity observed in this population (Brekke et al., 2013).

Although fertilisation failure is an important component of individual level hatching success, we found the most common cause of hatching failure in this population of hihi to be early embryo mortality. The sex ratio of embryos that died during the early stages of development was male biased, which has previously been linked to inbreeding depression in this species (Brekke et al., 2010). However, our results show that hatched individuals have a significantly higher inbreeding coefficient than those that died during embryonic development (particularly in the earliest stages)**.** These results are inconsistent with those of a previous study on hihi, which found that males embryos that died before hatching (excluding those that died in the earliest stages of development) had a higher inbreeding coefficient than those which survived (albeit with a smaller sample size than this study), and that male embryos that died were significantly more inbred than female embryos that died (Brekke et al., 2010). Our results

also do not concur with a wealth of literature documenting the general negative effect of inbreeding on reproductive success (Szulkin and Sheldon, 2008) and the hatching success of inbred mothers (Keller, 1998; Marr et al., 2006; White et al., 2015). Studies which look specifically at the effect of embryo inbreeding coefficient (Brekke et al., 2010; Pei (裴一凡) et al., 2020) or relatedness of parents (Hemmings et al., 2012) on survival probability also find that there are negative effects of inbreeding.

The lack of association between high inbreeding coefficients and embryo mortality observed in our results may be due to genetic purging, which is a reduction in frequency of deleterious recessive alleles with increased generation times in a population with inbreeding (Hedrick and Garcia-Dorado, 2016). However, if the deleterious alleles that cause embryo mortality in this population are being purged by purifying selection, we may expect to see a decrease in embryo mortality and an increase in hatching success throughout the 10-year period (2010 – 2020), which we do not. What is more feasible, is that that genetic purging had already taken place in the remnant population of hihi, during the 100 years between the original genetic bottleneck event (approx. 1880) and when individuals were removed for reintroduction and creation of the population used in this study (1995). Inbreeding levels in hihi are generally thought to be high as a result of multiple genetic bottlenecks, resulting from habitat destruction and invasive species introduction (Brekke et al., 2011; Innes et al., 2010). The remnant population of hihi has relatively high inbreeding levels as estimated through runs of homozygosity (F_{ROH} = 0.15, Duntsch et al., 2021), and has been the original source population for all reintroductions of hihi into protected sites (Brekke et al., 2011). However, measuring the effects of genetic purging on inbreeding depression is notoriously hard; without pre- and post-purge data for comparisons, we can only speculate on this possibility.

Our data also reveal an interesting bias in early embryo mortality towards extra-pair males, which co-varies with lower inbreeding coefficients because these two factors are highly associated. Offspring sired by extra-pair males have significantly and markedly lower inbreeding coefficients than those sired by within-pair males, likely due to the fact that females are significantly more related to within-pair males than they would be under random mating (Brekke et al., 2012). One might expect this to have negative effects on reproductive success, but our results show this is not the case, and embryos that hatch in fact have higher inbreeding coefficients than those that don't. One possible explanation for this result is that extra-pair males may be, on average, lower quality fathers than within-pair males. A significant proportion of extra-pair paternity comes from "floater males", who have been shown to be of lower quality: younger or older than the prime age, more inbred, and smaller than territorial males (Brekke et al., 2015a). Therefore, extra-pair offspring are more likely to be sired by low-quality fathers than within-pair offspring, meaning they themselves may be relatively low quality and less likely to

survive to hatch. Females may also invest less in eggs that have been fertilised by low quality/unpreferred males (Harris and Uller, 2009) by laying fewer eggs and/or clutches (de Lope and Møller, 1993), smaller eggs (Velando et al 2006), eggs with lower testosterone levels, or laying at a suboptimal time in the breeding season when breeding with low quality mates (Alonso-Alvarez et al., 2012, but see Horváthová et al., 2012). Lower female investment may increase the chances of embryo mortality; for example, smaller and lower quality eggs have been shown to have decreased hatching probability (McDaniel et al., 1979; Narushin and Romanov, 2002). Maternal stress may also influence egg investment; previous studies on zebra finch (*Taeniopygia guttata)* and quail *(Coturnix japonica)* found that elevated maternal stress, via experimentally manipulated corticosterone levels, leads to an increase in hatching failure (Khan et al., 2016; Schmidt et al., 2009). Maternal stress leads to higher corticosterone levels in laid eggs (Saino et al., 2005) which reduces their hatching success (Eriksen et al., 2003; Saino et al., 2005). Assuming last-male sperm precedence, which is the case in many bird species (Birkhead, 1998), it follows that females with eggs fertilised by an extra-pair male are also likely to have recently experienced stressful extra-pair copulations. If extra-pair copulations during the laying period lead to heightened maternal stress levels, eggs fertilised by extra-pair males may have higher corticosterone concentrations and therefore an increased risk of hatching failure. However, further studies which assess the corticosterone levels in eggs sired by extra-pair males are required to test this hypothesis.

It is worth noting the limitations of this study, which originate both from limitations of pedigree estimates of inbreeding and the difficulty of genotyping embryos which fail very early in development. The mean inbreeding coefficient for hihi in this study (F_{PED} = 0.023 ± 0.045, n=4,371) is lower than that estimated through genomic and microsatellite methods and has larger variation than those estimated with microsatellite markers, despite a larger sample size. The inbreeding levels of the population of hihi used in this study, on Tiritiri Mātangi, have been found previously to be f = 0.08 ± 0.009 (n = 89, Brekke et al., 2010) using microsatellite marker based inbreeding coefficients. The inbreeding coefficients in this study were estimated via a genetically reconstructed pedigree, which is not as accurate as genomic measures of inbreeding (Wang, 2016), despite outperforming microsatellite marker based methods of inbreeding estimation (Pemberton, 2004). Quantification of the inbreeding coefficients of failed embryos using genomic measures may allow further insight into why seemingly more inbred individuals have better survival. In addition, the genotyping of individuals which fail very early in embryonic development presents challenges due to the small quantity of DNA available in such a small number of cells and higher amplification failure rates than in blood samples.

In summary, we have shown that early embryo mortality is the primary cause of hatching failure in this population of hihi (*Notiomystis cincta)*, and that this mortality is biased towards the offspring of extra-pair males, possibly due to the impact of the low quality of extra-pair fathers on offspring quality or maternal investment. We find that rates of fertilisation failure are lower than previously assumed in this population, adding to an increasing number of studies which suggest that early embryo mortality is the most important cause of reproductive failure in threatened and endangered species (Hemmings et al., 2012; Savage et al., 2022). Despite this, infertility is a significant component of individual level hatching failure and is negatively associated with population level hatching success rate. We also find that small population sizes are more vulnerable to extreme male-biased sex ratios in this system, and that this has negative implications for female fertility, potentially driven by high rates of harassment of females by males.

CHAPTER 4.

EARLY-LIFE TELOMERE LENGTH PREDICTS LIFE-HISTORY STRATEGY AND SENESCENCE IN REPRODUCTION BUT NOT LIFETIME REPRODUCTIVE SUCCESS IN A THREATENED WILD SONGBIRD

This chapter has been submitted to the journal PNAS and is therefore presented in the journal's format, with the methods sections at the end.

ABSTRACT

Telomeres are well known for their associations with lifespan and ageing across diverse taxa. Early-life telomere length can be influenced by developmental conditions and has been shown to positively affect lifetime reproductive success in a limited number of studies. Whether these effects are caused by a change in lifespan, reproductive rate or perhaps most importantly reproductive senescence is unclear. Using long-term data on female breeding success from a threatened songbird (the hihi, *Notiomystis cincta)*, we show that the early-life telomere length of individuals predicts their future rate of senescence of key reproductive traits: clutch size and hatching success. In contrast, senescence of fledging success is not associated with early-life telomere length, which may be due to the added influence of bi-parental care at this stage. Early-life telomere length does not predict lifespan or lifetime reproductive success in this species. Females may therefore change their reproductive allocation strategy depending on their early developmental conditions, which we hypothesise are reflected in their early-life telomere length. Our results offer new insights on the role that telomeres play in reproductive senescence and individual fitness and suggest telomere length can be used as a predictor for future life-history in threatened species.

INTRODUCTION

Telomeres have been likened to a 'biological clock', with the gradual shortening of telomeres at each cell division ticking down to senescence and eventual cell death. This analogy has been consolidated by the many studies that have investigated the relationship between telomere length and ageing in humans (Cawthon et al., 2003), domestic animals, (Seeker et al., 2018; Sohn and Subramani, 2014), model organisms (Haussmann and Vleck, 2002; Heidinger et al., 2012; Muñoz-Lorente et al., 2019) and wild populations across taxa (Cherdsukjai et al., 2020; Froy et al., 2021; Sánchez-Montes et al., 2020) including birds (Bichet et al., 2020; Tricola et al., 2018; Vedder et al., 2021); concluding that telomere

length shortens with increasing age and predicts aspects of aging, including lifespan. However, the causality of telomere shortening in aging has been questioned, in humans (Simons, 2015) and birds (Horn, 2008). Comparative analyses across species are contradictory, with a negative relationship between telomere length and maximum lifespan in mammals (Pepke and Eisenberg, 2021), and the opposite relationship across bird species; where telomere shortening is a strong predictor of a species' lifespan (Tricola et al., 2018). Despite considerable research effort in this area, it is evident that the role of telomeres in ageing remains elusive.

Telomere length has not only been associated with lifespan but also with the second pillar of fitness: reproduction. Current theory on the evolution of ageing, including the disposable soma theory (Kirkwood and Holliday, 1979; Kirkwood et al., 1991) and the principle of allocation (Cody, 1966), predicts that reproduction should trade-off with longevity and future survival. There is empirical evidence to support these theories; for example a cross-taxa meta-analysis (Lemaître et al., 2015) and observational studies on birds find that higher reproductive output leads to increased mortality rate (Boonekamp et al., 2014b) and reduced future clutch size (Gustafsson and Pärt, 1990). If reproductive effort accelerates senescence, telomere shortening may provide a mechanism; increased reproductive effort has been associated with shorter telomeres in multiple species (e.g. common tern, *Sterna hirundo* (Bauch et al., 2013), blue tit, *Cyanistes caeruleus* (Sudyka et al., 2019, 2014), mice, *Mus musculus* (Kotrschal et al., 2007), reviewed in: Sudyka 2019). However, an experimental study on zebra finches, *Taeniopygia guttata*, which manipulated early life reproductive effort, found that while engaging in reproduction led to a significant reduction in telomere length (compared to non-breeding individuals) this difference was only apparent during the focal breeding attempt, with an absence of long-term effects of breeding on telomere length (Heidinger et al., 2012). There is also recent meta-analytic evidence questioning the classic theories of ageing, arguing that variation in individual quality masks the fitness costs of the reproduction-survival trade-off (Winder et al., 2022). Questions therefore remain about the long-term or lifetime effects of reproduction on telomeres and ageing.

Developmental conditions can shape future life-history decisions (Auer, 2010), individual quality (Hamel et al., 2009) and senescence (Balbontín and Møller, 2015), and these long-term effects have also been linked to telomere length (Eastwood et al., 2019; Heidinger et al., 2012). Early-life conditions impact telomere length; for example, early-life telomere length is related to the age and condition of parents (Asghar et al., 2015; Bennett et al., 2021; Marasco et al., 2019), and is negatively impacted by

environmental effects during development, including stress (Boonekamp et al., 2014a; Herborn et al., 2014; Stier et al., 2020), adverse weather (Eastwood et al., 2022) and heightened sibling competition (Cram et al., 2017; Nettle et al., 2013). Early-life telomere length is therefore a likely biomarker of individual quality. Indeed, longer early-life telomere length predicts a longer lifespan (Heidinger et al., 2012; van Lieshout et al., 2019) and, as a consequence, higher lifetime reproductive success (Eastwood et al., 2019; Heidinger et al., 2021). Few studies have examined these lifelong implications of early-life telomere length, and the life history consequences of early-life telomere length for an individual, in particular, are not well understood. Specifically, to our knowledge, there have been no studies of the effect of early-life telomere length on the rate of senescence of reproductive traits.

Reproductive senescence is widespread in mammals and birds (Lemaître and Gaillard, 2017; Vágási et al., 2021). An individual's rate of actuarial senescence has been shown to co-vary with reproductive investment (Nussey et al., 2006; Reed et al., 2008b) and environmental conditions during early life (Balbontín and Møller, 2015). Different reproductive traits can also vary in how they senesce (Berger et al., 2015; Hayward et al., 2013). Fitness will thus depend on how individual traits contribute to fitness and how these traits senesce. Hatching success, clutch size, fledgling production, breeding duration, laying date and number of breeding attempts are all traits that can significantly contribute to fitness, but can vary in their relative importance (Merilä and Sheldon, 2000; Teplitsky et al., 2009). We currently have limited understanding of the mechanisms that link life-history traits to ageing and fitness. Telomeres provide an attractive potential window into these mechanisms, but studies on telomeres covering the entire reproductive lifespan of individuals are lacking (Sudyka, 2019) and few studies take multiple reproductive traits into account when studying reproductive senescence (Vágási et al., 2021).

Here we examine how early-life telomere length relates to reproductive senescence and lifetime reproductive success in a threatened species of passerine bird, the hihi (*Notiomystis cincta*). The study population of hihi is closed, with no recorded migration, colour ringed, and closely monitored throughout the breeding season and with biannual population surveys. The early-life telomere lengths of 75 females were measured using qPCR (Cawthon, 2002), using blood samples taken at 21 days old. We test the relationship between early-life telomere length and i) lifespan, ii) patterns of senescence in multiple reproductive traits: clutch size, hatching success, and fledging success, and iii) four measures of lifetime reproductive success: the number of eggs laid, eggs hatched, chicks fledged, and recruits produced.

RESULTS

CLUTCH SIZE

The effect of early-life telomere length on clutch size senescence was tested using a com-Poisson model. There was a significant interaction between age and early life relative telomere length on a female's clutch size (Table 1), with females with longer telomeres showing a slower decline in clutch size across their lifespan (Figure 1). To visualise this interaction, we categorised females as having either "short" or "long" early life relative telomeres depending on whether telomere length fell below or above the 1st quartile telomere length, respectively (See Appendix 2, Supplementary Figure 1 for a version of Figure 1 separated by quartile and justification). The best fitting model was selected using AIC values (Appendix 2, Table S1) and included age as a quadratic term and linear term, with a significant interaction between the quadratic effect of age and telomere length, indicating a significant effect of telomere length on the peak clutch size of females.

Females with "short" early-life relative telomere length reached a peak mean clutch size of 4.6 eggs at 3 (mean = 4.52, se = 0.12) and 6 (mean = 4.57, se = 0.22) years of age, followed by a steep decline (from 3 years of age onwards, slope = -0.26, from 6 years onwards, slope = -0.63). Females with "long" early-life relative telomere length reached a lower peak mean clutch size of 4.2 (*se* = 0.14), at 3 years of age, followed by a shallower decline (slope = -0.036). Analysis of post-peak hatching success in females over 3 years of age revealed a significant negative effect of post-peak female age on clutch size in females with short early-life telomeres (Chisq = 11.71, df = 1, p = 0.00062), but a lack of significant effect in females with long early-life telomeres (Chisq = 0.17, df = 1, p = 0.68).

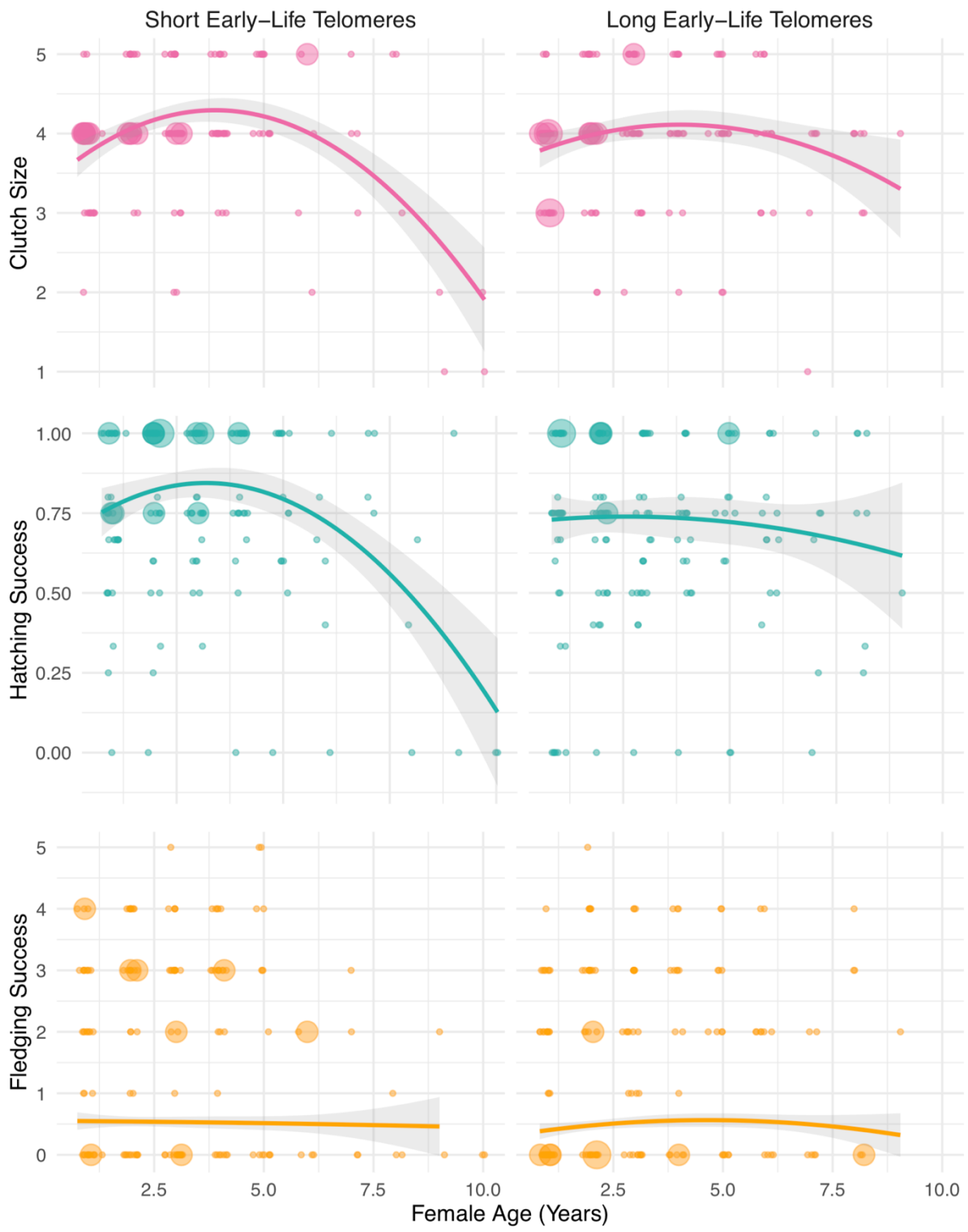

Figure 1) For data visualisation purposes, early-life telomere length was classed as "long" or "short" depending on whether it was above or below the 1st quartile. The 1st quartile was chosen after plotting female age against reproductive traits for all 4 quartiles of the range of early-life telomere length, showing that the 1st quartile seemed to be the critical length (see Appendix 2 for a plot of all 4 quartiles and details). Early-life telomere length was analysed as a continuous variable in all statistical models. The area of the point/bubble increases when the sample size at those X, Y values is greater. The rate of decline in A) clutch size and B) hatching success with female age varies with early-life relative telomere length. Females with very short telomeres (in the lowest quartile of the range) show faster rates of senescence in A) clutch size and B) hatching success. The rate of decline in fledging success with female age is not influenced by early-life telomere length.

HATCHING SUCCESS

Linear, quadratic and breakpoint relationships between female age, telomere length and hatching success were tested and the best fitting model was selected using AIC values (Appendix 2, Table S2). Females with longer telomere lengths show a slower senescence in hatching success during their lifespan (Figure 1). The best fitting model showed a significant interaction between the quadratic age term and telomere length on hatching success, indicating a significant effect of early-life telomere length on the peak hatching success of females.

Females with short early-life telomeres peaked in their mean hatching success at 4 years of age, with 86% (se = 0.05) hatching success, followed by a steep decline (slope = -0.1), females with long early-life telomeres peaked in their mean hatching success at 2 years of age with 80% (se = 0.04) hatching success, followed by a much shallower decline (slope = -0.017).Analysis of post-peak hatching success including females which were older than 4 years of age if they possessed short early-life telomeres, and females over 2 years of age if they possessed long early-life telomeres revealed a significant negative effect of post-peak female age on hatching success in females with short early-life telomeres (Chisq = 9.1, df = 1, p = 0.0026). There was a lack of significant effect of post-peak female age on hatching success in females with long early-life telomeres (Chisq = 1.49, df = 1, p = 0.22).

Table 1. The results of three separate generalised linear mixed models testing for the effect of early-life telomere length (TL) on the relationship between female age and her output in three reproductive traits. Lifespan was included in the model as a fixed effect to control for selective disappearance. Year, female ID and lay date (relative to the first lay date of the season) were included as random effects to control for repeat measures from females across the lifespan, temporal variation across years and timing within the breeding season.

