This is a repository copy of *Demographic drivers of reproductive failure in a threatened bird: insights from a decade of data*.

White Rose Research Online URL for this paper:
https://eprints.whiterose.ac.uk/216405/

Version: Accepted Version

Article:

Morland, F., Ewen, J.G., Santure, A.W. et al. (2 more authors) (2024) Demographic drivers of reproductive failure in a threatened bird: insights from a decade of data. Proceedings of the National Academy of Sciences of the United States of America, 121 (36). e2319104121. ISSN 0027-8424

https://doi.org/10.1073/pnas.2319104121

© 2024 The Authors. Except as otherwise noted, this author-accepted version of a journal article published in Proceedings of the National Academy of Sciences (PNAS) is made available via the University of Sheffield Research Publications and Copyright Policy under the terms of the Creative Commons Attribution 4.0 International License (CC-BY 4.0), which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/

Reuse

This article is distributed under the terms of the Creative Commons Attribution (CC BY) licence. This licence allows you to distribute, remix, tweak, and build upon the work, even commercially, as long as you credit the authors for the original work. More information and the full terms of the licence here: https://creativecommons.org/licenses/

Takedown

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

eprints@whiterose.ac.uk
https://eprints.whiterose.ac.uk/

PNAS

Main Manuscript for

Demographic Drivers of Reproductive Failure in a Threatened Bird: Insights from a Decade of Data

Fay Morland*[1,2,3], John G. Ewen[2], Anna W. Santure[4], Patricia Brekke†[2], Nicola Hemmings†[1]

1. Department of Biosciences, University of Sheffield, 2. Institute of Zoology, Zoological Society of London, 3. Department of Anatomy, University of Otago, 4. School of Biological Sciences, University of Auckland

†Joint senior authors

*Fay Morland

Email: fay.morland@otago.ac.nz

Author Contributions: Fay Morland, Patricia Brekke and Nicola Hemmings conceived the study. John G. Ewen oversaw fieldwork and long-term sampling and data collection with support from Patricia Brekke and Anna Santure. Patricia Brekke developed the microsatellite genotyping and pedigree used. Patricia Brekke and Anna Santure oversaw long term genotyping. Fay Morland carried out lab work (egg dissection, microscopy, and genotyping of early embryos) with support from Nicola Hemmings, Patricia Brekke and Anna Santure. Fay Morland wrote the manuscript with support from all other authors. All authors reviewed and approved the final manuscript.

Competing Interest Statement: We declare that no author has any competing financial, professional, or personal interest that might have influenced this manuscript.

Classification: Biological Sciences, Population Biology

Keywords: infertility, embryo mortality, sex ratio, population demographics, inbreeding

This PDF file includes:

Abstract

Hatching failure affects up to 77% of eggs laid by threatened bird species, yet the true prevalence and drivers of egg fertilisation failure versus embryo mortality as underlying mechanisms of hatching failure are unknown. Here, using ten years of data comprising 4,371 eggs laid by a population of a threatened bird, the hihi (*Notiomystis cincta)*, we investigate the relative importance of infertility and embryo death as drivers of hatching failure and explore population level factors associated with them. We show that, of the 1,438 eggs that failed to hatch (33% of laid eggs) between 2010 – 2020, 83% failed due to embryo mortality, with the majority failing in the early stages of embryonic development. In the most comprehensive estimates of infertility rates in a wild bird population to date, we find that fertilisation failure accounts for around 17% of hatching failure overall and is more prevalent in years where the population is smaller and more male-biased. Male embryos are more likely to die during early development than females, but we find no overall effect of the sex on the successful development of embryos. Offspring fathered by within-pair males have significantly higher inbreeding levels than extra-pair offspring, however we find no effect of inbreeding nor extra-pair paternity on embryo mortality. Accurately distinguishing between infertility and embryo mortality in this study provides new insight into the underlying causes of reproductive failure over a long-term scale and reveals the complex risks of small population sizes to the reproduction of threatened species.

Significance statement

Threatened species are often afflicted with small population sizes, elevated inbreeding levels, and high rates of reproductive failure. In threatened bird species, hatching failure is prevalent and caused by two, frequently conflated, mechanisms: fertilisation failure and embryo mortality. We investigate the impact of inbreeding and population demographics on these two discrete causes of avian reproductive failure in a threatened bird species. We find that embryos are most likely to fail in early development, but not due to inbreeding. We reveal a previously unidentified link between population demographics and fertilisation success; with higher fertilisation failure in years when the population is smaller and has a more male-biased sex ratio, offering fresh insights into the complex reproductive challenges faced by threatened species.