	Clutch Size			Hatching Success			Fledging Success		
Factor	Estimate	S.E	P	Estimate	S.E	P	Estimate	S.E	P
Age	0.025	0.03	**<0.001**	-0.04	0.059	0.5	0.12	0.12	0.36
Age^2	-0.011	0.002	**<0.001**	-0.039	0.017	**<0.05**	-0.003	0.05	0.94
TL	-0.025	0.011	**0.01**	-0.16	0.08	0.05	-0.07	0.12	0.54
Age^2*TL	0.0032	0.001	**<0.01**	0.022	0.008	**<0.01**	0.016	0.023	0.48
Lifespan	-0.0024	0.0058	0.68	0.064	0.045	0.15	-0.054	0.06	0.37

Fledging Success

The effects of female age and early-life telomere length on fledging success were tested using a zero-inflated binomial model, controlling for year, lay date and female identity and lifespan. There was no significant effect of a female's early life relative telomere length on the fledging success rate of her chicks, nor was there any strong evidence of an effect of female age or lifespan. There also was no significant interaction between early life relative telomere length and age on fledging success, suggesting that early-life telomere length does not influence the rate of senescence of this trait (Table 1). We attempted to control for the age of the social male partner in this model to account for the fact that a decline in fledgling success due to maternal senescence may be offset by the provisioning efforts of the male social partner. However, there was a significant correlation between the ages of the female and male in a social pair (df = 1294, r = 0.17, p = <0.001), leading to collinearity and a lack of convergence in the model. The correlation between ages of females and males in social pairs suggests that there is assortative mating by age in this population of hihi, as there is an absence of mate fidelity. The potential effect of male age on fledging success may have therefore confounded our ability to detect an effect of female senescence in this reproductive trait.

Lifetime Reproductive Success

Lifetime reproductive success was quantified only in females which were presumed dead, meaning that they had not been observed in breeding or survey data for 2 years prior to the last year of this study. We tested the effect of early-life telomere length on 4 measures of lifetime reproductive success (Table 3) using poisson/com-poisson models. We found no significant effect of early-life telomere length on any measures of lifetime reproductive success (Table 3). Lifetime reproductive success was instead driven by a female's lifespan (Table 4) and fledging success of her hatchlings. Neither the lifespan nor reproductive lifespan (measured from date of fledging to the lay date of the last breeding attempt) of female hihi have a significant correlation with her relative early-life telomere length (lifespan: df = 74, r = -0.12, p = 0.28, reproductive lifespan: df = 65, r = 0.075, p = 0.56). Fledging success of a female's nest was the only reproductive trait measured that significantly contributed to her overall lifetime reproductive success, estimated as the number of recruits (breeding individuals) produced in a lifetime (Table 4). The lack of a significant effect of early-life telomere length on lifetime reproductive success, despite the significant impact on senescence of two key reproductive traits suggests that females with relatively "long" and "short" early-life telomere lengths may be adopting alternative reproductive strategies. Those with shorter telomeres may invest more strongly prior to senescence, producing a greater number of recruits in their first few breeding years before senescent decline, whereas those

with relatively long telomeres may produce fewer recruits per year but over a longer period. This theory is suggested by visualisation of the data (Appendix 2, Supplementary Figure 2) and could be supported by an analysis of recruit production, but due to a large proportion of females producing no recruits every year, this requires a larger sample size than was available in this study.

*Table 3. The results presented below are from four separate models with four measures of lifetime reproductive output of females as response variables, with both early-life telomere length (*TL*) and lifespan included as fixed effects.*

	Early-life telomere length			Lifespan		
~ TL + Lifespan	Estimate	S.E	P	Estimate	S.E	P
Lifetime Eggs Laid	-0.022	0.04	0.58	0.27	0.02	**<0.001**
Lifetime Eggs Hatched	-0.034	0.045	0.44	0.27	0.02	**<0.001**
Lifetime Chicks Fledged	-0.045	0.87	0.17	0.22	0.018	**<0.001**
Lifetime Recruits	-0.096	0.13	0.44	0.19	0.069	**0.005**

Table 4. The contribution of three reproductive traits to lifetime fitness measured with Pearson's correlations Lifetime fitness is estimated as the number of recruits (breeding individuals) produced in a lifetime.

Correlation with lifetime fitness (recruits)	r	p
Clutch Size	0.03	0.17
Hatching Success	0.04	0.11
Fledging Success	0.09	**<0.001 *****
Lifespan	0.45	**<0.001 *****

DISCUSSION

Here, using long-term data on females of an intensively monitored population of a threatened passerine bird, we demonstrate that early-life relative telomere length, measured from blood samples

taken before fledging, predicts the presence and rate of senescence in key reproductive traits. Females with shorter early-life telomeres experienced a steeper decline in both clutch size and hatching success after the onset of senescence, compared to females with longer early-life telomeres. However, early-life telomere length does not predict the rate of senescence in fledging success rate, nor an individual's lifespan. In turn, early-life telomere length does not predict lifetime reproductive success, which is significantly influenced by an individual's lifespan and the fledging success rate of their nestlings rather than their clutch size or hatching success rate. These results suggest that females may adopt alternative reproductive strategies based on their early-life conditions, of which their telomere length may be representative.

Early-life Telomere Length Predicts Senescence of Multiple Female Reproductive Traits

In our study, we found that a female's early-life telomere length can predict the presence of senescence in both clutch size and hatching success, with shorter telomeres being associated with senescence in these two reproductive traits. This confirms our original prediction, which was made based on the "trade-off" concept of telomere attrition and the disposable soma theory of ageing. The disposable soma theory states that reproductive investment leads to a trade-off with bodily maintenance and therefore longevity (Kirkwood and Holliday, 1979), and telomere attrition provides a possible mechanism for this trade-off. Similarly, we predicted that short telomeres in early life are equal to less "trading power", resulting in a faster rate of senescence in later life when females start off with shorter early-life telomeres. In our study population of female hihi, early-life telomere length may be influencing reproductive strategy, since females with short early-life telomere length tend to have a higher peak in reproductive success in clutch size and hatching in early life followed by a faster rate of decline. Upon visual inspection of the data, it appears that females with short early-life telomeres may also have higher annual fitness in early life compared to females with long early-life telomeres (Appendix 2, Supplementary Figure 2), however this effect was not testable due to a high proportion of zero values for annual fitness, requiring a larger sample size. Our finding that early-life telomere length can predict rates of reproductive senescence are supported by previous findings, which show that early-life telomere length can predict lifetime reproductive success through its effect on lifespan (Eastwood et al., 2019), and that increased reproduction in early-life can lead to faster senescence in later life (Nussey et al., 2006; Reed et al., 2008b). However, ours is the first study to demonstrate a link between early-life telomere length and rates of senescence in specific reproductive traits, independent of lifespan.

The proposed mechanisms linking telomeres to reproductive ageing have primarily been researched in humans and model organisms such as mice (Chico-Sordo et al., 2021; Kalmbach et al., 2013). Mechanisms of female reproduction and fertility in birds are not well understood, due to a lack of studies on female fertility traits compared to those of males (Assersohn et al., 2021). However, the mechanisms underpinning telomere effects are likely to be comparable between mammals and birds. For example, telomere shortening is likely accelerated by the intensive cell replication that is required for follicle growth and ovulation in birds, as in humans (Chico-Sordo et al., 2021). Similarly, short telomeres can cause a number of problems during meiosis (Polonio et al., 2020) and increase the likelihood of embryo fragmentation in humans (Keefe et al., 2005), which if present in birds may feasibly lead to reduced clutch size or higher rates of hatching failure. However, it is worth considering that here we measure early-life telomere length from blood samples, and it has been shown in humans that tissue type can significantly impact the telomere length measured (Demanelis et al., 2020). Conversely, telomere length measurements across five tissue types in painted dragon lizards were significantly correlated (Rollings et al., 2019). Little is known about how telomere length and attrition vary across cell and tissue types in birds, although it would be reasonable to hypothesise that the telomere dynamics in cells of reproductive tissues may be more directly subject to DNA damage associated with reproduction.

Lifetime Reproductive Success is Not Predicted by Early-Life Telomere Length

Despite some indication that female hihi adopt differing reproductive strategies depending on their early-life telomere length, this does not appear to influence overall lifetime reproductive success, measured in this study as the number of breeding individuals recruited into the population. This could be explained by the fact that that lifetime reproductive success in female hihi is primarily determined by fledgling success, for which we found no evidence of an effect of early-life telomere length. It has previously been shown, using simulated data, that the fitness cost of reproductive failure at early stages (i.e. pre-birth/hatching) is significantly lower than reproductive failure at late stages (Lemaître and Gaillard, 2017). Alternatively, the small effect size of the interaction between early-life telomere length and age on reproductive traits observed in our models may suggest that the effect of senescence is not large enough to significantly impact lifetime fitness. This is supported by a previous study on great tits (*Parus major)* which finds the fitness costs of senescence to be negligible (Bouwhuis et al., 2012). Nevertheless, our results question the recent discussion of telomere length as biomarker of fitness (e.g. Eastwood et al., 2022), demonstrating that this idea is not universally applicable. It is also possible however, that the potential mechanisms linking short telomeres with high rates of reproductive failure

such as impaired follicle growth (Chico-Sordo et al., 2021) or meiosis (Polonio et al., 2020) are acting at the earlier stages of reproduction such as egg laying and embryonic development, and are not affecting the innate quality and therefore fledging success of chicks which do develop and hatch successfully.

Egg incubation is carried out solely by the female hihi, but bi-parental care is common once chicks are hatched. A female hihi's social male partner will provision chicks in the nest at variable rates depending on rates of extra-pair copulations by the female (Ewen and Armstrong, 2000), brood size, male age, and other factors (Low et al., 2006). Fledging success is therefore likely to be influenced not only by the mother, but also by male-mediated factors, including paternal age. Sexes can differ in patterns of actuarial senescence (Loison et al., 1999) and reproduction-induced telomere shortening rates (Sudyka et al., 2014). Similar to females, territorial male hihi show an increase in reproductive success with age up to a peak at 3 years old, followed by a decline (Brekke et al., 2015b), although this is not as steep as seen in females. This variation in male reproductive success with age creates the potential for females to offset the effects of their own senescence on reproductive success by pairing with younger males. However, we found a significant correlation between the age of females and males in social pairs, suggesting that there is assortative mating by age in this population. This correlation between female and male ages in social pairs, as well as the variable paternal contribution to chick care, potentially confounds our analysis of fledging success senescence in females.

Several environmental factors not tested here are more likely to influence fledgling success than clutch size or hatching success, potentially masking any small effects of female early-life telomere length on this trait. Weather conditions such as rainfall (Capilla-Lasheras et al., 2021), temperature (Chase et al., 2005) and storm events (Wallace et al., 2016) can impact the number of successful fledglings from a nest due to their effects on insect abundance and activity (Cox et al., 2019). Hihi adults feed their chicks with insects, so insect availability is particularly important for success during this stage of development. Nestling mortality rates in hihi are associated with low temperatures, with the leading cause of death being starvation (Rippon et al., 2011). It has also been shown before that temperature is more important for nestling survival than egg hatching in this species (Low and Pärt, 2009). More research is needed to account for factors such as environmental variations and male age/quality when studying female senescence in fledging success.

Female lifespan was by far the largest contributor to lifetime reproductive success. Surprisingly, female lifespan was not predicted by early-life telomere length in our study, which may also explain the lack of relationship between early-life telomere length and lifetime reproductive success. Our results contradict the very few studies which have found a relationship between early-life telomere length and lifetime reproductive success (Eastwood et al., 2019; Heidinger et al., 2021), and suggest that early-life telomere length is not under selection in females of this species. Instead, we hypothesise that the main drivers of early-life telomere length in this system are perhaps developmental conditions and maternal effects, as has been found in other species (Boonekamp et al., 2014a; Cram et al., 2017; Stier et al., 2020; van Lieshout et al., 2019).

There are few studies where the link between telomeres and reproduction is considered across an individual's lifespan (reviewed in Sudyka 2019). In this study we have demonstrated, using data on lifetime output in key reproductive traits, that females experience different rates of reproductive senescence depending on their early-life telomere length and that only females with short early-life telomeres experience a significant senescence in reproductive traits. The lack of an effect of early-life telomere length on lifespan and lifetime reproductive success, despite the clear effect on rate of senescence in reproductive traits, raises the possibility of reproductive strategies that differ with early-life telomere length, and questions the ubiquity of telomere length as an indicator of future lifespan and lifetime fitness. Although it has been previously suggested that telomeres shorten as a result of high reproductive investment (Bauch et al., 2013; Bichet et al., 2020; Ouyang et al., 2016), our results suggest that variation in reproductive traits is partially pre-determined by an individual's early-life telomere length.

METHODS

STUDY SYSTEM AND DATA COLLECTION

This study was carried out on a managed population of hihi on the island of Tiritiri Mātangi in northern New Zealand. The population is closed with no recorded migration of birds to mainland New Zealand or other islands, allowing for accurate estimations of natural lifespan. Each individual bird is colour ringed for identification purposes and has blood samples taken at 21 days old for paternity assignment through microsatellite analysis (details in 73). Hihi use nest boxes provided across the island, and during the breeding season (September-February), the population is monitored closely to record accurate dates for laying, hatching and fledging. Offspring are assigned a cohort depending on the breeding

season within which they hatched, which spans two years. Bi-annual constant effort population surveys: one pre-breeding survey in September, one post-breeding season in February, allows the monitoring of survival, lifespan, and non-breeding individuals. Clutch size, hatching success and fledging success are recorded for each nesting attempt. For this study, hatching success was measured as the proportion of eggs in a clutch that hatched and fledging success as the proportion of nestlings that fledged. Age of the female at the time of laying was measured as the number of days since the female fledged. The last date a female was observed in the breeding record was taken to be the fledging date of that nest attempt if chicks fledged, hatching date if no chicks fledged, and laying date if no eggs hatched. Females were recorded as "dead" if the last date they were observed in the breeding or census records was more than two years before the onset of the latest breeding season included in the analysis. Estimates of lifespan were only calculated for females classed as dead and were estimated as the days between a female's fledge date and the last date she was observed in the breeding or census records (whichever was later). Likewise, only dead females were included in the analyses including measures of lifetime reproductive success. A total of 75 females was included in the analysis, for which data on 344 clutches were included (an average of 4.5 clutches per individual). Females were distributed across 9 cohorts spanning the years 2005-2013.

Telomere Analysis

Blood samples were taken from individual chicks at 21 days old and stored in >80% ethanol. DNA was extracted from blood samples using the ammonium acetate method (Bruford et al., 1998) and eluted in LowTE. DNA quality and degradation was assessed with gel electrophoresis on a selection of 40 individual DNA samples representing all 9 cohorts; individuals were selected randomly within cohorts. DNA was found to be of consistently high molecular weight (23kb or over) and showed no signs of degradation. DNA purity was measured with a NanoDrop, and samples with A260/280 values outside the range of 1.7 – 2.6 or A260/230 values which fell outside the range 1.7 – 2.8 were cleaned with a bead clean-up protocol using AMPure PB magnetic beads. Samples were subjected to a maximum of 2 bead-cleans to achieve the target purity or were otherwise excluded from further analysis. DNA impurity was a problem for some of our samples, possibly because of the use of heparinised capillary tubes for blood sample collection. It proved important to check DNA purity, which was found to be extremely important to qPCR efficiency in trial plates. DNA quantity was assessed and normalised to 1 *ng/µl* using a Qubit fluorometer.

Telomere analysis was performed using a singleplex quantitative PCR method (Cawthon, 2002; Criscuolo et al., 2009) which provides a relative telomere length or T/S; the ratio of telomere repeat copy number relative to a non-variable copy number control gene. The qPCR was performed using a Quantstudio 12K Flex Real-Time PCR System. We used Tel1b (5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′) and Tel2b (5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3′) to target telomere sequences, and RAG1-F (5′-CAGCACATAAACAAAGATCAGGCAG-3′) and RAG1-R (5′-AAGTCCCTGCCTATTGCCCA-3′) to target the non-variable copy number control gene. The RAG1 primer was designed by using BLAST to find identical regions between *Corvus monedulocides* RAG1 mRNA sequence data and hihi RAG1 partial coding region sequence (CDS). *Corvus monedulocides* was selected because it had the highest percentage agreement with the hihi RAG1 sequence (95.96%). The primers were then designed to fall within an identical overlapping region of the *Corvus monedulocides* RAG1 mRNA sequence with the hihi RAG1 partial coding region (CDS) sequence, mRNA sequence data was necessary to allow the designed primer to span an exon-exon junction, ensuring that the primer was specific to cDNA.

Samples were run in triplicate, with telomere and reference primers run on separate plates. Primers were not run on the same plate, as is preferred (Barrett et al., 2012), because the optimum annealing temperature for the 2 primers differed. A between-plate calibrator was used to account for inter and intra plate variability; every plate included 3 negative control wells and a 1:4 serial dilution (10ng, 2.5ng, 0.625ng, 0.156ng) of the same standard ('golden') sample in triplicate for each primer. Every reaction contained 1ng of DNA, 10µl of SYBR Select Master Mix and 9µl of a primer/ddH_2O mix to a concentration of 0.7µmol for Telomere primers and 0.2µmol for RAG1 primers. Telomere plates were run using the following method: 50°C (20 seconds), 95°C (20 seconds) followed by 40 cycles of 95°C (1 second), 56°C (20 seconds). Rag1 plates were run with the following method: 50°C (20 seconds), 95°C (20 seconds) followed by 40 cycles of 95°C (1 second), 61°C (20 seconds).

The CT threshold was set to 1.45 for analysis of Telomere primer reactions and 0.7 for analysis of Rag1 primer reactions. The CT thresholds were selected as the mean of the automatic thresholds as set by Quantstudio for the multiple plates. Changing the automatic CT threshold to a standardised CT threshold did not substantially change the R^2 or Efficiency values of any standard curve (to be within or outside of the acceptable ranges for analysis). Amplification efficiencies, provided by the Applied Biosystems Quantstudio programme which corrects for baseline fluorescence, were 95.5-96.8% for

RAG plates and 102.6-106% for TEL plates. R^2 values, also provided in the Applied Biosystems Quantstudio were 0.99 for all plates. Relative telomere lengths were calculated using Ct values determined by QuantStudio software using standard curve efficiencies, and by Linregpcr, a software which uses individual sample reaction efficiencies to calculate Ct values. The ΔΔCt (or relative telomere length/RTL) was calculated as follows (Joglekar et al., 2020):

ΔΔCt = (Sample Average RAG1 Ct - Sample Average Telomere Ct) - (Control Average RAG1 Ct - Control Average Telomere Ct)

The "control average" was calculated from the standard curve, which was included in triplicate on each plate using known concentration DNA from a control individual. The relative telomere lengths were calculated using Ct values produced in Linregpcr and were used in all subsequent analyses.

Data Analysis

Does Early-life Telomere Length Affect Rate of Senescence in Reproductive Traits?

Early-life relative telomere length was tested as a continuous variable in models summarised in Table 1. For data visualisation and summary statistics, early-life relative telomere lengths were categorised as "short" or "long early-life telomeres" depending on whether they fell below or above the 1st quartile telomere length. Female age was tested as a continuous variable and was rescaled from days to years to improve model convergence. All models included year, female identity and lay date (relative to the first lay date of the season) as random factors, the latter because of its previously found importance in the success of the hatchling to fledgling transition (de Villemereuil et al., 2019a). Lifespan and individual ID were included in all models as a fixed effect to control for selective disappearance (Nussey et al., 2006) i.e., the fact that individual quality covaries with the likelihood that the individual will disappear from the population (van de Pol and Verhulst, 2006). Specifically, the selective disappearance hypothesis assumes a correlation between age of last reproduction and individual quality. Final models was selected using AIC values (details of models in Appendix 2). Age and telomere length were scaled using mean centring.

The effects of female age and early life relative telomere length on clutch size were tested using a com-Poisson model from the *glmmTMB* package, which is designed to deal with under-dispersed count data (Shmueli et al., 2005). Dispersion was assessed with the *P__disp* function in the *msme* package.

The effects of female age and early life relative telomere length on hatching success and were tested using generalised linear mixed-effects models with binomial or beta-binomial errors from glmmTMB (Brooks et al., 2017) and lme4 packages (Bates et al., 2015) in R version 3.5.2. (R Core Team, 2021). The following relationships between female age and hatching success were tested: linear, quadratic and breakpoint. Breakpoint models (otherwise called piecewise, segmented or broken-stick models) are regression models where the relationship between the independent and dependent variables is partitioned into two or more segments which are modelled separately by linear regression (Zeileis et al., 2002). Breakpoint models are useful when the relationship is expected to change through time (i.e. with age in this case) or when a "threshold" is expected to be reached which changes the nature of the relationship. The breakpoint estimated with the package *strucchange* (Zeileis et al. 2002; 2003), were placed at 0.42 years; however, this breakpoint did not explain the data as well as breakpoints at 2 and 5 years, identified visually by plotting the data (Figure 1).

The age of peak clutch size/hatching success was the age (in years) with the highest mean clutch size/hatching success and was calculated separately for females with "short" and "long" early life telomeres. If two age categories had mean clutch size/hatching success which were not significantly different, determined by overlapped standard errors, we selected the first peak as the peak age for downstream post-peak analysis. Linear models of post-peak clutch size/hatching success were limited to data from females older than the age of peak clutch size/hatching success for the relative early-life telomere lengths, and included female ID, year and relative lay date as random factors. Linear models were constructed in lme4.

The effects of female age and early life relative telomere length on fledging success were tested with zero-inflated binomial model using the *glmmTMB package* due to the disproportionate number of clutches which fledged zero chicks. Linear and quadratic relationships between fledging success and female age were tested. Male age was an additional random factor included in this model to control for the contribution of the male partner to provisioning during incubation. However, due to the

correlation between female age and male age in a pair, male age was removed from the final model of fledging success.

Does Early-life Telomere Length Predict Lifetime Reproductive Success?

The effect of early life relative telomere length on lifetime reproductive success was tested with 4 separate models for different reproductive traits: number of eggs laid, eggs hatched, chicks fledged, and recruits produced in a female's lifetime Recruits were classed as individuals who went on to breed at least once. Only females classified as "dead" (not seen in the two years prior to the last breeding season included in the study) were included in analyses of lifetime reproductive success. Female lifespan was controlled for in all models by being included as a random effect, models were run again with female lifespan as a fixed effect to demonstrate the relative importance of a female's lifespan on lifetime reproductive success. All models were of the poisson or com-poisson family and the model for the lifetime number of recruits produced included a zero-inflation component due to the disproportionate number of females who produced no recruits in their lifetime. The contribution of reproductive traits to lifetime fitness (total number of recruits) was estimated using Pearson's correlation coefficients from the *cor.test* function.

CHAPTER 5.

HISTORIC PLANTING STRATEGY AFFECTS RESTORED FOREST HABITAT DIVERSITY AND MICROCLIMATE: IMPLICATIONS FOR INCUBATION BEHAVIOUR AND REPRODUCTIVE SUCCESS OF A REINTRODUCED THREATENED BIRD POPULATION

ABSTRACT

Habitat restoration is the most common conservation action carried out in terrestrial ecosystems. Assessing the impact of habitat restoration on future habitat and resident birds is essential for informing conservation strategies that target bird populations. Here we assess how the incubation behaviour and reproductive success of a threatened bird, the hihi (*Notiomystis* cincta), is affected by habitat characteristics, which have been moulded by the historic planting of Tiritiri Mātangi forest sanctuary. We show that planting more diverse tree species, and particularly bird attracting tree species with high nectar yields, creates areas of future habitat with an increased diversity of fruiting and flowering trees, a taller canopy and cooler microclimate. The diversity of food source tree species in the immediate nest box habitat does not directly influence hihi reproductive success. However, a cooler nest site microclimate leads to improved hatching success and fledgling production at hihi nests, despite plasticity observed in female incubation behaviour in response to microclimate. Our results highlight the importance of successful planting strategies for future restoration projects and raises concerns about the effect of rising global temperatures on the reproductive success of hihi.

INTRODUCTION

Habitat loss is considered a major driver of extinction and one of the leading causes of biodiversity loss (Brook et al., 2008), due to the reliance of animals on suitable habitat for every aspect of their survival and reproduction. Habitat restoration projects are a major part of conservation strategy across the world, with vegetation planting being the most common conservation action in marine and terrestrial ecosystems (Hale et al., 2019). Habitat restoration is seen as a major solution to biodiversity loss (Banks-Leite et al., 2020) and, as a result, the Bonn Challenge – a major global effort to restore 350 million hectares of forest by 2030 – was launched in 2011 (Dave et al., 2018). The goal of habitat restoration is to not only enhance the local vegetation, but also benefit animal species that are dependent on the habitat being restored. Understanding how the characteristics of restored habitat impact reproductive success is vital for determining the effectiveness of conservation practices.

Habitat quality is an important determinant of reproductive success in wild animal populations. Habitat complexity predicts predation risk in red-backed voles (*Clethrionomys gapperi*) (Andruskiw et al., 2008) and arthropods (Frey et al., 2018), and survival in cheetah (*Acinonyx jubatus*) cubs (Gigliotti et al., 2020). Predation poses a direct risk to individual reproductive success, but there are also many indirect ways in which the habitat can impact reproductive success. For example, habitat suitability for prey species can indirectly affect the reproductive success of their predators (Hofer, 1988; Valkama et al., 1995), and it has been proposed that a lack of food sources can limit reproductive output in low quality habitats (Griffen and Norelli, 2015). Habitat characteristics can also affect the microclimate of breeding sites (Kim and Monaghan, 2005), which can be important for reproductive success, particularly in oviparous species where offspring development takes place outside of the body and is therefore more directly affected by environmental temperature and other climatic variables. The nest microclimate has been found to be as a key determinant of life history traits, including clutch size and hatching asynchrony across bird species (Lundblad and Conway, 2021a). Between individuals, the microclimate of birds' nests and local weather conditions have been shown to influence embryonic development rates (Griffith et al., 2016), juvenile survival (Sandvig et al., 2017) and behavioural traits of both offspring and parents, such as clutch size (Wiebe, 2001), incubation duration, nestling begging, parental provisioning and fledging success (Mueller et al., 2019). Parents can adjust their incubation behaviour according to the atmospheric temperature of a breeding site, in order to create a suitable nest microclimate; for example, parents have been shown to reduce nest attentiveness in favourable atmospheric temperatures (Londoño et al., 2008; Lundblad and Conway, 2021b) and increase nest attentiveness in unfavourable temperature conditions (Sharpe et al., 2021). Plasticity in incubation behaviour for achieving an optimum nest microclimate has been demonstrated experimentally (Maziarz, 2019), as has the effectiveness of this plasticity for buffering against environmental conditions (McClintock et al., 2014), highlighting this as a potential mechanism by which birds could adapt to a changing climate.

Birds often select nest sites based on habitat characteristics. Female song sparrows (*Melospiza melodia*), for example, select nest sites based on habitat characteristics including food abundance and microclimate, and females occupying these preferred sites have higher reproductive success by producing more recruits (Germain et al., 2015). Lesser black-backed gulls (*Larus fuscus)* select nest sites with more surrounding vegetation, which positively impacts hatching success. The abundance and diversity of food sources are key determinants of habitat quality, directly influencing key fitness traits. The availability and energy content of food has been shown to influence reproduction in a range of species. The provision of supplementary sugar water has been shown to reduce time between clutches laid (Castro et al., 2003) and increase fledgling production (Doerr et al., 2017) in a nectar feeding bird

(*Notiomystis cincta)*. However, the nutritional quality and composition of an organism's food supply may be more important for reproductive success than energy content alone. For example, provision of nutritionally complex food increases clutch size in kākāpō (*Strigops habroptilus*) (Houston et al., 2007). The importance of the nutritional complexity of food on fecundity and hatching success is likely driven by the role of micronutrients, such as zinc (Amen and Al-Daraji, 2011) carotenoids (Mangiagalli et al., 2010) and vitamins (Nawab et al., 2019) in fertility and egg production.

Since habitat quality is so crucial to individual fitness, it is perhaps no surprise that habitat loss – particularly the loss of forest habitat (Pimm et al., 2006) – is the most important cause of species extinction risk for birds (Stattersfield et al., 2000). In New Zealand, habitat destruction and degradation has been one of the major causes of population decline for many endemic bird species (Innes et al., 2010). Restoration of suitable habitat is therefore of great importance. Endemic birds are a primary focus of New Zealand's conservation strategy, and their protection is of conservation and public interest due to their taxonomic uniqueness (Driskell et al., 2007) and importance in Māori culture, with many endangered and vulnerable bird species listed as taonga ("treasured possession") in the Ngāi Tahu Claims Settlement Act (1998).

Aside from their cultural role, birds also play an important role in the restoration of New Zealand forest habitat. Native birds are important pollinators in forests, particularly for flowering tree species (Andrews et al., 2022; Kelly et al., 2010). Studies which have experimentally excluded bird pollination from flowering plants have shown that endemic birds significantly improved the percentage fruit set of flowering species (Anderson, 2003). Furthermore, native birds are the dominant seed dispersers for New Zealand forests, dispersing significantly more seeds than the invasive possum (Wyman and Kelly, 2017) and aiding dispersal of over 70% of tree species found in regenerating forests (Reay and Norton, 2002). Attracting birds to regenerating forest is therefore likely to facilitate the colonisation of tree species and natural forest regeneration. The resources and conditions that the habitat provides birds is therefore closely interwoven with the role that birds play in their habitat. Understanding the links between habitat restoration strategies, habitat characteristics, and the impact that habitat has on resident bird species is vital to inform more effective restoration practices (Hale et al., 2019).

In this study, we assess how current habitat characteristics have been influenced by forest restoration techniques, and how this affects the reproduction of a resident population of hihi, *Notiomystis cincta*. The study population of hihi was reintroduced to the restored island sanctuary of Tiritiri Mātangi after an intensive habitat restoration project. Tiritiri Mātangi is an excellent example of a successful community restoration project with an opportunistic planting regime, resulting in a mosaic of forest

characteristics across the island. As such, the island provides an opportunity to assess the effectiveness of different planting techniques. Due to their feeding ecology, hihi are also an ideal species to use as an indicator of forest maturity and health. Hihi feed primarily on nectar and fruits borne by native shrubs and tree species, and since they are smaller and less dominant than other native nectivores, hihi are often displaced from food sources (Rasch and Craig, 1988) and rely on a wide diversity of fruiting and flowering trees. The hihi is the most threatened of New Zealand's nectar feeding birds and is extremely vulnerable to habitat loss and degradation (BirdLife International, 2016). Therefore, hihi represent an important bio-indicator for the maturity and suitability of forest to host endemic avian nectivores. On Tiritiri Mātangi, the hihi population is provided with a supplementary food source of unrefined sugar water at fixed sites, allowing us to separate the effects of energy rich food availability from those of nutritionally diverse food availability, in a wild managed system. Using a combination of historic data on planting regimes, modern habitat surveys, and long-term data on hihi reproductive success, this study aims to (1) assess the role of historic planting regime in shaping current availability of natural food sources and habitat microclimate, and (2) explore the importance of these current habitat characteristics for female incubation behaviour and reproductive success. The importance of various food supplementations for clutch size (Castro et al., 2003), nestling growth and survival (Walker et al., 2013), body weight and adult survival (Armstrong and Perrott, 2000) of hihi has previously been investigated, with mixed conclusions. This study instead focuses on the importance of natural food sources at the breeding sites of female hihi for reproductive output, while controlling for the effect of supplementary food sources. Since hihi have also been shown to have little adaptive potential in many fitness related traits (de Villemereuil et al., 2019b; Duntsch et al., 2020) including the timing of breeding (de Villemereuil et al., 2019a), which may leave temperature sensitive processes such as embryonic development vulnerable to the effects of climate change, we also look at the potential for plasticity in incubation behaviour as a mechanism for buffering microclimate variation.

METHODS

STUDY SYSTEM, BREEDING DATA & INCUBATION BEHAVIOUR

All data were collected on Tiritiri Mātangi, an island reserve in the Hauraki Gulf on which a habitat restoration project took place between the years 1982 and 1994. The island is 2.2 km^2 in size and is split into 26 bush/forest sections assigned at the beginning of planting (Figure 1). Tiritiri Mātangi currently hosts a mosaic of restored forest and some remnant forest habitats, and populations of several endemic protected bird species including hihi. The focal population of hihi on Tiritiri Mātangi Island are monitored during the breeding season to record life history data including the timing of egg

laying and hatching, clutch size, hatching success and fledging success. All hihi on Tiritiri Mātangi are colour ringed for individual identification purposes and accurate recording of life history data. Data on hatching success (the proportion of eggs hatched per clutch), clutch size, and fledgling success from breeding records were included in the analyses. Hihi have a 14 day incubation period and incubation is carried out solely by the female. The incubation behaviour of female hihi was recorded during the breeding seasons of 2019/2020 and 2020/21 using a mixture of in person observations and video recordings. Incubation behaviour was recorded during 90-minute observations at 3 points in the incubation period, early (day 1 – 2), mid incubation (day 6 – 8) and late incubation (day 13) resulting in 3 x 90-minute observations (1 early, 1 mid and 1 late) per female/nest. The effectiveness of video recordings was verified using videos recorded alongside in-person observations at the beginning of the study. Off-bouts are categorised as the time between the female leaving and entering the nest box.

Planting Data

Data on the number, species, location (bush section) and date of trees planted was collected from historical planting maps and planting diaries dating 1982–1992, which were digitised by the Supporters of Tiritiri Mātangi. The records from which data were collected are not exhaustive of all trees planted during the restoration project due to omissions in the available data. However, the data spans 10 out of the 12-year planting period (1984 to 1992) and comprises 60% of the estimated 280,000 trees planted during the entire restoration project. We therefore assume that the data analysed is largely representative of the overall planting regime. The following summary characteristics of the species planted were calculated for each of the 26 bush sections: (i) Shannon species diversity and species richness of trees planted, and (ii) the dominant/most abundant tree species planted. The Shannon species diversity was calculated using the diversity function in the vegan package in R (Dixon, 2003) and is defined as:

$$\mathrm{H} = \sum_{i=1}^{S} p_i \, log_b \, p_i$$

Where p_i is the proportion of species i and S is the number of species, so that $\sum_{i=1}^{S} p_i = 1$ and b is the base of the logarithm (Jost, 2007).

Modern Habitat Data

Hihi on Tiritiri Mātangi almost exclusively use nest boxes for breeding. In the breeding season, female hihi occupy nest boxes situated across the 26 bush sections (Figure 1). The habitat survey methods were developed after Makan (2014) and surveyed the surrounding area of each of 93 hihi nest boxes

as follows: five 20m transects were carried out per nest box, starting at the nest box, and radiating out at 0°, 72°, 144°, 216°, 288°. The number and species of vegetation was recorded every 4m along each transect in ascending vertical segments as follows: 0cm-30cm, 30cm-2m, 2m-4m, 4m-6m and so on, to the top of the canopy.

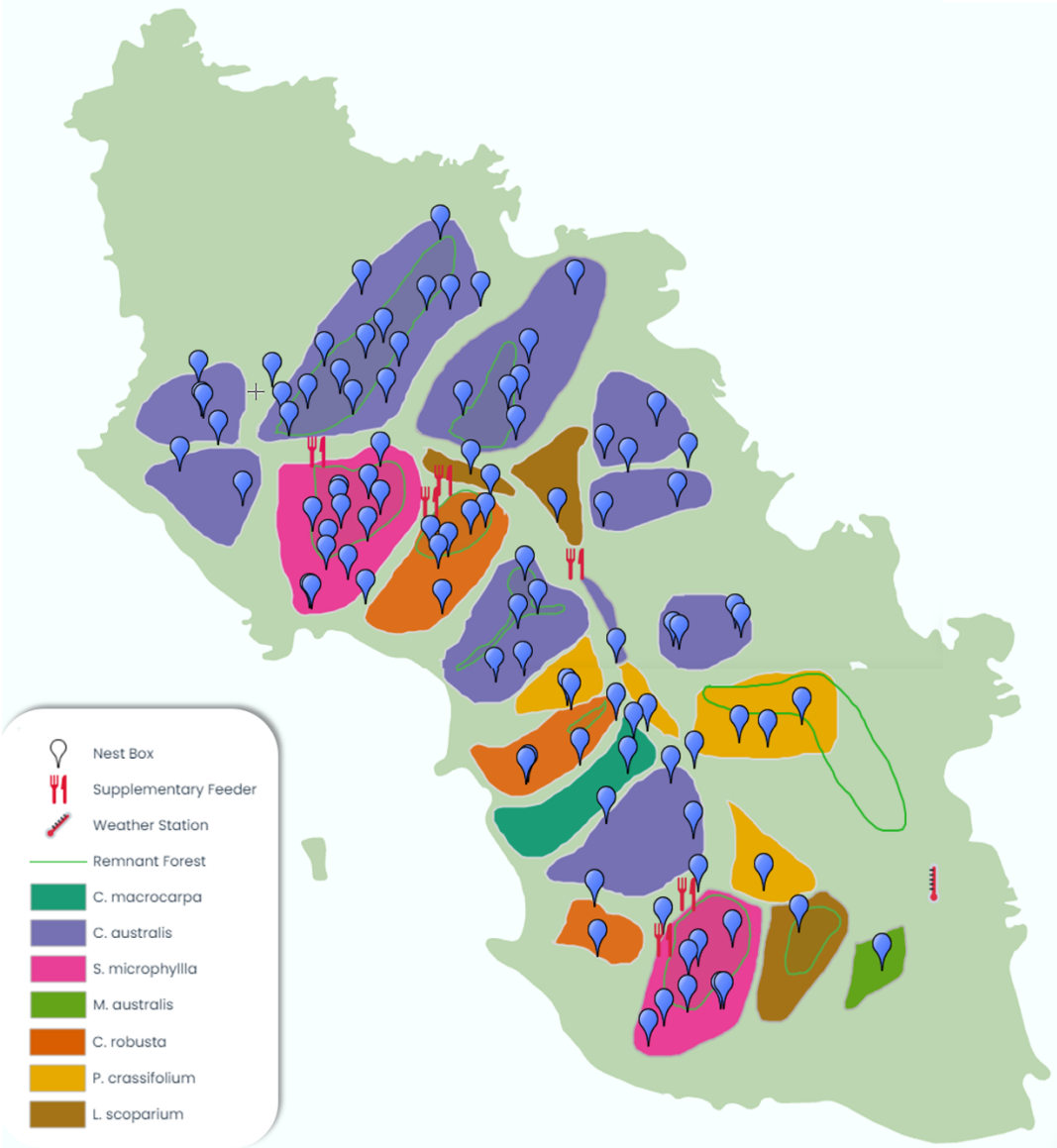

Figure 5. A map of Tiritiri Mātangi island planting, locations of habitat surveys and other points of interest relevant to the study. Bush sections/planting areas are indicated by polygons coloured by the most abundant tree species planted in that section. Remnant forest areas, nest boxes which had habitat surveys carried out, supplementary feeders and the weather station are plotted.

A total of 30 plant species of interest were identified due to their production of flowers or fruit that are fed on by adult hihi. Species richness (the number of species present) and species diversity (using the Shannon Index of diversity) of these 30 food source species was calculated for each of the 93 nest box

territories. All habitat surveys were carried out between 2016 – 2020 by two surveyors. Between surveyor differences in surveys carried out at a subset of 10 nest box sites in 2016, 2018 and 2020 were estimated, and species counts did not significantly differ between surveyors/years (Chisq = 51.2, df = 41, p = 0.13).

Microclimate Data and Incubation Behaviour

Microclimate data was collected in the breeding season of 2019-2020 using iButton temperature data loggers. Loggers were fixed to the lids of 23 nest boxes (1 logger per nest box) as close to the incubation start date as possible to obtain a biologically relevant measure of the nest location microclimate. Temperature loggers stayed in place until incubation was complete at 14 days but provided varying amounts of data (3–14 days) due to variable logger storage size. Average maximum and minimum temperatures were calculated from the entire range of data collection. To control for the variation in temperature across the season, the difference between logger daily maximum and minimum temperatures and atmospheric daily maximum and minimum temperatures were calculated on every day that a logger was recording. Atmospheric daily maximum and minimum temperatures were recorded at the Tiritiri Mātangi weather station and obtained from the CliFlo database on the National Institute of Water and Atmospheric Research (NIWA) website.

Data Analysis

All data analysis was carried out in R (R Core Team, 2021). Due to the nature of the tree planting on Tiritiri Mātangi, with the bush sections all receiving unique planting "treatments", the effect of some aspects of the planting treatment on the current habitat characteristics is confounded by potential spatial effects of bush section. Although we have no reason to believe that the bush sections have innate differences that may affect current habitat characteristics, we cannot completely rule this out within our statistical analysis. To explore the effect of past planting regime on current habitat characteristics of nest box areas, we used Spearman's correlations to assess the relationship between the species diversity, richness and number of trees planted on the current species diversity and richness of fruiting and flowering trees. We were able to assess the effect of the most abundant tree species planted in a bush section (Figure 1) on the current species diversity and richness of trees and shrubs, whilst controlling for potential spatial variation between bush sections using a MCMCglmm model including bush section as a random factor. This was possible because there were 5 species that were planted most abundantly across the 23 bush sections. When determining the most abundant tree species planted in an area, we excluded one species, *Metrosideros excelsa* (pōhutukawa), because although *Metrosideros excelsa* was planted heavily during the early restoration project, most of these saplings died (Supporter of Tiritiri Mātangi, personal communication, 2022). Accordingly, 70% of all the

trees recorded as planted in our data were *Metrosideros excelsa,* but this species makes up only 19% of trees recorded in modern habitat surveys.

The effect of current species diversity and richness on reproductive success was estimated using MCMCglmm models. Data from breeding attempts spanning 2012 – 2020 (totalling 442 clutches laid by 181 individual females), were used in our analyses. Since there were no observable differences between habitat characteristics in the 4 years between the first (2016) and the last (2020) habitat surveys carried out, we deemed it reasonable to assume that habitat characteristics also did not change significantly in the 4 years prior to that period (2012 – 2016). We therefore included data from breeding attempts from 2012-2020. Six separate models were specified, with clutch size, hatching success and number of fledglings produced as the dependent variables, and species diversity and richness as the explanatory variables (these variables were highly correlated; hence they were tested in separate models). To test the effect of supplementary feeding on hatching success, we then expanded the model to include proximity to the nearest supplementary sugar feeder as a random factor. All MCMCglmm models included female ID, female age and year as random factors and used weakly informative parameter expanded priors. The relationship between habitat characteristics and incubation behaviour was tested using MCMCglmm with weakly informative parameter expanded priors, including the factors of average maximum temperature and day of incubation as random effects. Principle component analysis was performed with the vegan package (Dixon, 2003) to explore associations between planting regime and multiple habitat characteristics and included 19 variables describing current habitat characteristics (the diversity and richness of current tree species and the foliage density at ascending heights) and planting characteristics (the number planted of the 5 most abundantly planted species across the island and the diversity and richness of all tree species planted), these are detailed in Appendix 3 (Supplementary Table 1).