Introduction

Reproductive failure signifies a fitness cost for individuals, particularly for females who often invest more heavily than males in reproduction (1–3). In birds, females incur a large physiological cost from the production of eggs (4), which are formed and laid in response to phenological changes (5, 6) regardless of whether the ovum is fertilised or contains a viable embryo. The number of eggs laid by female birds is limited physiologically and dependent on a trade-off with future condition and survival (7, 8); therefore, unhatched eggs represent not only wasted fitness potential but also wasted energy and resources. Average rates of hatching failure across all birds are around 17% (9), but this is much higher in threatened bird species, with rates of up to 77% in small genetically isolated populations (10) and 89% under harsh conditions (11). Identifying the drivers of hatching failure is therefore an important goal for bird conservation (12).

Understanding 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 ovum fail to fuse, while embryo mortality can occur at any stage of development from fertilisation to hatching. It has been shown that the majority of failed eggs contain embryos that have died very early, and therefore show no macroscopic signs of development (13, 14). 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 and are therefore overlooked in population genetics studies (13, 16). Many previous studies do not attempt to distinguish between embryo mortality and fertilisation failure as causes of hatching failure or fail to do so accurately (17–21). Studies that do address the issue of embryo mortality directly often still fail to include early embryo mortality and assume that all undeveloped eggs (with no obvious sign of an embryo) are unfertilised (22, 23). Those that do attempt to assess egg infertility often make the same assumption, overestimating the incidence of egg infertility (10, 24–28).

The underlying mechanisms leading to either fertilisation failure or embryo mortality are likely very different, therefore it is important to distinguish between them to fully understand the causes of reproductive failure and to better direct conservation efforts. There are many possible mechanisms of fertilisation failure, originating from male fertility disorders (e.g., low sperm numbers (29), azoospermia (30)), female fertility disorders (15, 31, 32), and pair incompatibility (12, 33); all of which could be addressed by artificial insemination (34) or switching incompatible pairs (35). On the other hand, early embryo death may result from a range of extrinsic and intrinsic factors. Extrinsic factors such as stress (36), temperature (37–40), rainfall (41), humidity (42), physical damage and resulting microbial infection (43–45) can be addressed by artificial incubation strategies (46). Intrinsic factors such as chromosomal abnormalities (47) or low-quality male (48–50) or female (51, 52) gametes, may be addressed by tactics such as sperm screening and (53) supplementary feeding (54) respectively. In small populations, hatching success is often negatively impacted by the inbreeding level of the embryo itself (17, 18, 55, 56), of its parents (17, 57), and of the population as a whole (58), although whether the failure of these eggs is mostly due to fertilisation failure or embryo death has not been explicitly tested.

Overlooking the occurrence of early embryo mortality also means that there is a lack of understanding about the individuals that die at this stage of development, such as their sex and paternity, and the factors that may be influencing their mortality, such as inbreeding. For example, extra-pair paternity has been proposed to lead to higher overall hatching success (59), and higher survival probability of individuals (60), but whether this proposed benefit of female promiscuity extends to fertilisation success and/or embryo survival remains to be investigated. In wild bird populations the average adult sex ratio (tertiary sex ratio) is typically male-skewed, and more heavily so in threatened populations, whereas the average sex ratio of hatchlings (secondary sex ratio) is balanced between males and females (61, 62). Previous studies have found embryo mortality to be male biased (22, 63, 64), suggesting that the primary sex ratio (sex ratio at fertilisation) may also be male biased in order for the balanced secondary sex ratio seen in hatchlings to occur. Sex biases in embryo mortality in birds could result from sex-biased inbreeding depression (22, 65) or differences in optimal incubation temperature between the sexes (38). However, 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 may over- or under-estimate the degree of sex bias in embryo mortality.

Sex-biased embryo mortality may be contributing to male-skewed adult sex ratios but, conversely, the strong male bias observed in many small, threatened bird populations (61, 66–68) may also be contributing to hatching failure. Population demographic factors, such as sex ratio, are more susceptible to stochasticity in small populations (69), and 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 (67, 68) or females (70). Theory predicts small population size to negatively impact individual reproductive success via Allee effects (71) and research in experimental systems has proven that the adult sex ratio can affect individual reproductive success (72, 73). One consequence of a biased sex ratio is an increase in sexual competition within the more numerous sex, which has been shown to bring costs for the rarer sex; such as a reduction in female fecundity (74), survival and reproductive success as a direct result of male harassment and aggressive behaviours in populations with male-skewed adult sex ratios (75, 76). Yet little is known about the influence of demographics on reproductive failure in wild populations, and even less is known about the potentially varying effects of adult sex ratio on fertility and embryo mortality.