The effect of microclimate on incubation behaviour was tested with six MCMCglmm models, with number of incubation off-bouts per hour, mean duration of off-bouts, and total percentage time spent off the nest as the dependent variables, and average maximum microclimate temperature as the explanatory variable, controlling for female ID. The effect of microclimate on reproductive success was tested with six MCMCglmm models, with clutch size, hatching success rate and the number of fledglings produced as dependent variables, and the average difference between (i) the microclimate maximum and atmospheric maximum temperatures and (ii) the microclimate minimum and atmospheric minimum temperatures as two separate explanatory variables (this approach allowed us to test the microclimatic properties of the habitat, rather than the absolute temperature). Breeding data was included from one year pre- and one year post-microclimate data collection, as this was within the

time-span of the habitat surveys and it is therefore assumed that the microclimatic properties of the forest did not change significantly in that time.

RESULTS

The mean Shannon diversity of fruiting and flowering trees in a nest box habitat was 1.67 (SD = 0.4), the mean species richness was 8.9 (SD = 2.66). There was little evidence that the diversity, number or richness of species planted in each bush section between 1984 – 1992 significantly impacted the current (2016 – 2020) diversity (Figure 2) or richness of tree and shrub species present in nest box habitats in the same bush sections (planting and current diversity, rho = -0.05, S = 197934, p = 0.57; planting and current species richness, rho = -0.015, S = 190309, p = 0.88; number of trees planted and current species diversity, rho = -0.067, S = 199946, p = 0.5).

However, the current species diversity of fruiting and flowering trees did vary significantly depending on the most abundant tree species planted in that area (Figure 3). Pairwise comparisons using Wilcoxon tests revealed that the historic planting of *Pittosporum crassifolium* has led to a significantly more diverse modern habitat than *Coprosma macrocarpa* (p = 0.005) and *Coprosma robusta* (p = 0.039), and that planting greater numbers of *Cordyline Australis* leads to a significantly more diverse modern habitat than *Coprosma macrocarpa* (p = 0.026). When controlling for bush section using a MCMCglmm model, we find some agreement with the results of Wilcoxon tests, in that areas planted with mostly *Leptospermum scoparium* (Post. mean = 0.67, 95% CI = 0.059, 1.37) and *Pittisporum crassifolium* (Post. mean = 0.56, 95% CI = 0.048, 1.1) have significantly higher current diversity than areas planted mostly with *Coprosma macrocarpa.* When including *Metrosideros excelsa* in the quantification of the most abundant tree planted in a bush section, there was no significant difference in current species diversity or richness between sections planted mostly with *Metrosideros excelsa* and sections planted with any other tree, which is likely due to the low survival of *Metrosideros excelsa* saplings.

Principal component analysis (PCA) revealed a number of other habitat characteristics that are associated with the historic planting regime (Figure 4). Two principal components (Appendix 3, Supplementary Table 1) were required to explain 55% of the variation in habitat and planting characteristics, and these highlight the following patterns. PC1: Areas that were subject to more rich and diverse tree plantings have more tall trees (over 6m), lower understorey density (foliage under 4m), and cooler maximum and minimum microclimate temperatures at the nest box site. PC2: Areas that were planted with fewer *Pittosporum crassifolium* (Karo) trees have a less diverse current habitat and lower number of saplings (0-30cm) and young understorey plants (30cm-4m).

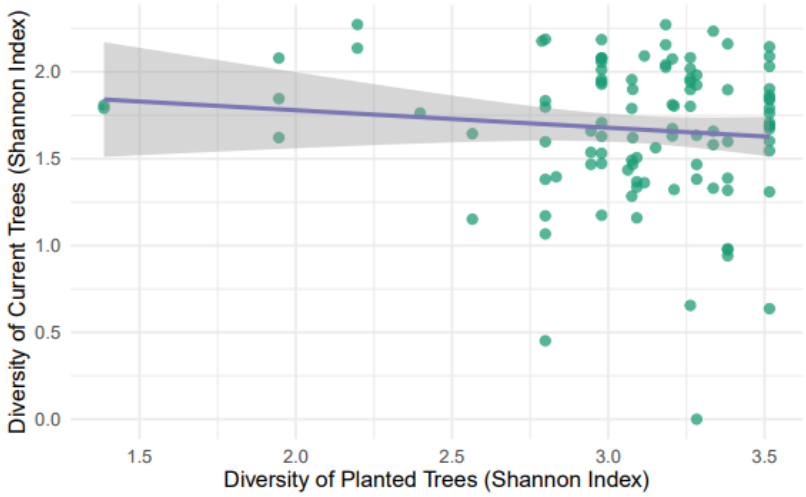

Figure 2. The species diversity of trees and shrubs planted in bush/forest patches during a habitat restoration project is not significantly associated with the current species diversity of trees and shrubs recorded in modern habitat surveys in those areas. Each point represents a habitat survey covering an area of 20m² within those forest patches, with multiple surveys being carried out per forest patches.

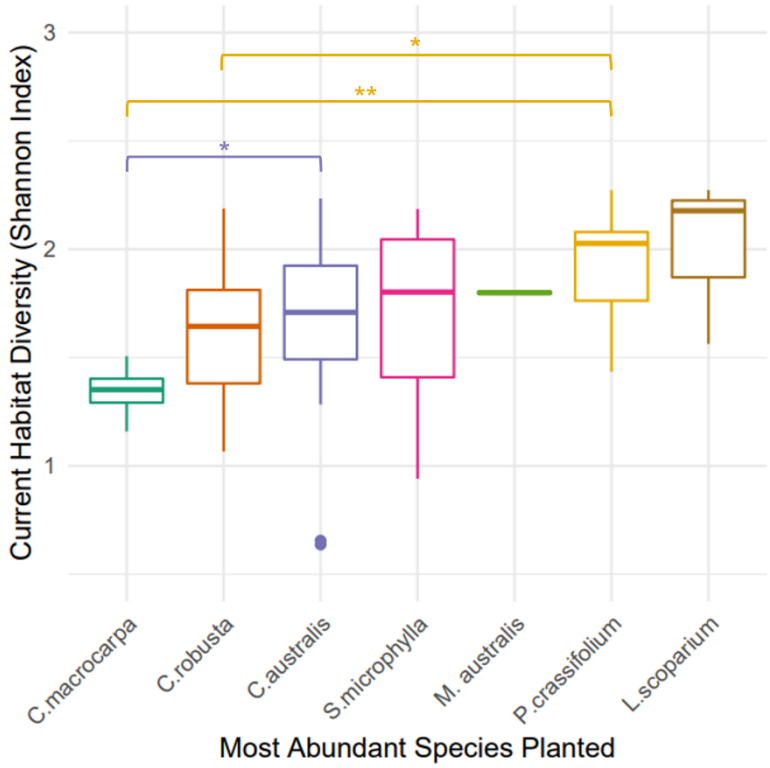

Figure 3. Current (2016-2020) diversity of tree and shrub species in areas of historic (1984-1992) habitat restoration planting. The centre of the boxplot represents the median and the outside of the boxes (hinges) represent the 1st and 3rd quartile, the whiskers extend to the smallest and largest values, no further than 1.5 x the interquartile range, values outside that range, or outliers, are represented as points. The current species diversity of the habitat varies with the most abundant tree planted in the area during habitat restoration. Species are (left to right): Coprosma macrocarpa (coastal karamu), Coprosma robusta (karamu), Cordyline australis (cabbage tree), Sophora microphylla (kōwhai), Myrsine australis (māpou), Pittosporum crassifolium (karo), Leptospermum scoparium. Significance levels indicate results from paired Wilcoxon tests.

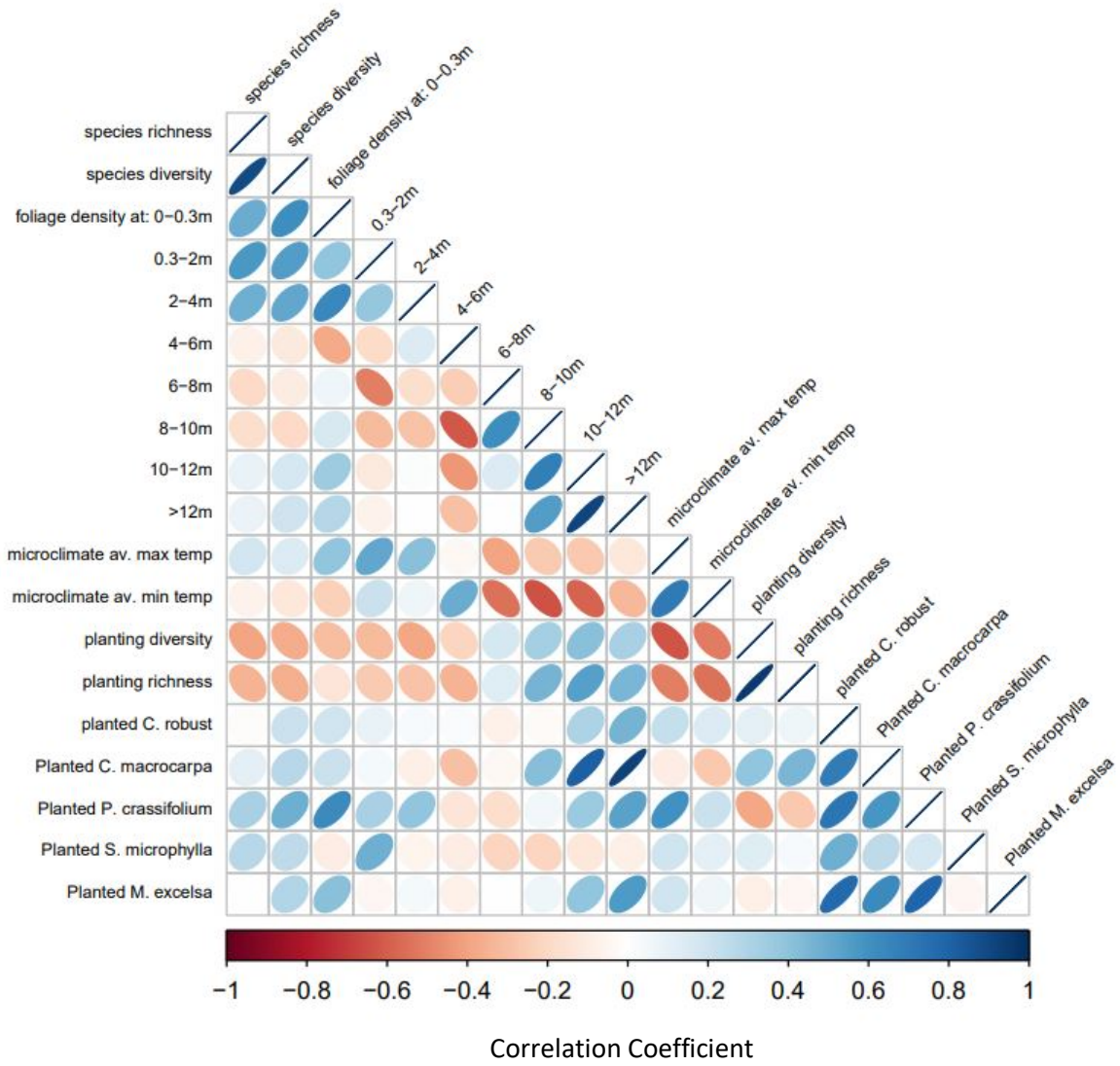

Figure 4. A correlation plot of habitat characteristics associated with species diversity: abundance of 5 key hihi food source tree species: Hangehange (Elaeocarpus dentatus), Karamu (Coprosma macrocarpa), Ti Kouka (Cordyline australis), Mahoe (Melicytus ramiflorus), Matipou (Myrsine australis), foliage density at different heights in the forest and microclimate maximum and minimum temperature averages. The orientation and size of the plotted ovals indicate the direction and strength of the correlation, as well as the colour (refer to x-axis), red ovals with a downwards left to right direction indicate negative correlations, blue ovals with an upwards left to right direction indicate positive correlations, narrower ovals indicate stronger correlations.

Daily average microclimate temperatures measured at nest box locations were consistently higher than the temperatures measured at the weather station (Figure 5). Incubation behaviour of female hihi was impacted by the microclimate of the nest, and this effect was dependent on the stage of incubation (Figure 6).

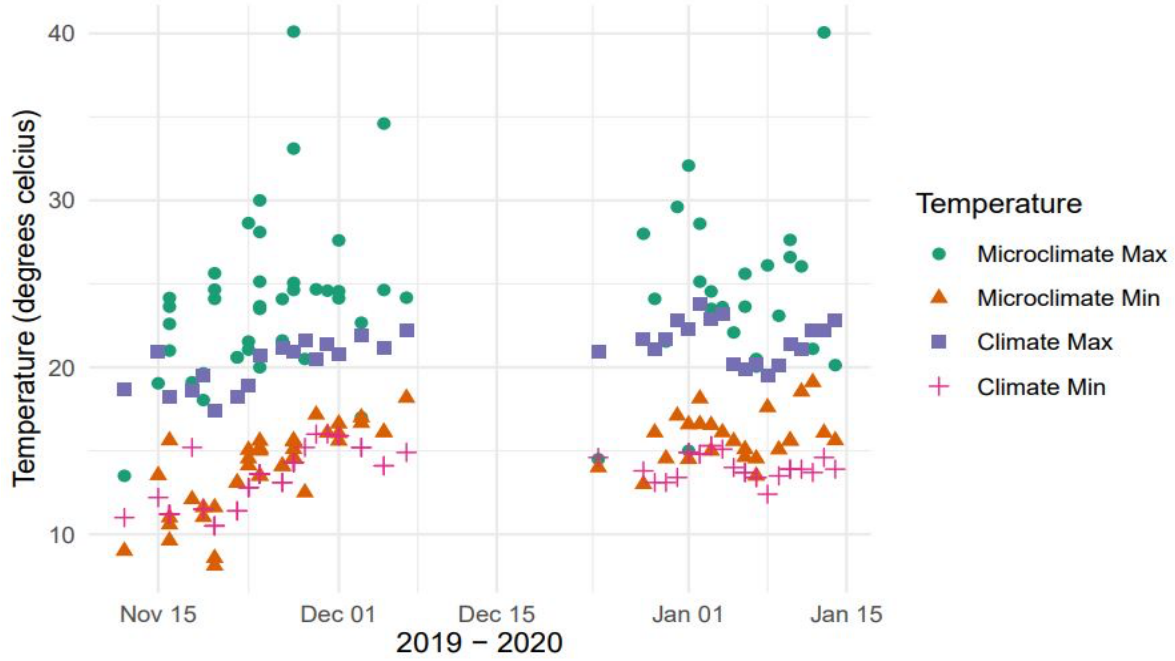

Figure 5. The differences between the climatic maximum and minimum temperatures, measured at a weather station, and the maximum and minimum microclimate temperatures measured at nest box habitats on the same day.

Females left the nest more frequently when the average maximum temperature of the microclimate reached higher temperatures (Post. mean = 0.68, 95% CI = 0.06, 1.17). Females spent significantly less time off the nest during the late incubation stages than the early incubation stages (Post. mean = -42.78, 95% CI = -79.82, -3.66) and the effect of the average maximum microclimate temperature on time spent off the nest differed significantly between late and early incubation stages (Post. mean = 1.6, 95% CI = 1.64, 0.16), with females spending less time off the nest at high maximum microclimate temperatures during the early stages of incubation. The effect of microclimate maximum temperature on the duration of the off-bouts also differed significantly between the early and late incubation stages (Post. mean = 46.23, 95% CI = 5.9, 89.37), with higher temperatures leading to shorter off-bouts in the early stages of incubation and but longer off-bouts during the late stages (Figure 6). In general females took significantly shorter off-bouts during the late incubation stages (Post. mean = -1,109.95, 95% CI = -2,160, -54.19). The overall trend therefore was for females incubating in microclimates with higher maximum temperatures to take more frequent, shorter breaks during the early stages of incubation, and longer, less frequent breaks during the late stages of incubation (Figure 6).

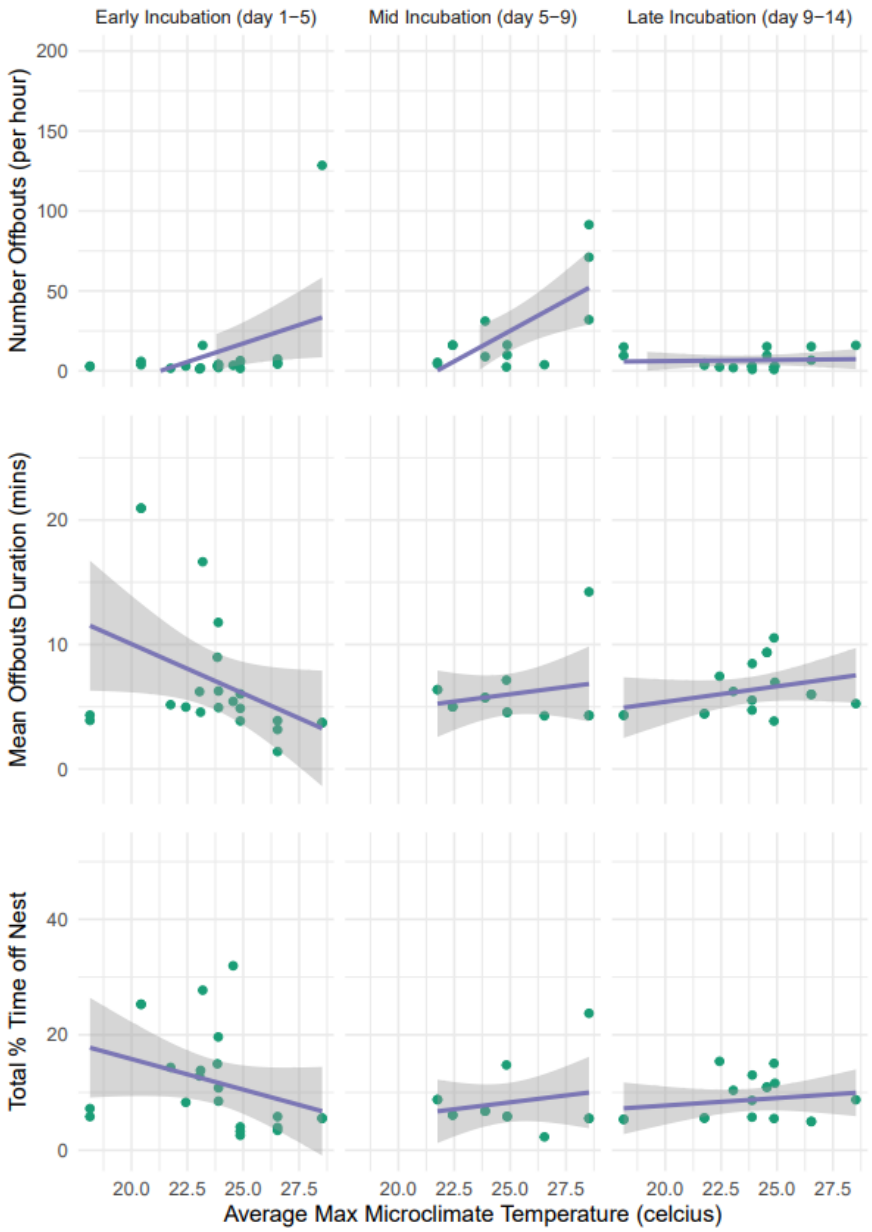

Figure 6. Variation in female hihi incubation behaviour with the stage of incubation and the microclimate temperature experienced at the nest site.

Female reproductive success was measured in three ways: clutch size, hatching success and number of fledglings produced. We found no evidence that the diversity of species richness of fruiting and flowering trees and shrubs impacts any of these measures of hihi reproductive success (Table 1). Two measures of female reproductive output, hatching success and number of fledglings produced were affected by the microclimate at the nest site (Table 1). There was evidence that a warmer nest site microclimate (i.e., a larger positive difference between the temperature at the nest site and the atmospheric temperature taken at the weather station), led to significantly lower hatching success and fewer fledglings produced (Figure 7, Table 1).

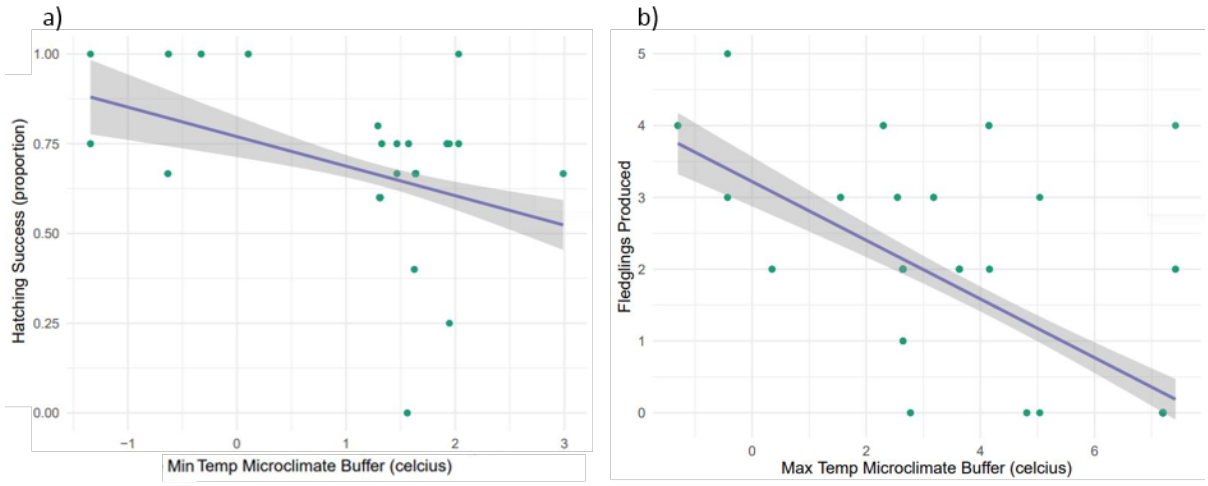

Figure 7. The microclimate of the nest site habitats affects the reproductive output of hihi females. a) Hatching success and b) number of fledglings produced both decrease significantly when the minimum temperature of the nest site microclimate is increasingly higher than the atmospheric temperature taken at the Tiritiri Matangi weather stations.

Table 1. The results of 12 separate MCMCglmms analysing the effect of species diversity and richness of flowering and fruiting tree and shrub species and the microclimatic properties of nests on the reproductive success of hihi, measured as clutch size, hatching success, and the number of fledglings produced. All models included female ID, female age, year and distance to the nearest sugar water feeder as random effects.

	Clutch Size			Hatching Success			Fledglings Produced		
	Posterior Mean	Lower 95 % CI	Upper 95% CI	Posterior Mean	Lower 95 % CI	Upper 95% CI	Posterior Mean	Lower 95 % CI	Upper 95% CI
species diversity	-0.014	-0.15	0.12	0.1	-0.5	0.63	0.14	-0.2	0.45
species richness	-0.0022	-0.022	0.018	0.0029	-0.075	0.085	0.017	-0.031	0.064
Min. Temp Microclimate Buffer [1]	-0.02	-0.2	0.13	**-0.57**	**-1.12**	**-0.087**	-0.3	-0.74	0.09
Max. Temp Microclimate Buffer [2]	0.039	-0.037	0.1	-0.064	-0.33	0.20	**-0.21**	**-0.36**	**-0.05**

[1] The difference between the minimum daily temperature at the nest microclimate and the daily minimum temperature recorded at the NIWA weather station at Tiritiri Matangi Lighthouse. Positive values indicate higher microclimate temperatures than atmospheric temperatures.

[2] The difference between the maximum daily temperature at the nest microclimate and the daily minimum temperature recorded at the NIWA weather station at Tiritiri Matangi Lighthouse. Positive values indicate higher microclimate temperatures than atmospheric temperatures.

DISCUSSION

Using historical habitat restoration data combined with surveys of current habitats from Tiritiri Mātangi open sanctuary, we show that the prevalence of specific tree species in planting regimes has important consequences for overall species composition, more so than the number, richness and diversity of tree species planted, indicating that future habitat diversity may be most efficiently boosted by concentrating planting efforts on key species. However, we also show that the reproductive output of hihi is not significantly affected by the richness and diversity of fruiting and flowering tree species in the immediate nesting habitat. Instead, the nest site microclimate seems to be the most important factor of those measured here influencing hihi reproductive success. The microclimate of the nest site significantly influences the incubation behaviour of female hihi across the incubation period, with females incubating in hotter microclimates taking more frequent, shorter breaks during the early stages of incubation, and longer, less frequent breaks during the late stages of incubation. However, this plasticity in incubation behaviour does not appear to be sufficient to reduce the impact of high microclimatic temperatures on reproductive success, with both hatching success and the number of fledglings produced being significantly lower at warmer nest sites.

Forest Restoration Planting & Current Habitat Characteristics

Planting a more diverse array of trees was found to be associated with (i) higher current species diversity in flowering and fruiting trees, (ii) more tall trees and (iii) a more favourable microclimate in terms of reproductive success. However, contrary to our predictions, many aspects of the planting regime during historic habitat restoration on Tiritiri Mātangi did not appear to significantly affect current habitat characteristics. The lack of an association between planting regimes and current habitat characteristics may be explained by the profound importance of seed dispersing birds for habitat restoration. Birds play a large role in pollination and seed dispersal in New Zealand forest (Anderson, 2003; Kelly et al., 2010) and beyond (e.g. Clark et al., 2001; Ingle, 2003; Medellin and Gaona, 1999), being responsible for a large component of dispersed seeds in restoring and restored forest sites worldwide (Carlo and Morales, 2016; Reay and Norton, 2002; Wunderle, 1997). Therefore, the activity of birds in regenerating forest areas is likely to have a strong influence on habitat restoration, perhaps more so than the planting regime of said habitat restoration. For example, it has been shown that even the planting of non-fruiting trees during habitat restoration can promote bird-driven seed dispersal through the provision of perching sites for seed dispersing birds (McClanahan and Wolfe, 1993; Robinson and Handel, 1993), with 71% of regenerating tree species at a site planted with non-fruiting trees being bird-dispersed species, rather than planted species (Reay and Norton, 2002).

However, it has also been shown that the species of trees present in forest restoration plots can influence bird behaviour. For example, the abundance of endemic bird species has been found to be higher in native kanuka forest as opposed to forest dominated by the non-native gorse bush (Williams and Karl, 2002). Seed dispersing birds pay more frequent and longer visits to certain tree species (Fink et al., 2009), perhaps because of their food availability. It is possible that the planting of tree species that attract birds may encourage natural forest regeneration and diversification – for example, the attraction of birds to fruiting trees may lead to a higher abundance of dispersed seeds in the soil (Guevara et al., 1986; Uhl et al., 1982). Our data show that the most dominant species planted in an area can have a significant effect on future habitat diversity, with the planting of some species leading to higher future diversity than others. The most likely explanation for this is that certain plant species are more likely to attract birds and other pollinators to the area. It is also important to recognise that due to the nature of the habitat restoration planting on Tiritiri Mātangi, the effect of species diversity, richness and number of trees planted on current habitat characteristics may be confounded by spatial variation across bush sections. Therefore, the lack of evidence found here for an association between planting characteristics and current habitat characteristics needs to be interpreted cautiously. Confounding factors and pseudo-replication are common issues in ecological studies (Davies and Gray, 2015; Oksanen, 2001), and in this case are unavoidable due to the use of retrospective, historic planting data. This is likely to be the case in other assessments of habitat restoration success, where restoration is carried out opportunistically and without an experimental approach.

The plant species we identified as most effective at boosting future habitat diversity was *Pittosporum crassifolium* (karo). Karo trees receive more bird visits when flowering, and attract a greater number of bird species when fruiting, than another tested tree species on Tiritiri Mātangi (Anderson et al., 2021). Flowering karo trees have also been shown to have one of the largest standing crop of nectar in a study of 8 endemic flowering tree species: karo produced 23 Joules of nectar, compared to 1.5-6.7 Joules per 24hrs produced by the 6 other species tested (Castro and Robertson, 1997). This high nectar production results in a higher energy uptake by nectivorous birds, including hihi, when feeding on karo compared to other flowering trees (Castro and Robertson, 1997). These results suggest that planting bird-attracting plants, particularly those which produce large quantities of nectar and fruit, may boost natural regeneration and, importantly, diversification, of forests through the behaviour of pollinating and seed dispersing bird species. *Metrosideros excelsa* (pōhutukawa) also produces large amounts of flowers and nectar (Castro and Robertson, 1997), and they were planted in great numbers across Tiritiri Mātangi, making up 70% of all trees planted in the historic planting data we collected. However, many of the pōhutukawa saplings did not persist long after planting (Supporters of Tiritiri Mātangi, personal communication, 2022), meaning that they now make up only 20% of hihi food source tree species

recorded in modern habitat surveys. The reason for their failure is unknown, but may be linked to the fact that, after the removal of livestock from Tiritiri Mātangi, the vegetation did not accommodate the high light or mycorrhizae requirements of *Metrosideros excelsa* establishment (Mitchell, 2013). The results presented here suggest that care should be taken to consider the food preferences of local seed dispersing species when choosing dominant tree species to be planted in forest restoration projects. In addition, our results suggest that forest restoration projects aimed at supporting nectivorous bird species may need not waste resources specifically on increasing species richness/diversity of trees and shrubs planted in an area, since we found no evidence that the diversity of trees planted in an area was important for future habitat diversity. The current study was not, however, able to test the impact of planting diversity on other aspects of forest restoration, such as the speed of plant growth, so further research is required in this area.

Habitat Diversity and Reproductive Success

We found no evidence that species richness or diversity of fruiting and flowering trees/shrubs in the nesting habitat of hihi had a positive effect on reproductive success. At the fledging stage, this may be due to the invertebrate-dominated diet of hihi nestlings. Between hatching and fledging, hihi chicks are provisioned with insects by parents, the diversity and abundance of which may not be dependent on the diversity of the habitat (Corcos et al., 2018). In addition, the lack of effect we see on reproductive success may be because the forest on Tiritiri Mātangi is of a sufficient maturity and diversity to provide high nutrient quality food, which is to say that hihi reproductive success is not limited by nutrition in this population. However, it was found in a study on hihi reproductive success on a population in a mature forest reserve that the provision of supplementary sugar water has positive effects on hihi fledgling success (Doerr et al., 2017), casting doubts on that theory. It has been found previously that maternal investment in egg carotenoids can reduce the cost of ectoparasitism in hihi nestlings (Ewen et al., 2009), highlighting the importance of maternal nutrition in this species. A previous experimental study found that nutritional supplementation of hihi nestlings had only short-term effects on survival and that there were complicated interactions between nutritional components and sex (Walker et al., 2013). Therefore, the roles different nutritional compounds play in hihi reproduction are still not well understood. It is also possible that the area surveyed in the habitat surveys is not large enough to cover the foraging range of a female hihi. Female hihi are not know to be territorial like male hihi (Brekke et al., 2015b) and may range into other nest box areas in search of fruiting and flowering trees. It is thought that female hihi range across the island, as they are observed using feeders far away from their nest boxes (Brekke, personal communication, 2022). Additional comparative studies including multiple populations of hihi, taking larger scale habitat characteristics into account, may reveal population-level differences in reproductive success driven by habitat.

Studies of the effect of adult nutritional intake on reproduction in wild bird species are few in number (Assersohn et al., 2021). However, the use of a new nutritionally rich food supplement was found to increase clutch size in the endangered kākāpō, highlighting the importance of nutritional content over energy content for egg production in this species (Houston et al., 2007). A number of studies on insects have also shown that higher nutritional quality of food positively impacts fecundity, hatching success and overall reproductive success (Geister et al., 2008; Harvey et al., 2012; Wittmeyer et al., 2001). The importance of nutrition (Cooper et al., 2005), such as trace minerals (Amen and Al-Daraji, 2011) carotenoids (Mangiagalli et al., 2010) and vitamins (Nawab et al., 2019), for improved male and female fertility is well established in poultry species.

Another possible explanation for the lack of effect of diversity and richness of fruiting and flowering trees on reproductive success is the availability of high energy supplementary food. Although there is evidence that the availability of energy rich food is not as important for reproductive success as nutritional quality (Geister et al., 2008; Houston et al., 2007), it is possible that the supplementary feeding is compensating for any lack of natural nectar availability in nesting habitats. Previous studies on hihi have shown that the use of supplementary sugar feeding increases clutch size (Castro et al., 2003) and significantly increases fledgling success and reduces whole nest failures even in a mature forest reserve (Doerr et al., 2017). In addition, fledging success of hihi chicks has been shown to be positively impacted by habitat complexity, including the complexity of nectar producing trees, in areas with no supplementary feeding (Makan et al., 2014). However, it has also been found the condition and survival of adult hihi is not affected by supplementary sugar water feeding (Armstrong and Perrott, 2000), again highlighting the complex role of energy resources and nutrition in life history. It is possible that the constant provision of low-quality supplementary food encourages a reliance on this energy source, thereby decreasing the birds' use of natural nectar resources. An experimental study found that the provision of supplementary food decreased flower visitation rates by nectar feeding birds, despite increasing their abundance in the area (Du Plessis et al., 2021). A more far-reaching effect of supplementary feeding is reduced pollination rates of bird visited flower species (Avalos et al., 2012) which can lead to fewer seeds produced (Arizmendi et al., 2007). This highlights the caution with which supplementary feeding should be used in the maintenance of populations of nectivorous birds in regenerating forest.

Habitat Microclimate, Incubation Behaviour and Reproductive Success

The microclimate of the nest habitat temperature was found to be consistently higher than the atmospheric temperature recorded at the Tiritiri Mātangi weather station. This may be because atmospheric temperature is measured in complete shade in an open space exposed to airflow,

compared to the sheltered, enclosed nature of the forested nest box habitats, which may also be subject to some sun exposure. It is not unusual for the microclimate within forests to differ to the microclimate in open locations; however, this is often with a pattern of cooler maximum and warmer minimum temperatures (De Frenne et al., 2021). The pattern observed here perhaps reflects the immaturity of the Tiritiri Mātangi forest; canopy closure is known to increase with forest age, or time since disturbance (Nilson and Peterson, 1994; Valverde and Silvertown, 1997), therefore immature forest is more likely to have open canopy and increased sun exposure to the forest interior where nest boxes are located.

Nest site microclimates which were consistently warmer than the atmospheric temperature were associated with higher rates of hatching failure and lower numbers of fledglings produced. This is despite plasticity observed in female incubation behaviour in response to the microclimate of her nest site, with females showing less nest attentiveness in warmer microhabitats during early incubation. This result raises a concern for reduced hihi reproductive success and population growth with increasing global temperatures (Pörtner et al., 2022), which is particularly alarming due to the lack of adaptive potential in lay date (de Villemereuil et al., 2019a) and the ineffectiveness of behavioural plasticity in incubation behaviour for improving hatching success and fledging success found in this study. The normal development of embryos requires a narrow temperature window, and variation around this can cause abnormal development and hatching failure (Webb, 1987). The maximum temperatures observed at nest site locations were approximately 28°C, which is below the temperatures expected to cause heat stress and abnormal development in embryos (Webb, 1987), but above physiological/developmental zero temperature (24-27°C) (Beissinger et al., 2005) which can cause development to be initiated before incubation and at sub-optimal temperatures, resulting in abnormal development and potential embryo mortality (Deeming and Ferguson, 1991). Female hihi crucially only start incubating eggs once the whole clutch is laid, meaning that some eggs are subject to ambient temperatures. The egg viability hypothesis predicts that eggs with prolonged exposure to ambient temperatures, i.e. the first eggs of the clutch, are more likely to fail when there is no parental care during laying (Veiga, 1992). There is evidence for this across species (Cooper et al., 2005), but the ambient temperature is thought to be critical to the relevance of this hypothesis; only applying when temperatures exceed developmental zero or are very low (Stoleson, 1999; Stoleson and Beissinger, 1999). The increased hatching failure associated with warmer microclimates found in this study may be due to increased exposure to ambient temperatures above developmental zero prior to the onset of incubation. Further studies in this system which take the laying order of eggs into account may be able to test whether this increased hatching failure constitutes support for the egg-viability hypothesis. High temperatures have more generally been found to reduce hatching success and nestling survival

(Lundblad and Conway, 2021a) and reduce nestling weight gain and delay fledgling in both extreme heat (Cunningham et al., 2013) and in temperate climates (Greño et al., 2008; Salaberria et al., 2014). High temperatures can cause reduced immune function in poultry (Mashaly et al., 2004) and dehydration in nestlings (Salaberria et al., 2014), two factors which may feasibly reduce nestling survival.

Our data do not allow us to separate the direct effects of heat exposure on embryos/chicks from potential indirect effects of microclimate, such as the abundance of invertebrates (Bernaschini et al., 2020; Peng et al., 1992) which are an important component of the hihi diet (Walker et al., 2014). Principle component analysis revealed that warmer nest site microclimates are associated with lower tree species diversity and richness, and a lower number of tall trees. Therefore, perhaps the observed effect of microclimate on hatching success and fledgling numbers reflects a more general effect of habitat quality on reproductive success. Indeed, the lower nest attentiveness observed in early incubation by females nesting in warm microclimates may be driven by a need to travel further for food due to a lower local diversity of food source trees, rather than a direct response to heat. This theory aligns with the "energetic-bottleneck" theory that incubation is energetically constrained and that time spent on or off the eggs is motivated by individual condition rather than temperature control of the egg (Engstrand and Bryant, 1999). There is experimental evidence that incubation behaviour is determined by energetic constraints, from a study that reduced energetic cost of incubation for females by artificially heating nest boxes. The study found that females in artificially heated nest boxes actually spent more time incubating and maintained higher egg temperatures than those in unheated boxes (Ardia et al., 2009). In contrast, it has also been found that females nesting in preferred habitats take longer off-bouts, which has been described as a more "efficient" incubation technique (Germain et al., 2015). The results presented in our study are consistent with that of Germain et al. (2015), who showed that females nesting in higher quality habitat (i.e., cooler microclimate which is also associated with higher tree species diversity), take longer off-bouts, but only during early incubation. Investigation of the microclimate inside the nest and the temperature of eggs and fledglings may reveal whether thermal effects are driving the observed reproductive failure associated with hotter microclimates. Other potential avenues for further research include testing for an association between abundance of invertebrate food sources and habitat characteristics/microclimate and investigating whether female condition can be shaped by the quality of the habitat in close vicinity of the nest box.

CONCLUSION

Here we demonstrate that planting techniques, most importantly the species of most abundant tree planted, can influence future habitat diversity to some extent, but that this resulting habitat diversity

does not directly influence hihi reproductive success. We show that certain planting techniques and current habitat characteristics are associated with nest site microclimate, and that the microclimate can influence the incubation behaviour of female hihi. Despite plasticity in incubation behaviour, warmer nest site microclimates are associated with significantly lower hatching success and numbers of fledglings produced. We suggest that forest restoration projects concentrate their efforts on the selection of high-value tree species, prioritising species that are particularly attractive to seed dispersing birds. Our results indicate that this approach will be more important to future habitat species diversity than the species diversity or richness of trees initially planted, likely due to the importance of animal-driven seed dispersal in natural regeneration. We also suggest that habitat restoration planning considers the goal of restored habitat in a holistic way, taking into account the multitude of habitat characteristics (such as microclimate) that may influence the reproductive success of threatened populations.

CHAPTER 6. GENERAL DISCUSSION

AIMS AND MAIN FINDINGS

Successful reproduction is crucial for individual fitness. Despite this, reproductive failure is common across taxa and individuals vary greatly in their ability to reproduce. This is hardly surprising, considering the range of factors that may impact reproduction, from parental compatibility, maternal quality and individual embryo genetics, to social and ecological environments. The delicate and complex processes of fertilisation and embryonic development are particularly vulnerable to of these factors, yet there has been limited research on how the drivers of these two processes differ, particularly in wild populations. In this thesis, I set out to investigate why females vary in their ability to reproduce, in a threatened bird species, the hihi (*Notiomystis cincta*), which has high rates of hatching failure. I focused primarily on early stages of reproduction, including fertilisation, clutch size, and embryo development, and investigated how these are influenced by genetics, demographics and ecology. The hihi provided an ideal system to study questions about female reproductive failure, due to the wealth of individual level data on reproductive traits available, its genetically resolved pedigree, and it's extremely promiscuous mating system which allows separation of paternal and maternal effects and a focus on female factors. In addition, this species provides an ideal example of a threatened bird species, with a history of genetic bottlenecks, high inbreeding levels and reliance on conservation management, traits which are shared with many threatened species across the world. The results presented in this thesis may therefore inform conservation approaches not only for hihi, but for other threatened bird species facing similar issues with reproductive failure.

In chapter 2, I developed new methods for obtaining DNA from embryos that died during the early stages of development and had undergone post-mortem incubation due to being left in the nest until the clutch hatch date. This post-mortem incubation period is relevant for studies of reproductive failure in wild populations of threatened birds, because disturbance during the incubation period is not desirable. I outlined methods to (i) safely collect unhatched, undeveloped eggs of threatened birds in the field and store/transport them for further analysis, (ii) determine whether those unhatched eggs were fertilised, (iii) obtain cell samples from embryos that died at an early stage of development, (iv) extract as high DNA yields as possible from the cell samples, and (v) apply microsatellite genotyping techniques to these early embryo DNA samples for sex-typing and paternity analysis, revealing for the first time genetic information from the "invisible fraction" of embryos that die before sampling. I found that both the yield of DNA obtained from embryos that die early, and the success of microsatellite genotyping, was lower than when working with blood samples from live birds. The DNA yield and microsatellite success rate also depended on the sample type taken from the unhatched egg, with

samples that included the perivitelline layer yielding more DNA but having higher microsatellite amplification failure rates, possibly due to protein inhibition. I also found that species-specific microsatellite primers had a much higher success rate than cross-species primers, possibly due PCR inhibitors accentuating the lack of complete specificity of cross-species primers.