This study aims to examine patterns of hatching failure and infertility in a long-term monitored population of hihi (*Notiomystis cincta*), which is under conservation management on the island reserve Tiritiri Matangi. The hihi is a threatened, New Zealand endemic passerine bird which, following population decline and subsequent conservation efforts, exists as a remnant population along with numerous reintroduced populations (77). The species therefore has a history of multiple genetic bottlenecks, and all its populations are of small size, with high inbreeding levels (22, 78). Hihi have consistently high rates of hatching failure (33%); however, based on macroscopic examination of unhatched eggs, the infertility rate of these unhatched eggs is unknown. The population on Tiritiri Matangi is small and therefore susceptible to increased stochasticity, skewed sex ratio and lower reproductive success of individuals. Hihi have extremely high levels of extra-pair paternity, with females experiencing forced extra-pair copulations from non-pair males, which become more frequent with an increasingly male biased sex ratio (79). The adult sex ratio in this population has been found to 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 (80). However, the impact on other aspects of female reproduction such the high incidence of hatching failure, has not been investigated.

Using a long-term dataset spanning 10 years, including a genetic pedigree, reproductive data, population demographic estimates, and the accurate fertilisation and developmental status of 1,437 unhatched eggs, we assess the mechanisms underlying the high incidence of hatching failure in this species, and how they are linked to key features shared across many threatened species: small population size, skewed sex ratio and high inbreeding levels. We use microscopic techniques for determining the fertilisation status of eggs, allowing us to accurately determine whether an egg failed to hatch due to fertilisation failure or embryo mortality. This study provides the most accurate estimates of infertility rates in a wild bird population to date and utilises a previously unsampled subset of individuals that die very early during embryo development to assess potential causes of embryo mortality at all stages of development.

Results

Fertilisation failure vs embryo mortality

Of a total of 4,371 eggs recorded as laid in the Tiritiri Matangi population of hihi between 2010 and 2020, 1,470 failed to hatch (33.6%), we were able to identify the fertilisation status and/or developmental stage of 1,437 of these eggs (See Table S3 in the supplementary information for detailed sample sizes), with 33 eggs (2.2% of unhatched eggs) being excluded due to nest abandonment, damaged samples, or missing records. The importance of these 33 missing eggs for the results was tested and deemed to be insignificant (Supplementary Information). To determine the mechanism of hatching failure for each egg, they were macroscopically and/or microscopically examined to assess whether hatching failure was a consequence of fertilisation failure or embryo death (12, 16, 81). Dead embryos were assessed for their stage of development based on the Hamburger and Hamilton's (HH) (82) chick developmental series and classified as early, mid or late failures. The most common cause of hatching failure was embryo mortality at an early stage of development (Figure 1.b), prior to HH stage 8 and within the first two days of hihi embryo development, when 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.

The overall proportion of hihi eggs that failed to hatch due to fertilisation failure across the 10 years was 17.8%, with an annual mean of 16.7% ± 13.3% SD (range: 2.2%-17.6%). These values are significantly lower than estimates of fertilisation failure based on macroscopic assessment (between 26% and 74%; average of 26.3% ± 8.5% SD of unhatched eggs thought to be infertile, average of 46.6% ± 9.3% SD of unhatched eggs with unknown fertilisation status) (Figure 1.a; paired Wilcoxon test, p = 0.003). To determine if fertilisation failure was a significant component of hatching failure, we tested the relationship between annual fertilisation failure rate and annual hatching failure rate of females, controlling for repeated measures of females across years. The annual hatching failure rate of females was significantly related to their annual infertility rate (glmm conditional model (number of hatched eggs): estimate = -0.507, std. error = 0.036, p = <0.001, zero-inflation model (probability of zero hatched eggs): estimate = 0.22, std. error = 0.1, p = <0.05), suggesting that fertilisation failure is a significant contributor to hatching failure.

Patterns in Embryo Mortality

All 133 mid and all 240 late stage failed embryos were genotyped using microsatellites allowing sexing and parentage assignment using a genetic pedigree (83). Genotyping was also attempted on 145 of the 803 early failed embryo samples (16) (i.e., those that were frozen in years 2019 and 2020; we were not able to extract DNA from samples from prior years because they were formalin-fixed).