Chapter 2 addresses a gap in the methodological literature for the assessment of hatching failure, and has clear conservation applications e.g., for assessing inbreeding depression or genetic signatures of early embryonic mortality in threatened species. However, the methods I developed also have application outside the field of conservation, for example in studies concerned with primary sex ratio, sex allocation, and extra-pair paternity. Previous studies in these areas have not considered the "invisible fraction" of early embryo mortality (e.g. Brekke et al., 2010; DuRant et al., 2016; Eiby et al., 2008; English et al., 2014; Whittingham and Dunn, 2001), meaning our understanding of life-history trade-offs, natural selection, and population biology is incomplete. I have provided step-by-step methods for expanding studies of this nature in the future. Further improvements to these methods could involve trialling a DNA clean-up stage to remove potential PCR inhibitors, which may reduce the failure rate of microsatellite amplification and improve genotyping success. In addition, future work could also assess the DNA quality, for example using purity assessments and gel/automated electrophoresis to assess protein contamination, DNA fragment size and DNA degradation. Although I found no association between the length of the microsatellite marker and its failure rate, and therefore little evidence of problems with DNA degradation in these samples, quantification of the molecular weight of the DNA obtained from embryos that have died early would be useful for assessing the potential for further genomic applications, such as whole genome sequencing or other molecular techniques that require long-read DNA.

Employing the methods developed in chapter 2, I then went on investigate patterns of fertilisation failure and embryo mortality in hihi in chapter 3. First, I aimed to determine the relative importance and causes of fertilisation failure and embryo mortality as causes of female reproductive failure. Fertilisation failure was much lower than previously thought, based on macroscopic determination of egg fertility, and early embryo mortality was found to be the primary case of hatching failure. This result aligns with the few previous studies that investigated the prevalence of true fertilisation failure as a cause of hatching failure in threatened bird species, which also found fertilisation failure to be rare (Hemmings et al., 2012; Savage et al., 2022). However, although fertilisation failure was rare, it was still a significant component of hatching failure (on average 15%) and was influenced by two key population

demographic factors: sex ratio and population size. These two factors are intrinsically linked in this population, with years where populations are smaller also exhibiting more skewed sex ratios (the hihi sex ratio is consistently male skewed). Our results corroborate and demonstrate in real time this demographic pattern that has been observed in bird populations before, particularly those which are threatened or have small populations (Dale, 2001; Donald, 2007b). I found that in years when the sex ratio was more male-biased and the population size was smaller, the fertilisation failure rates of eggs was higher. I propose that this is due to the stress caused by high levels of harassment towards females by extra-pair males (Castro et al., 1996a; Ewen et al., 2004; Low, 2005), which may lead to elevated maternal circulating corticosterone levels and a disruption of hormonally controlled aspects of female fertility such as sperm release (Ito et al., 2011). Heightened maternal corticosterone activity due to forced extra-pair copulations may also be the mechanism behind the high rates of extra-pair paternity found in this chapter among embryos that died early in development. An interesting avenue for future research would be to establish a blood sampling regime for females during the breeding season, to assess the physiological impacts of male-biased sex ratios on circulating stress hormone levels. Although the survival of female hihi in this population has been found not to be related to a male bias in the adult sex ratio (Ewen et al., 2011b), the fitness effects of male-biased adult sex ratio found for females in this chapter are likely to have important long-term consequences.

Moving on from the population-level focus in chapter 3, chapter 4 narrowed in on the intrinsic causes of reproductive failure on the between and within individual level. In this chapter, I aimed to investigate how individual females experience reproductive failure variably across their lifespan, and whether this varies between individuals due to their early-life telomere length. I found that early-life telomere length of a female can predict their life-history strategy and senescence in their reproductive traits. Females with shorter early-life telomere length showed increased reproductive investment in early-life, followed by a significant, steeper decline in reproductive output with age, than females with longer early-life telomeres, who did not show any significant senescence in reproductive traits. Studies of the link between telomeres and reproduction across the entire lifespan of an individual are lacking, as highlighted by Sudyka (2019) and this is one of the first studies to examine the life-history and reproductive senescence implications of between individual early-life telomere length variation, alongside a recently published study on Seychelles warblers (*Acrocephalus sechellensis*) which found that the early-life telomere length of individuals did not predict certain measured life-history traits: age at first reproduction or the number of offspring produced at first reproduction (van de Crommenacker et al., 2022). In chapter 4, I used individual level lifelong data on all breeding attempts, and considered multiple reproductive traits, which are known to senesce at different rates (Berger et al., 2015; Hayward et al., 2013). An interesting avenue of future research could involve linking the findings of chapter 3

and chapter 4, by examining the effect of maternal stress on early-life telomere length. Short early-life telomere is often an indicator of poor developmental conditions (i.e. exposure to sibling competition (Cram et al., 2017; Nettle et al., 2013) and stress (Boonekamp et al., 2014a; Herborn et al., 2014; Stier et al., 2020) or parental age/quality (Asghar et al., 2015; Bennett et al., 2021; Marasco et al., 2019). A study of the effect of maternal corticosterone levels on the early-life telomere length of offspring might reveal a physiological mechanism behind variation in early-life telomere length and life-history strategy between individuals. Negative effects of maternal stress on offspring early-life telomere length has been observed in humans (Marchetto et al., 2016) and by experimentally increasing corticosterone levels of yolks (Haussmann et al., 2012). However, a direct link between naturally occurring female stress and early-life telomere length is yet to be investigated.

In chapter 5, I investigated reproductive failure in the context of the wider environment of the study species. In this chapter, I aimed to examine the habitat level effects, specifically the availability and diversity of natural food sources in the nest box habitat and the microclimate of the nest box habitat, on reproductive output and behaviour. The habitat of the study population is mostly restored forest, which is the result of a large-scale planting project in the 1980s. I also aimed to assess the impact that this planting project had on the current habitat, and on the reproductive success of one of the bird species, the hihi, which was reintroduced into the restored forest sanctuary. I found that the planting techniques impacted current habitat characteristics, with the most abundant tree species planted in an area having a significant effect on the current tree species diversity, possibly through attracting seed dispersing bird species. The planting of more diverse tree species is associated with taller trees and a cooler microclimate in the current forest. Hihi reproductive success is significantly impacted by the microclimate of the habitat: nests with warmer microclimates have lower hatching success and lower fledgling production. This is despite plasticity in the incubation behaviour of female hihi in response to microclimate conditions, with females leaving the nest more frequently but for shorter periods (resulting in less time spent off the nest in total during early incubation) when the microclimate is warmer.

To determine whether warmer microclimates are directly impacting hatching and fledgling failure, temperature loggers could be placed inside the nest box during incubation and nestling development. Internal nest box temperature would likely be a better indicator of egg and nestling heat exposure, and could be considered in relation to specific causes of embryo and nestling death such as excessive water loss from eggs (Reid et al., 2000) and nestling dehydration (Salaberria et al., 2014). Future studies could investigate the holistic idea of habitat quality further, by considering additional habitat characteristics

not considered here, such as the geographic characteristics of the nest box location, orientation of the nest box, and the canopy cover (Rambo and North, 2009).

CONSERVATION IMPLICATIONS

The focal species of this thesis, the hihi, is a threatened New Zealand endemic species, classified as vulnerable on the IUCN redlist (BirdLife International, 2016), with an estimated 1,629 – 3,629 adult individuals remaining. As is the case with many threatened New Zealand birds, this species has undergone multiple, drastic genetic bottlenecks, primarily caused by invasive predator introduction and habitat loss. The Tiritiri Mātangi Island population of hihi was started by a reintroduction to the restored forest sanctuary in the early 1990s, and is the most successful of the 6 reintroduced populations in terms of population growth, commonly used as a source population for new reintroduction programmes or to supplement existing populations. Therefore, the success of the hihi population on Tiritiri Mātangi is integral to the success and growth of the species as a whole. In this thesis I highlight some key conservation risks to hihi and will now interpret those findings to make recommendations for conservation management. These findings are likely to have implications for other threatened birds species with similar barriers to reproductive success.

Firstly, I found that early embryo mortality is the primary cause of hatching failure in this population. The causes of early embryo mortality in hihi therefore require further investigation. This may include, for example, identifying specific deleterious alleles causing early embryo mortality and investigating the effect of historic inbreeding events on early embryo mortality using genomic inbreeding measures. In addition, an investigation into the effects of maternal stress hormone levels on early embryo mortality could reveal physiological consequences of heavily male-skewed sex ratios.

Secondly, I showed that small populations of hihi are particularly at risk from heavily male-biased adult sex ratios and that this may have negative impacts on female fertility and reproduction. I therefore recommend that the population demographics of hihi should continue to be closely monitored, with an increased appreciation of the risks of small population size and male-biased sex ratios for reproductive outcomes. The sex ratio of birds used in translocations or reintroductions should also be considered in this light.

Finally, I show that when restoring habitat intended for the establishment of threatened bird populations, including hihi, planting regimes must be carefully considered. Planting diverse tree species and tree species with high nectar and/or fruit loads that attract seed dispersing and pollinating birds is likely to result in higher future habitat quality, including a higher diversity of fruiting and flowering trees, taller canopy and cooler microclimate. My results highlight the importance of a cooler microclimate for

improving hihi reproductive success and suggest that this should be considered when assessing habitat suitability and deciding on nest box placements. The negative effects of a warm microclimate on hihi reproductive success raises serious concerns about the response of hihi to climate change, especially in light of the low potential for genetic adaptation (de Villemereuil et al., 2019b, 2019a; Duntsch et al., 2020) and behavioural plasticity to mitigate these changes. The negative effects of warmer nesting temperatures should be investigated further to confirm whether these effects are due to direct heat exposure or general habitat quality, in order to properly inform future conservation management.

CONCLUSION

Successful reproduction is a common goal of all organisms and is under strong selection. However, at every stage of reproduction, from copulation and fertilisation, to embryonic and postnatal development, a complex combination of optimal conditions and functioning processes are required, which are all subject to a range of stressors and disturbances. In this thesis, I have assessed the drivers of female reproductive failure in a threatened bird species from multiple angles: intrinsic genetic drivers, demographic drivers and ecological drivers. I have also investigated the mechanisms of female reproductive failure on multiple levels: fertilisation failure, reduced clutch size, embryonic mortality throughout the developmental period, and nestling mortality. This thesis has concentrated on investigating the causes of early embryo mortality and fertilisation failure, due to the previous misclassification and underrepresentation of these causes of hatching failure in the literature, and in doing so has highlighted the "invisible fraction" of mortality which has been overlooked in previous studies. The work presented therefore offers novel insights into the ultimate causes of early-stage reproductive failure which have so far gone undetected, and provides new methods to explore this subject further. Overall, the findings of this thesis have important implications for our understanding of evolutionary dynamics in wild populations, as well as direct application to the conservation management of threatened species.

APPENDIX 1

WHY DO EGGS FAIL? CAUSES OF HATCHING FAIURE IN THREATENED POPULATIONS AND CONSEQUENCES FOR CONSERVATION

K. Assersohn, A. F. Marshall, F. Morland, P. Brekke, N. Hemmings

I am joint first author on this paper, which was published 1st February, 2021. I co-wrote this review paper during my PhD programme, and it touches on many issues which are relevant for my thesis, including the major drivers and mechanisms of hatching failure in threatened birds, such as hihi. The paper highlights gaps in the literature discussed and addressed in this thesis, chiefly the distinction between fertilisation failure and early embryo mortality, and the causes of embryo mortality in threatened bird species.

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REVIEW

Why do eggs fail? Causes of hatching failure in threatened populations and consequences for conservation

K. Assersohn[1]*, A. F. Marshall[2,3]*, F. Morland[1,2]*, P. Brekke[2†] & N. Hemmings[1†]

1 Department of Animal & Plant Sciences, University of Sheffield, Sheffield, UK
2 Institute of Zoology, Zoological Society of London, London, UK
3 Department of Genetics, Evolution and Environment, University College London, London, UK

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Correspondence
Dr Nicola Hemmings, Department of Animal & Plant Sciences, University of Sheffield, UK.
Email: n.hemmings@sheffield.ac.uk

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*These authors contributed equally to this work.
†Joint senior authors

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Abstract

Reproductive failure is ubiquitous. However, research on the mechanisms underpinning reproductive failure is still lacking in most species. This gap in our understanding has particularly strong repercussions for threatened species and it hinders our ability to establish effective interventions to improve survival. In this review, we focus on why eggs fail to hatch – one of the most critical and understudied aspects of bird reproduction. We identify the main drivers of hatching failure in threatened populations of birds and the key mechanisms that cause failure at different stages of development inside the egg. We then discuss the importance of management interventions aimed at reducing hatching failure in species of conservation concern. Our review highlights the need for a better understanding of the mechanistic basis of hatching failure in non-model bird species and identifies the methodological tools necessary to achieve this.

Introduction

Around 40% of all bird species have declining populations and 13% are threatened with extinction (BirdLife International, 2020). One of the most common and important problems for threatened bird species is the failure of eggs to hatch. Many eggs are lost as a result of consumption, damage, or disturbance by humans and other animals, but even beyond these losses, some threatened bird populations experience up to 75% hatching failure as a result of indirect anthropogenic or other causes (Jamieson & Ryan, 2000; Ferreira *et al*., 2005). High rates of hatching failure not only influence individual reproductive success but can have strong repercussions for population growth and species recovery (e.g. Jamieson & Ryan, 2000; Ferreira *et al*., 2005; Brekke *et al*., 2010; White *et al*., 2015). However, the drivers of hatching failure are complex and poorly understood. In this review, we highlight the key factors associated with high levels of hatching failure beyond the impacts of predation, damage, desertion and exploitation. We then explore the underlying reproductive problems linked to hatching failure and how these are influenced by ecological and behavioural factors. We argue that a lack of understanding of the mechanistic basis of hatching failure can lead to flawed conclusions about how and why it occurs, with important implications for our understanding of avian ecology and conservation.

Major drivers of hatching failure in threatened birds

Inbreeding depression

Threatened bird populations are generally small and isolated, resulting in high levels of inbreeding and low genetic diversity (Keller & Waller, 2002). Threatened and invasive species that have undergone single or multiple bottleneck and founder events associated with low levels of genetic diversity and high inbreeding, have significantly higher levels of hatching failure (Briskie & Mackintosh, 2004; Heber &

Briskie, 2010). A large number of studies in laboratory systems and non-threatened species across multiple taxa also support that inbreeding depresses hatching success (e.g. Morrow, Arnqvist, & Pitcher, 2002; Spottiswoode & Møller, 2004; Slatyer *et al*., 2012), and a few suggest that genetic recovery (in populations of common species) can improve hatching rates (Ortego *et al.*, 2010, Lindsay *et al*., 2020). Both parental and embryonic inbreeding depresses hatching success (Briskie & Mackintosh, 2004; Heber & Briskie, 2010) and egg viability (Brekke *et al*., 2010; Hemmings, Slate & Birkhead, 2012), but the effects of parental inbreeding on fertilization and embryo development are poorly understood. Although most studies show that inbreeding depresses hatching (e.g. White *et al*., 2015), some have found that parental inbreeding has no effect (e.g. Brekke *et al*., 2010) or, in a few exceptional circumstances, has a positive effect on hatching success (e.g. Weiser *et al*., 2016). Research on the effects of maternal inbreeding on fertility, egg traits and egg number in wild threatened populations is sorely lacking, despite considerable evidence of these effects in non-threatened species (e.g. Keller, 1998).

Inbreeding depression also varies with the development stage (Keller & Waller, 2002). Mutations in early acting genes that are functionally critical are generally thought to be lethal or at least highly detrimental (Keller & Waller, 2002), so the impact of inbreeding depression due to the expression of genetic load should be strongest at early stages of development (Brekke *et al*., 2010). However, our inability to correctly measure the impact of inbreeding at early stages of embryo development in birds (Hemmings, West & Birkhead, 2012) could have repercussions for the management and recovery of threatened species, as vital information on the magnitude and severity of inbreeding depression is unreliable (Grueber *et al*., 2015). This is particularly important in wild populations, where the effects of inbreeding can be exacerbated by changing environmental conditions (Keller & Waller, 2002).

Climate change

Climate change effects on hatching success in small populations are complex and confounded by other factors, such as disturbance, habitat degradation, lack of habitat connectivity, food supply and synchrony in phenology (de Villeremuil *et al.*, 2019). Environmental stress as a result of climate change has, however, been shown to influence a number of different reproductive traits across a wide range of species. Changes in lay-date as a response to climate change, for example, seem to be ubiquitous (Dunn, 2019), and such shifts may have a knock-on influence on hatching success. However, the evidence for this in threatened species is unclear. In the New Zealand Hihi (Stitchbird, *Notiomystis cincta*), for example, lay-date has not shifted to match changes in climate, showing little adaptive potential (de Villeremuil *et al.*, 2019).

Experimental evidence has shown that fertility and egg viability decline with rising temperatures (Lara & Rostagno, 2013). For example, in the threatened Florida Scrub Jay (*Aphelocoma coerulescens*), females with larger clutches that experienced longer periods of pre-incubation exposure to ambient temperature had reduced hatching success (Aldredge, Leclair, & Bowman, 2012). Increased frequency of extreme weather events such as drought has also led to increased hatching failure in the Lesser Prairie Chicken (*Tympanuchus pallidicinctus*), as incubating females are unable to maintain microclimate conditions in the nest, exceeding lethal limits to embryo development (Grisham *et al*., 2016).

In species where anthropogenic incubation disturbance is frequent, the impact of environmental change may also be compounded. In ground-nesting seabirds that breed in highly vulnerable coastal regions, like in the critically endangered Tara iti (Fairy Tern, *Sternula nereis*) human disturbance and extreme weather events are the main drivers of hatching failure (Ferreira *et al*., 2005; Supplementary Material). Rising temperatures have also impacted hatching success and population sex ratios in megapodes, a family of birds in which half of species are at risk of extinction (IUCN, 2020). Megapodes rely on environmental sources of heat for incubation, and high incubation temperatures lead to male-biased mortality in the Australian Brush-turkey (*Alectura lathami*) (Eiby, Wilmer, & Booth, 2008).

Pollution

Pollution is known to interfere with reproductive function and egg viability in birds, and has been associated with widespread adult mortalities, species declines and extinctions (Giesy *et al*., 2003). Over 90,000 chemicals have been released into the environment by humans, and the vast majority of these have not been tested for their effects on humans or wildlife (Patisaul, Fenton, & Aylor, 2018). Pollutants currently known to affect bird reproduction include persistent organic pollutants (particularly chlorinated hydrocarbons such as DDT, PCBs and BFRs), non-halogenated pesticides (e.g. organophosphorus) and metal toxins (e.g. lead, mercury, cadmium, selenium) (Fry, 1995; Giesy *et al*., 2003).

Several hundred anthropogenic pollutants are known to be Endocrine-Disrupting Compounds (EDCs) – substances that interfere with normal hormone function (Borgeest *et al*., 2002; Patisaul, Fenton, & Aylor, 2018). Known EDCs include many organic pollutants and metal toxins, as well as phytoestrogens, PAHs, alkylphenols and phthalate esters (Borgeest *et al*., 2002; Giesy *et al*., 2003). Many EDCs are highly toxic to birds and can have severe effects on fertility, embryo viability and mating behaviour (Fry, 1995; Giesy *et al*., 2003). Embryonic exposure to pollution can occur through maternal deposition into the yolk, with significant implications for egg quality and embryo development (Ottinger *et al*., 2005). When EDCs are passed on to developing embryos, they can reduce egg quality (e.g. through eggshell thinning), disrupt development, cause abnormalities of the reproductive tract, and result in sterility or even embryo death (Leighton, 1993; Fry, 1995). The effects of the organochlorine insecticide DDT and its primary metabolite DDE is a widely known example. DDTs led to the demise of many birds of prey in the 20th century, primarily due to

eggshell thinning and embryo malformations that resulted from exposure. Despite their ban in the 1980s, DDT (and similar pesticides such as MXC) still affects wild bird reproduction today (Borgeest *et al*., 2002; Helander *et al*., 2002, Burnett *et al*., 2013, Hernández *et al*., 2018, van Oosten, 2019). Understanding the consequences of EDCs on avian reproductive physiology and fertility is crucial for conservation efforts; however, the mechanisms underpinning the effects these chemicals have on birds are not fully understood (Giesy *et al*., 2003). We also have little to no knowledge of how the majority of anthropogenic pollutants affect wildlife (Patisaul, Fenton, & Aylor, 2018), and few long-term studies have monitored the effects of EDCs on fertility in wild birds (Bernanke & Köhler, 2009).

Emerging environmental contaminants that are likely to impact avian reproduction and hatching success are those from human and veterinary health care pharmaceuticals (Espín *et al*., 2018). The last two decades have seen a rise in the effects of veterinary pharmaceuticals on avian scavenger populations (Cuthbert *et al*., 2014). Avian scavengers frequently eat medicated dead livestock, either opportunistically or when it is provided during supplementary feeding for conservation purposes (Cuthbert *et al*., 2014; Blanco *et al*., 2017). Fluoroquinolones are one of the most commonly used antimicrobial veterinary drugs for livestock (Margalida & Bogliani, 2014), and the ingestion of fluoroquinolones and other pharmaceuticals can influence embryo development and reduce hatching success (Espín *et al*., 2016; Hruba *et al*., 2019). With livestock carcasses still being commonly used at supplementary feeding stations (Blanco *et al*., 2017), understanding the impact of pharmaceuticals on hatching success in wild birds remains a priority.

Mechanisms of hatching failure

Despite ample evidence that environmental change is driving increased rates of hatching failure in threatened birds, a clear understanding of the mechanistic drivers of egg failure remains elusive. The first step towards resolving this issue is to identify whether hatching failure is due to (1) fertilization failure, or (2) failure of a fertilized egg to develop into a hatched chick (i.e. embryo mortality). These two types of failure can have very different causes, so distinguishing between them is essential if we are to identify (and act upon) the ecological and/or behavioural drivers of hatching failure. Only a handful of studies distinguish between fertilization failure and embryo mortality as causes of reproductive failure in birds, and confusingly, ornithologists often universally refer to any undeveloped eggs as 'infertile' (e.g. Wetton & Parkin, 1991; Morrow, Arnqvist & Pitcher, 2002).

Fertilization failure

Fertilization is the process of sperm and egg pronuclei fusing to form a viable zygote (syngamy). Therefore, an infertile egg is one where the female pronucleus has not fused with a male pronucleus. However, infertility is often used interchangeably to describe both embryo mortality and fertilization failure, possibly due to historic difficulties in distinguishing between the two. Birkhead *et al*. (2008) described a method by which fertilization failure and early embryo mortality can be unequivocally distinguished in unhatched bird eggs, by microscopically examining the egg contents for (1) sperm on the perivitelline layer surrounding the ovum, (2) penetration points in the perivitelline layer indicating the entrance of sperm into the egg, and (3) embryonic cells/tissue in the germinal disc of the ovum, indicating the onset of development (Figure 1). This method has been used on a range of bird species and demonstrated to be relatively robust to egg degradation (Hemmings, West, & Birkhead, 2012), making it well-suited for use on eggs of endangered wild birds, which must typically be left in the nest until after other eggs hatch to eliminate the risk of removing a viable egg. We have developed step-by-step protocols and video demonstrations of this method that are openly available via https://www.zsl.org/practical-resources-for-identifying-the-causes-of-hatching-failure-in-birds. For clarity, we define infertility as fertilization failure (i.e. no syngamy) in this review, and when we talk about the causes of infertility, we refer to any processes contributing to fertilization failure.

In birds, fertilization failure is commonly assumed to be the result of a lack of sperm (Hemmings & Birkhead, 2015) or poor sperm function (Brillard, 1990; Lifjeld *et al*., 2007), that is a problem with the male. However, there is little evidence explicitly linking sperm traits with hatching success in birds. Fertilization failure could also be female-mediated;

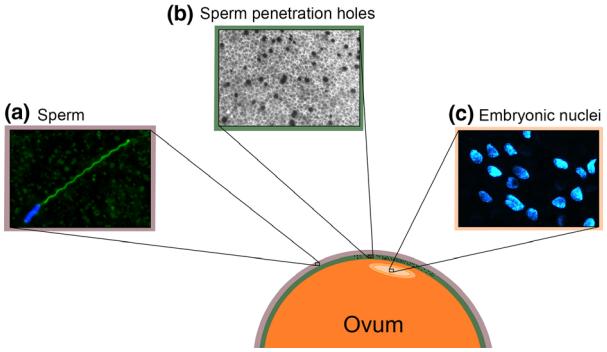

Figure 1 Microscopic examination of undeveloped eggs allows us to distinguish between fertilization failure and embryo mortality as the cause of hatching failure. (a) Zebra Finch (*Taeniopygia guttata*) sperm stained with fluorescent dyes and imaged at 200x magnification. Sperm can be found on the PVL of unhatched eggs several weeks after failure. (b) Penetration points left by sperm that have entered the ovum, imaged with darkfield microscopy at 200× magnification. (c) Embryonic cells after 24 hours incubation, stained with a fluorescent dye and imaged at 400x magnification. Cell division begins approximately 2 hours after fertilization, and by the time the egg is laid, the germinal disc typically contains thousands of embryonic cells. Polyspermy (where multiple sperm enter the ovum) is part of the normal process of fertilization in birds and is required for normal early embryo development. Diagram not to scale [Colour figure can be viewed at zslpublications.onlinelibrary.wiley.com.]

recent research has revealed that females exert far more control over post-copulatory processes than was previously assumed (Pizzari & Birkhead, 2000; Hemmings & Birkhead, 2017), and that variation in female reproductive traits may have a substantial impact on fertilization success. For example, the avian vagina is thought to be highly selective, with only 1% of sperm successfully passing the vagina and entering storage. Therefore, sperm selection in the female reproductive tract (cryptic female choice) can influence which sperm are available during fertilization (Sasanami *et al*., 2013). Ideally, this process would ensure only good quality sperm can fertilize the egg, theoretically improving fertilization success but also potentially enhancing offspring quality. It has been found in other taxa, for example, that cryptic female choice improves both egg fertilization rate and embryo survival (Rosengrave *et al*., 2016). The exact mechanisms of sperm selection are still unclear in birds, but some females are known to preferentially eject the sperm of undesirable males (Pizzari & Birkhead, 2000), and immunological activity within the vagina can influence sperm viability and transport (Bakst, Wishart, & Brillard, 1994). If these processes are too selective, insufficient sperm may reach the site of fertilization (Hemmings & Birkhead, 2015). In domestic birds, fertilization failure has also been shown to be associated with female age (Bramwell *et al*., 1996), female reproductive disorders (Srinivasan *et al*., 2014), aspects of the female's environment (such as diet and stress) (Lewis, 2004; Walzem & Chen, 2014) and genetic factors that may influence the receptivity of the oviduct and/or egg to sperm (Bernier, Spencer, & Swartwood, 1951). Fertilization failure may also result from behavioural incompatibilities between males and females that impede successful courtship and copulation.

Embryo mortality

If an ovum is successfully fertilized, hatching failure may still occur as a result of embryo mortality. Embryo mortality can occur at any stage of development (including prior to oviposition) and for a variety of reasons (Figure 2), although deaths are more common during the early and late stages (Romanoff, 1949). Early embryo mortality (within 72 hours of fertilization) is commonly associated with lethal genetic factors, such as chromosomal abnormalities (Shook, Stephenson, & Biellier, 1971). Genetic perturbations are more likely in inbred individuals, and accordingly, inbreeding has been shown to significantly depress early embryo survival (Hemmings, Slate, & Birkhead, 2012). However, the mechanisms by which inbreeding depresses embryo development remain largely unknown. While most genetic problems manifest early in development, some result in death at a later stage of development, typically due to gross morphological abnormalities (Romanoff, 1949).

Although sperm quality is more typically expected to influence fertilization success, prolonged sperm storage in the male or female reproductive tract before fertilization has been shown to increase the incidence of early embryo mortality (Lodge, Fechheimer, & Jaap, 1971). This effect may be explained by age-related deterioration of sperm and/or a reduction in the number of sperm surviving to reach and penetrate the ovum (Eslick & McDaniel, 1992). Fewer viable sperm may limit the scope for physiological polyspermy, which is essential for normal early embryo development in birds (Hemmings & Birkhead, 2015).

In the early stages of development, embryos are vulnerable to fluctuations in ambient climatic conditions (particularly elevated temperatures) and trans-shell infections during the period between oviposition and incubation onset (Meijerhof, 1992). In many species that lay a clutch of eggs, incubation does not begin until the end of the egg-laying period to ensure synchronous hatching, so eggs laid earlier in the clutch have a longer pre-incubation exposure time. Early embryo mortality also appears to be more common when (1) females are younger (Fairchild *et al*., 2002); (2) females have greater body weight (Coleman & Siegel, 1966) and (3) eggs are small and/or poor quality (including the eggshell) (Lerner *et al*., 1993), which can be the result of poor female condition or stress/disturbance during egg production (Reynard & Savory, 1999).

Mid-development embryo mortality is relatively infrequent, although hyperthermia at this stage can result in developmental arrest or malformations (Christensen, 2001). The nature of these malformations depends on the stage at which the embryo is exposed to high temperatures. For example, around day 3 of incubation, during early brain formation, elevated temperatures can lead to abnormal brain and neural tube development (Alsop, 1919), whereas a week or so into development, high temperatures are more likely to lead to circulatory system failure, for example heart enlargement and cardiac arrest. Romanoff (1949) identified a critical period in the mid-stage development (at 12–14 days of incubation) of Domestic Fowl (*Gallus gallus domesticus*) when embryo mortality can peak if the maternal diet during egg production is deficient in animal protein, vitamins and minerals. Towards the end of development, high or low temperatures, as well as insufficient egg turning, can increase the incidence of embryonic malpositioning, limiting the ease with which the developed chick can successfully break out of the egg.

Although it is relatively easy to identify by eye whether a freshly laid egg is fertilized (Christensen, 2001), the structure of the blastoderm degenerates rapidly following early developmental arrest, particularly in the warm conditions of a nest. Therefore, if an embryo from a wild nest dies within the first 72 hours of development, and several days or weeks elapse before it is collected, the egg can be mistaken as unfertilized upon macroscopic examination (Birkhead *et al*., 2008). Using fluorescence microscopy methods, Hemmings & Evans (2020) found that early embryo deaths were mistaken for fertilization failure in 52% of Blue Tit (*Cyanistes caeruleus*) and 33% of Great Tit (*Parus major*) eggs left in the nest for 2 weeks after hatching. The fact that early embryo mortality can be so easily mistaken for fertilization failure in wild populations is of particular concern, given that the majority of embryo mortalities may happen during these early stages of development (Christensen, 2001).

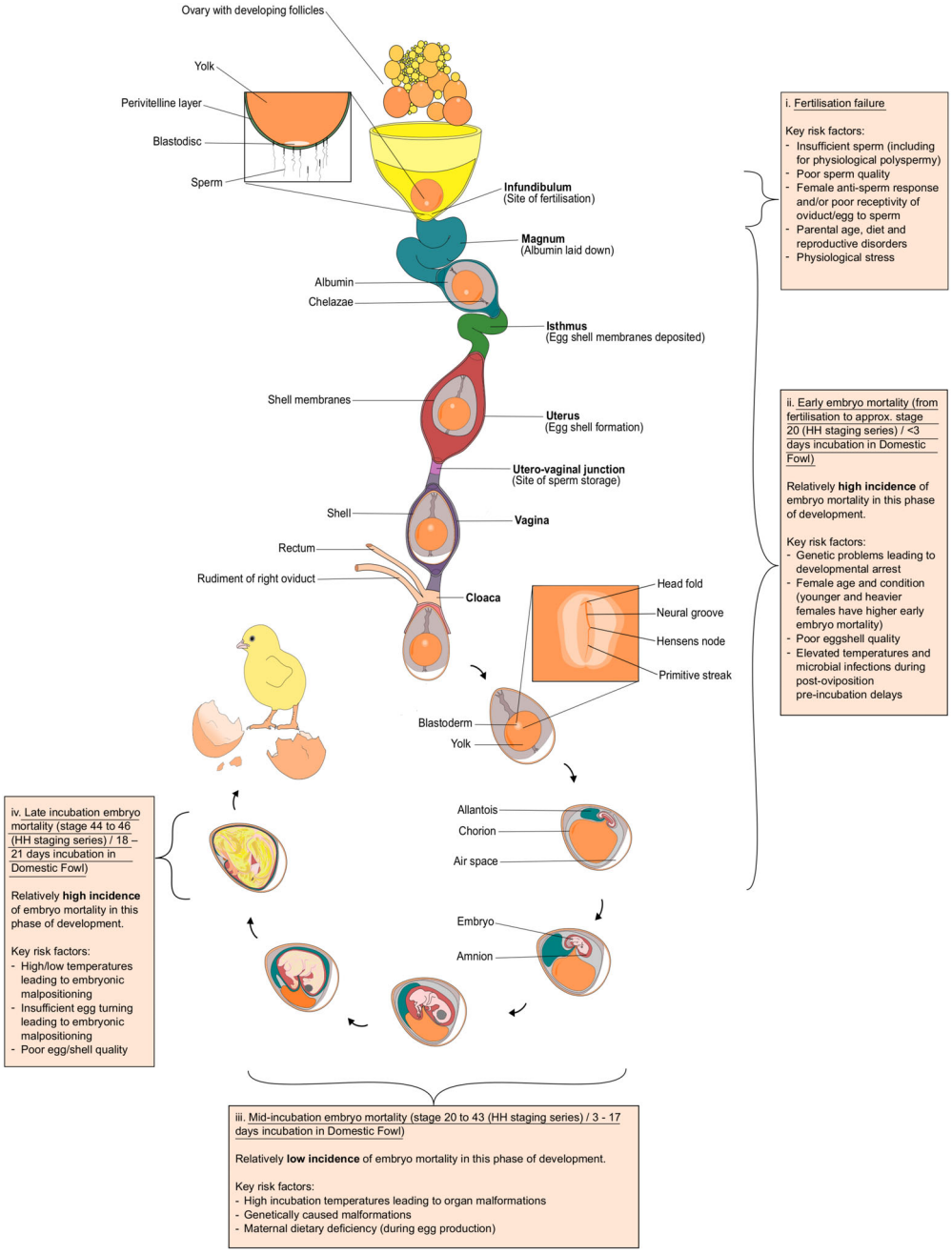

Figure 2 Key risk factors associated with egg failure at different stages of egg formation and embryo development. I. Fertilization failure refers to factors that reduce the likelihood of sperm reaching and penetrating the ovum; II. Early embryo mortality refers to embryo death occurring between fertilization and approximately developmental stage 20 (~3 days incubation in Domestic Fowl). III. Mid-incubation mortality refers to embryo death during developmental stages 20-43 (~3-17 days incubation in Domestic Fowl); IV. Late incubation mortality refers to embryo death during developmental stages 44-46 (~18-21 days incubation in Domestic Fowl). Developmental stages and incubation phases are provided as a guide but vary depending on developmental rate and mode of different species. Embryo death is most common during the early (<3 days incubation) and late (pre-hatch) stages of development. Diagram not to scale [Colour figure can be viewed at zslpublications.onlinelibrary.wiley.com.]

Hatching failure in wild populations

Despite differences in the mechanisms that cause fertilization failure versus embryo mortality, the majority of studies of hatching failure in wild birds consider only whether or not eggs hatch, without investigating the underlying cause of failure and/or the stage at which the embryos died (e.g. Spottiswoode & Møller, 2004). Of those studies that have attempted to look at embryo mortality rates in wild birds, most have assumed undeveloped eggs to be unfertilized and therefore restricted their analyses to analysing mid- and late-term embryos (Jamieson & Ryan, 2000; Brekke *et al*., 2010). Results from the limited number of studies that have distinguished between fertilization failure and early embryo death as causes of hatching failure in wild birds suggest that early embryo mortality is more common (Hemmings & Evans, 2020). Hemmings, West, & Birkhead (2012) microscopically examined eggs classed as 'infertile' from five endangered species and found that only 26% of these eggs were truly unfertilized. If extrapolated to another study such as Jamieson & Ryan (2000), which compares infertility rates in New Zealand endangered species, this suggests that infertility may be strongly overestimated, while the incidence of early embryo mortality is underestimated (Figure 3).

Recognizing the role of early embryo mortality in the hatching failure of wild populations can improve conservation research but is also important for studies in other fields. For example, a study on a wild population of Eurasian Tree Sparrows (*Passer montanus*) – one of the few studies that has accurately discriminated between unfertilized eggs and early embryo mortality – found that the female-biased secondary sex ratio in this population was due to higher mortality of male embryos, most (62%) of which occurred at the early embryo stage (Kato *et al*., 2017). Previous studies have attributed skewed sex ratios to temperature-dependent sex-biased embryo mortality (Eiby, Wilmer, & Booth, 2008) and biased parental investment (Spelt & Pichegru, 2017), but failure to consider individuals that die very early in the population creates a potential bias in these studies.

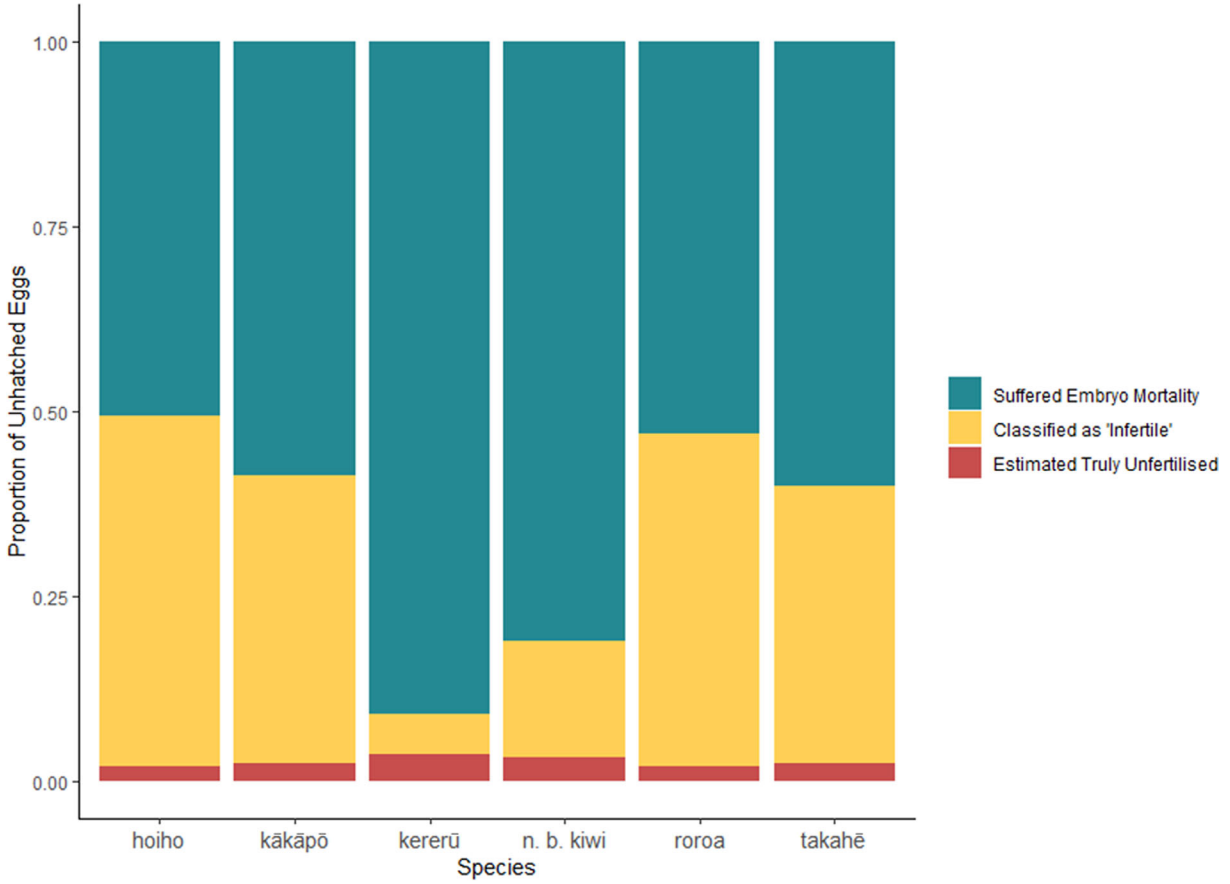

Figure 3 The proportion of failed eggs classified as infertile in six endangered bird species (Northern Brown Kiwi (*Apteryx mantelli*), Roroa (Great Spotted Kiwi, *Apteryx haastii*), Kākāpō (*Strigops habroptilus*), Kererū (*Hemiphaga novaeseelandiae*), Takahē (*Porphyrio hochstetteri*), and Hoiho (Yellow-eyed Penguin, *Megadyptes antipodes*; data from Jamieson & Ryan (2000), Table 1), and estimated proportion of truly infertile eggs based on results of Hemmings, West, & Birkhead (2012), who found on average 74% of undeveloped eggs from endangered species that were classed as unfertilized by ornithologists actually showed evidence of fertilization/development [Colour figure can be viewed at zslpublications.onlinelibrary.wiley.com.]

Accurate monitoring of early embryo mortality in wild populations can also provide important and formerly lacking data on extra-pair paternity. The role of extra pair paternity in connection with reproductive success is controversial; there is some evidence that engaging in extra-pair copulations is a female strategy for directly improving fitness via decreased hatching failure (Yuta *et al*., 2018). However, there is opposing evidence for whether within-pair or extra-pair offspring have higher fitness themselves (Sardell *et al*., 2012; Hsu *et al*., 2014) and meta-analyses have come to contradictory conclusions about the correlation between extra-pair paternity and hatching success rates across species (Morrow, Arnqvist, & Pitcher, 2002; Reding, 2015). The paternity assignment of early embryos, previously assumed to be unfertilized eggs, provides more accurate data on paternity and reopens lines of enquiry on this issue – not only on the occurrence of extra-pair paternity, but also the consequences for hatching and survival of extra-pair offspring, their distribution in the laying order, differential parental investment and other related questions.

Conservation management interventions for hatching failure: benefits and challenges

Birds that are bred in captivity for conservation management purposes often suffer notably high levels of hatching failure. However, unlike in the wild, where unhatched eggs tend to be fertilized but suffer early embryo mortality, fertilization failure may be a common cause of hatching failure in captive birds (Hemmings, West, & Birkhead, 2012). While captive birds benefit from medical care, a stable food supply and absence of predation (Mason, 2010), captivity can also be stressful due to frequent human disturbance and handling, unnatural or inadequate environment (e.g. artificial lighting), atypical group sizes, and forced mate pairing or separation (Morgan & Tromborg, 2007; Griffith *et al*., 2017; Fischer & Romero, 2019). Such captive stress could lead to fewer breeding attempts, reduced parental investment/abnormal parental behaviour, and overall, reduced production of successful eggs. For example, in Houbara Bustards (*Chlamydotis [undulata] macqueenii*) hatching failure is higher in captivity than in the wild (Saint Jalme *et al*., 1996), and captive (domesticated) Zebra Finches (*Taeniopygia guttata*) experience around twice the level of hatching failure reported for their wild counterparts (Hemmings, Slate, & Birkhead, 2012). Elevated rates of hatching failure impact the effectiveness of captive-breeding programmes, so it is important that management techniques are implemented to counteract these issues and improve egg hatchability (Supplementary material).

Egg manipulations are commonly used by conservation programmes of endangered birds to improve hatching success and population growth. A common conservation management practice for both captive and wild populations is 'egg pulling' – removal of eggs from nests for artificial incubation and/or fostering. These eggs are then either returned to the wild at a later stage of incubation or hatched in captivity, with the chicks being captive-reared and either retained for breeding programmes or released into the wild as juveniles or adults. Egg pulling may be employed if there are 'surplus' eggs, for example in the Whooping Crane (*Grus americana*) where two eggs are typically laid but only one chick usually survives (Supplementary Material; Kuyt, 1996). Eggs may also be pulled if they are at risk in the nest, for example to prevent incubating Peregrine Falcons (*Falco peregrinus*) from accidentally smashing eggs that were thin-shelled due to DDE contamination (e.g. Burnham *et al.*, 1988). Alternatively, eggs may be removed to encourage the breeding pair to lay a replacement clutch, increasing the overall number of eggs laid in the population (e.g. Wood & Collopy, 1993). However, egg fertility, hatchability and quality have all been shown to decline in replacement clutches (e.g. Jones *et al*., 1994) and forced re-clutching may negatively impact fledgling survival (e.g. Parmley *et al*., 2015) and/or future reproductive success of adults (e.g. Wood & Collopy, 1993). This indicates that egg pulling can have important costs as well as benefits for breeding management, and accordingly, some conservation protocols enforce limitations on the number of replacement clutches that can be laid in a season.

Although removing eggs for artificial incubation is generally considered the safest option for conservation managers, artificially incubated eggs often experience lower hatching success than eggs left in the wild, and therefore represent an important source of mortality in captive-breeding programmes (e.g. Sancha *et al*., 2004). While many aspects of the artificial incubation environment can be tightly controlled, what is lacking is the fine-scale control and adjustments that may be provided by parent birds throughout development as they respond to the developing embryo's requirements (Tong *et al*., 2013). Artificial incubation will also inevitably lack factors that natural nesting environments and parental incubation provide such as growth of beneficial microbes, periodic cooling, natural turning patterns and stimulation provided by parental and sibling vocalizations (Deeming, 2002). One technique that has been shown to increase the hatchability of wild eggs in artificial incubation is delaying the removal of eggs from the nest (e.g. Burnham, 1983), suggesting that allowing a small amount of early incubation by the parent may be beneficial. However, delayed egg removal can also increase predation risk and exposure to adverse climatic conditions and may reduce the likelihood that parents will lay a replacement egg or clutch, which is often the primary objective of this intervention.

Artificial incubation is also used for eggs produced by birds in captive breeding programmes. Hatching success of captive-laid eggs under artificial incubation is often lower than that of wild-laid eggs (e.g. Burnham, 1983), but this may reflect lower rates of fertilization success in captive birds. Indeed, wild-laid Whooping Crane eggs (Supplementary Material) had greater hatching success than captive-laid eggs even when they were both naturally incubated (by foster parents; Kuyt, 1996). Differences between wild and captive-laid eggs may also be a consequence of the presence or absence of pre-collection incubation, respectively (see above), and/or health problems affecting egg/embryo quality in the captive

population. For example, a sudden increase in late-incubation embryo deaths in captive Kakī (Black Stilt, *Himantopus novaezelandiae*) eggs, but not wild-laid eggs subjected to the same artificial incubation environment, indicated differences in egg quality between captive and wild birds. This was subsequently shown to be the result of iodine deficiency in the captive population (Sancha *et al*., 2004).

A major risk to eggs during the incubation period are trans-shell microbial infections, which can lead to embryo mortality. Parental incubation has been shown to limit bacterial and fungal growth on eggshells relative to unincubated eggs, reducing the risk of infection and increasing hatching success (Cook *et al*., 2005). However, the precise mechanisms underpinning this effect remain unclear. In the absence of parental incubation, cleaning eggs with alcohol has been shown to reduce trans-shell infection and increase hatching success (Cook *et al*., 2005), and egg-cleaning before artificial incubation is common practice within some areas of the poultry industry (Rideout, 2012). However, support for egg-cleaning is mixed, since resulting damage to the shell cuticle could potentially reduce natural barriers against microorganisms (Baggott & Graeme-Cook, 2002).

Fostering of eggs is sometimes used in breeding management practices in combination with, or as an alternative to, artificial incubation. A study comparing parentally incubated, fostered and artificially incubated wild-laid Killdeer (*Charadrius vociferous*) eggs showed that hatching success was similar after parental incubation and fostering (in this case by another species, Spotted Sandpipers *Actitus macularia*) (Powell & Cuthbert, 1993). Artificial incubation resulted in significantly higher hatching success than both parental incubation and fostering, but this was primarily because a large proportion of wild nests were predated rather than due to failure in artificial incubation. While fostering by both conspecifics and heterospecific parents has been successful (e.g. Byrd *et al*., 1984), fostering by heterospecifics carries the risk of incorrect imprinting (e.g. Butler & Merton, 1992) and inter-species disease transfer (e.g. Snyder et al., 1985). Hence, fostering by conspecifics is generally preferred where possible.

Conditions in captivity may influence reproductive behaviour, ultimately resulting in decreased fertilization success (Saint Jalme *et al*., 1996; Hemmings, West, & Birkhead, 2012). Captive birds are often kept in pairs or small groups, limiting the potential for mate choice and extra-pair copulations, and potentially leading to a higher incidence of incestuous and/or same-sex pairings than found in the wild (Driscoll, 2008). Commonly in captive breeding programmes, unsuccessful individuals are separated and provided with alternative mates, a technique which may also be used to manage genetic diversity. However, multiple studies in both wild and captive populations have indicated that birds that retain their mates over multiple seasons have greater reproductive success than those that 'divorce' and change mate (e.g. Yamamoto *et al*., 1989), and several studies of captive birds (albeit with small sample sizes) have shown that reproductive success – particularly fertilization success – improves with increasing time spent as a pair (e.g. Brosset, 1981). Hence, there is a trade-off in terms of management decisions between allowing sufficient time for captive pairs or groups to establish normal socio-sexual behaviour, gain experience and improve their reproductive success, and avoiding the risk of wasted mating opportunities. This is particularly important in seasonal and/or unpredictable breeders.

To address issues with reproductive behaviour and timing, artificial insemination has been introduced in many captive populations, and in the special case of the free-living Kākāpō (*Strigops habroptilus*) (Supplementary Material). Artificial insemination can compensate for a lack of copulation, an absence of extra-pair copulations, and/or unsuitable or unsuccessful pairings. For example, in a captive-bred population of Houbara Bustard, 'natural breeding' scenarios yielded 20-50% fertility (in this study, fertility refers to eggs that showed an obvious sign of embryonic development), while artificial insemination achieved up to 85% fertility (Saint Jalme *et al*., 1996). It has been shown that even when fertility levels are high (80-85%) they can be improved by an additional 5-10% through artificial insemination, with the best results being obtained from repeated deep inseminations of a large volume of semen as soon as possible after collection (Gee *et al*., 2004). The application of artificial insemination can be expanded through the use of frozen semen, which removes the temporal and spatial constraints imposed by the decline in sperm function over time post-ejaculation (Lodge, Fechheimer, & Jaap, 1971). However, the use of frozen semen results in lower egg fertilization rates (e.g. Parks & Hardaswick, 1987; Gee *et al*., 2004) and improvements in cryopreservation methods are essential to make this a viable management approach. Despite its benefits, artificial insemination is labour intensive and invasive, hence many programmes continue to focus on improving fertilization success in natural breeding.

Conclusions and guidelines for best practice

Hatching failure is one of the most crucial factors limiting the recovery of threatened bird populations. Here we have highlighted the key drivers of hatching failure and explored how these might differ between wild and managed/captive populations. Our overarching conclusion is that a better understanding of the mechanistic causes of hatching failure is required in order to ensure conservation management interventions are appropriately targeted. Distinguishing accurately between infertility and early embryo death and the rates at which each of these occur will enable bird conservation managers to adapt their approaches and provide more tailored solutions to egg failure. We have developed a set of openly available protocols and video demonstrations to facilitate the integration of egg examination techniques into conservation management (https://www.zsl.org/practical-resources-for-identifying-the-causes-of-hatching-failure-in-birds), and we advocate the use of these methods for the following reasons. First, these techniques allow us to establish if sufficient sperm are reaching eggs. The absence of sperm on the perivitelline layer of unhatched eggs strongly indicates a male

sperm production or copulation problem, facilitating quick intervention. In captivity, for example, unsuccessful males (no sperm reaching eggs) can be removed to allow the female to form a new pair bond with a male of proven fertility within the same breeding season. Alternatively, females could be artificially inseminated with sperm from a proven male. Either approach would maximize the production of fertilized eggs within a season. Second, the identification of male fertility status from the presence/absence of sperm on eggs provides crucial information for translocation decisions – inclusion of an infertile male could potentially threaten the successful establishment of a small founder population. Third, if undeveloped eggs are fertilized but suffer early embryo death, management interventions can shift focus to incubation conditions and maternal health/nutrition to ensure optimal conditions for early embryo survival, as well as considering the genetic compatibility of the parents. Methods for examining unhatched eggs have so far been used to inform the management of a small number of captive and managed bird populations (e.g. Hemmings, West, & Birkhead, 2012; Croyle, Durrant, & Jensen, 2015). We hope that conservation practitioners will make use of the open resources now available (https://www.zsl.org/practical-resources-for-identifying-the-causes-of-hatching-failure-in-birds), and that examinations of unhatched eggs will be widely adopted in the future to maximize our understanding of avian reproductive failure.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

File S1. Three examples of threatened bird populations under intensive management with high levels of hatching failure.

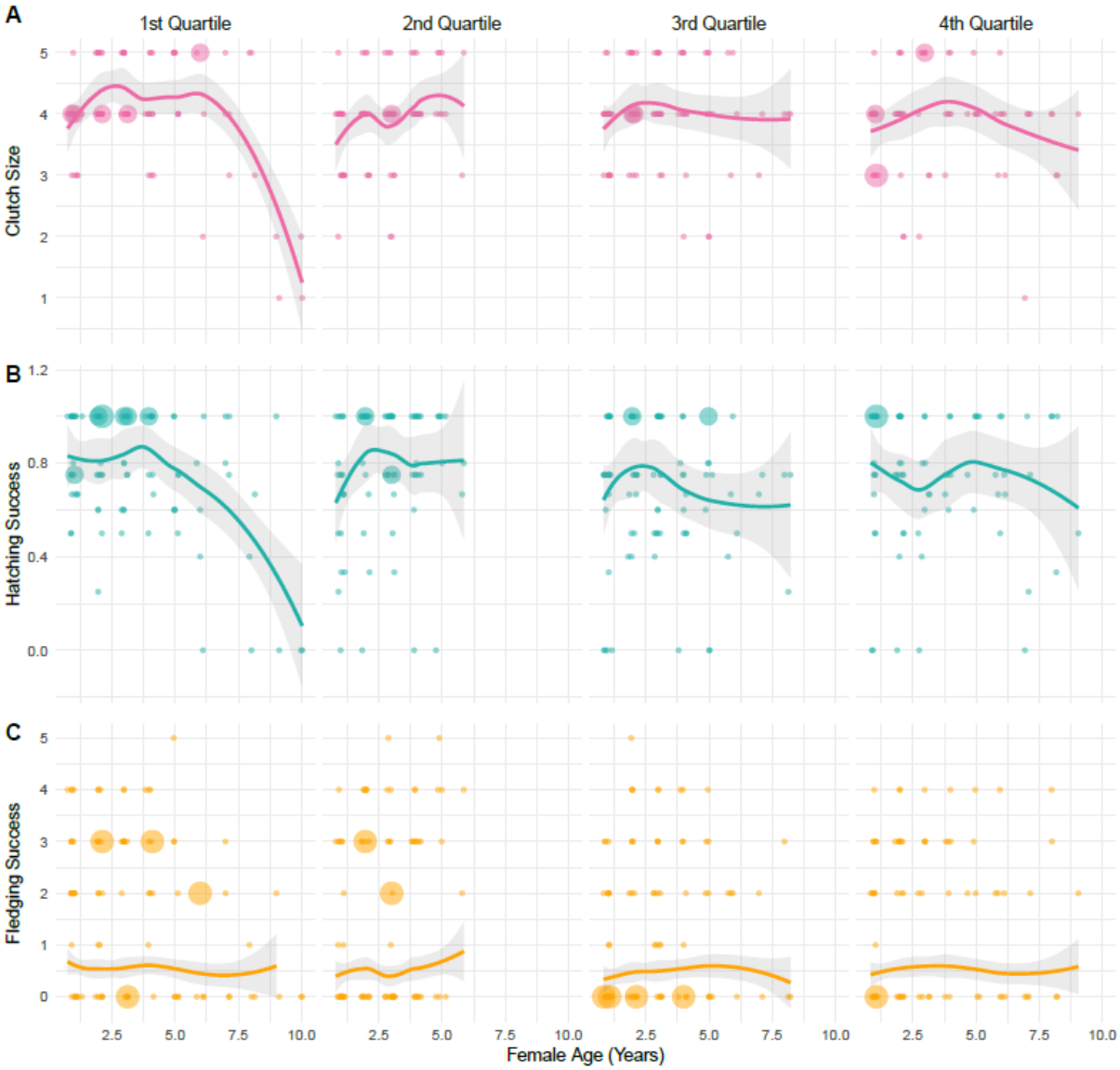
APPENDIX 2

Table S1. AIC values were used to select the best model for the effect of female age and female ID on clutch size, the best fitting model included a quadratic relationship between age and clutch size. Linear, quadratic and breakpoint models were tested but breakpoint models failed to converge. All models had a Conway-Maxwell Poisson or Poisson error distribution.

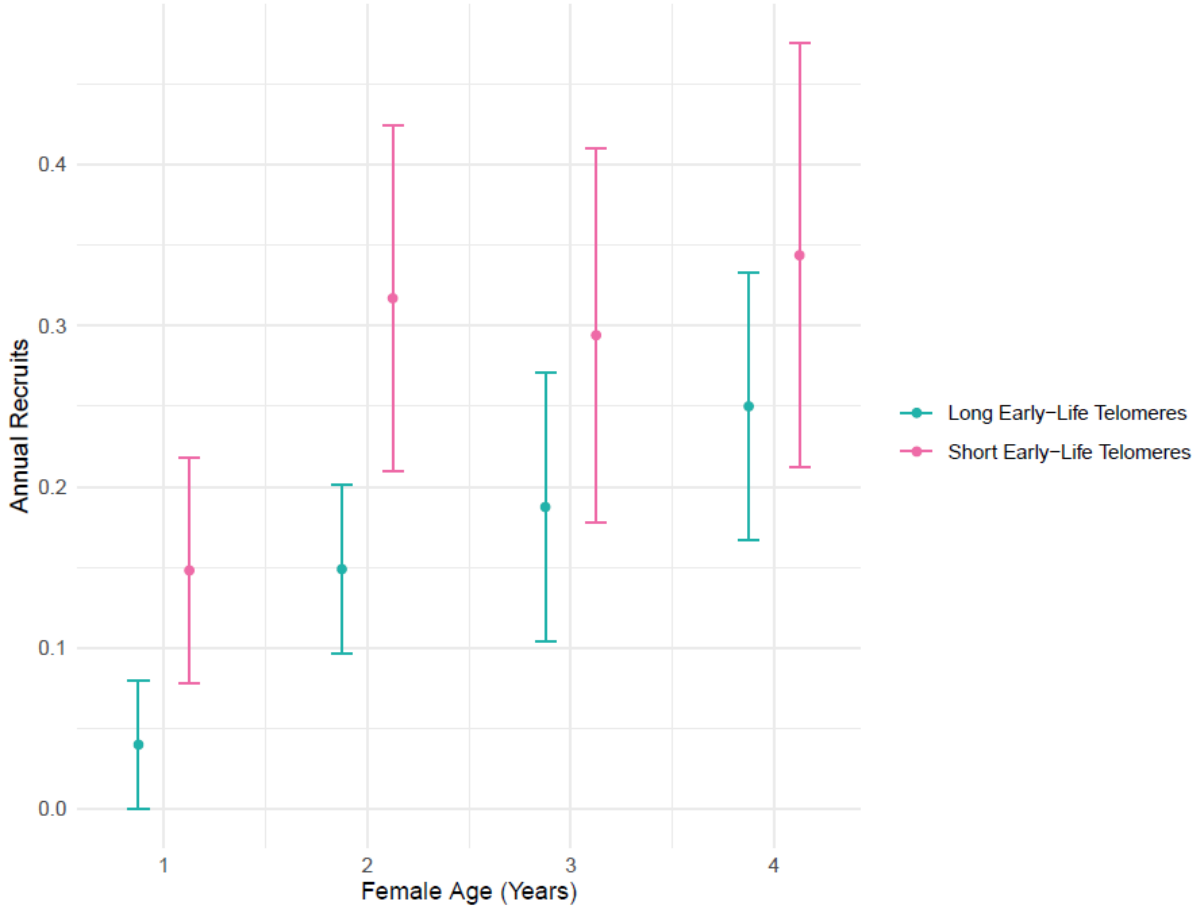
Model formula for clutch size	AIC	ΔAIC
clutch size ~ age^2 * TL + age + lifespan + (1 | year) + (1 | ID) + (1 | lay date)	787.9	0
clutch size ~ age^2 + TL * age + lifespan + (1 | year) + (1 | ID) + (1 | lay date)	789.2	1.3
clutch size ~ age^2 * TL * age + lifespan + (1 | year) + (1 | ID) + (1 | lay date)	789.2	1.3
clutch size ~ age^2 + TL + age + lifespan + (1 | year) + (1 | ID) + (1 | lay date)	794.6	6.7

Table S2 . AIC values were used to select the best model for the effect of female age and female early-life telomere length on hatching success. Linear, quadratic and breakpoint models were tested. All models had either betabinomial or binomial error distributions.

Model formula	AIC	ΔAIC
hatching success ~ age^2 * RTL + age + lifespan + (1 | year) + (1 | ID) + (1 | lay date)	883.3	0
hatching success ~ age^2 + RTL * age + lifespan + (1 | year) + (1 | ID) + (1 | lay date)	887.0	3.7
hatching success ~ age^2 + RTL + age + lifespan + (1 | year) + (1 | ID) + (1 | lay date)	889.8	6.5
hatching success ~ age (with breakpoints at 2 and 5 years)*ΔRTL + lifespan + (1 | year) + (1 | ID) + (1 | lay date)	902.8	19.5
hatching success ~ age (with breakpoint at 0.42 years)*ΔRTL+ lifespan + (1 | year) + (1 | ID) + (1 | lay date)	911.9	28.6



Supplementary Figure 1. The rate of decline in A) clutch size and B) hatching success with female age varies with early-life relative telomere length. The plot is divided here into quartiles of early-life telomere length: 1st quartile = min (-3.17) to 1st quartile (-0.75), 2nd quartile = 1st quartile to the median (0.127), 3rd quartile = median to 3rd quartile (0.81) and 4thquartile = 3rd quartile to the max early-life telomere length (3.1). Females with very short telomeres (in the lowest quartile of the range) show faster rates of senescence in A) clutch size and B) hatching success. For the first quartile appears to be the "tipping point" for the effects we see of early-telomere length on reproductive senescence, therefore the 1st quartile was selected at the separation point in the main text figure.



Supplementary Figure 2. The mean annual recruits produced by females with "short" and "long" early-life telomere lengths (classified as below or above the first quartile respectively, due to this length being a significant "tipping point" for an effect on senesence to be observed – see supplementary Figure 1). Females with short early-life telomere length experience a faster senesence in clutch size and hatching success and appear here to have higher reproductive output in early life, particularly the first two breeding seasons. However, this effect was not testable due to the sample size.

APPENDIX 3

Supplementary Table 1. A summary of the two principal components that explain 55% (PC1 = 28% and PC2 27%) of the variation in habitat characteristics and planting regime.

Habitat Characteristic	PC1	PC2
Species richness	-0.27	-0.60
Shannon diversity	-0.16	-0.74
Foliage Dens. 0 - 30cm	0.04	-0.74
30 – 2m	-0.41	-0.55
2 – 4m	-0.35	-0.52
4 – 6m	-0.49	0.22
6 – 8m	0.44	0.25
8 – 10m	0.79	0.01
10 – 12m	0.83	-0.41
> 12m	0.73	-0.53
Microclimate max temp.	-0.56	-0.52
Microclimate min temp.	-0.73	-0.08
Planting diversity	0.74	0.34
Planting species richness	0.79	0.23
Planted *C. robusta*	0.24	-0.62
Planted *C. macrocarpa*	0.69	-0.59
Planted *P. crassifolium*	0.03	-0.92
Planted *S. microphylla*	-0.11	-0.28
Planted *M. excelsa*	0.28	-0.66

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