This resulted in successful paternity analysis of 400 failed embryos, pedigree estimated inbreeding coefficients for 286 failed embryos, and the sexing of 436 failed embryos, allowing us to test the effects of the paternity (extra-pair vs within-pair), sex and inbreeding coefficient of an embryo on its chances of successfully developing and hatching. Early embryo mortality, the most common cause of hatching failure, was male biased (males/females = 1.4, Figure 2.a).

A cumulative link mixed model (clmm) of the effect of inbreeding coefficient (F_{PED}), sex and paternity on embryo outcome found a significant effect of inbreeding coefficient (F_{PED}) on embryo outcome (clmm: estimate = 6.58, std. error = 3.13, p = 0.036); unexpectedly, individuals which hatched had significantly higher inbreeding coefficients than individuals that failed at the early embryonic stage (glmm: estimate = 0.75, std. error: 0.38, p = 0.046). The average inbreeding coefficient (F_{PED}) of failed embryos was 0.19 ± 0.38 SD and of hatchlings was 0.23 ± 0.47 SD. There was no significant interaction between the effects of inbreeding coefficient (F_{PED}) and sex on embryo outcome, suggesting that the inbreeding level and the impact of inbreeding depression in male and female embryos is similar.

There was no significant overall effect of an embryo's sex, or paternity (within-pair versus extra-pair) on its outcome (i.e., early, mid, late embryo mortality or successful hatching) (Figure 2. a, b & c). Individuals fathered by within-pair males had significantly higher inbreeding coefficients than those fathered by extra-pair males (Figure 2.e, glmm: estimate = 0.3, std. error = 0.063, p = <0.001), but this does not seem to have negative impacts on their likelihood of survival to hatching; the rate of extra pair paternity for hatched individuals (60%) and individuals which suffered embryo mortality (58%) was similar (Figure 2.c).

Demographic Effects on Fertilisation Failure and Embryo Mortality

Using estimates of population size and sex ratio calculated from biannual transect surveys (84), we explored the long-term trends in population demographics and their association with overall hatching failure rate, embryo mortality and fertilisation failure rate. The average primary sex ratio (pre-hatch males/females = 1.01 ± 0.16 SD) and secondary sex ratio (hatched males/females = 1.01 ± 0.15 SD) were found to be practically equal, suggesting that although early embryo mortality is sex-biased towards males, mortality across all stages of embryonic development is not sex biased. However, the adult/tertiary sex ratio is consistently male biased in this population (Figure 1.c), with an average sex ratio (males/females) of 1.38 ± 0.27 SD. Population size and sex ratio were found to be significantly correlated in this population (Figure 1.c, r = -0.76, df = 9, p= 0.007) and therefore the effect of these demographics on fertilisation failure and embryo mortality was tested in separate models.

The probability of an egg being unfertilised significantly increased in years when the size of the population was smaller (Figure 1, glmm: estimate = 1.21, std. error = 0.32, p = <0.001) and the sex ratio of the population was more male biased (Figure 2, glmm: estimate = -3.58, std.error = 1.36, p = <0.001). However, the overall hatching success of eggs was not significantly affected by population size (glmm: estimate = 0.29, std. error = 0.23, p=0.21) nor the adult sex ratio (glmm: estimate = -1.07, std. error = 0.87, p=0.21), despite the appearance of a trend towards higher hatching failure in more male-biased populations (Figure 2.c).

Discussion

In this study we aimed to determine the contribution of fertilisation failure and embryo mortality to the high incidence of hatching failure observed in a reintroduced population of an inbred, threatened, endemic New Zealand passerine, the hihi (*Notiomystis cincta*)*,* and how these two modes of hatching failure are linked to key characteristics typical of threatened species: fluctuating population sizes and sex ratios, and high inbreeding levels. We show that 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 sex bias. Fertilisation failure 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 several bird species, which have found infertility to be less prevalent than was previously thought (14, 85) and adds to the body of evidence suggesting that research and conservation efforts should be focused on reducing embryo mortality rates in threatened species. However, the fertilisation failure rate (16.7% of unhatched eggs) was greater than has been recorded for the small number of other wild bird populations in which this has been assessed accurately (13, 14, 85, 86). While the fertilisation failure of eggs is less common in this population than previously thought, fertilisation failure rates are nonetheless substantial, vary across years according to fluctuations in population size and sex ratio, and are significantly correlated with hatching failure rates. Population size and sex ratio are intrinsically linked in this population, with smaller population sizes having a more male-biased adult sex ratio, and when the sex ratio is more extremely male-biased, infertility rates are higher.

The relationship between population size and sex ratio found in this study reflects a common pattern found in birds, particularly those with threatened (61) and/or small, isolated populations (66). Sex biases in small populations are proposed to be driven by lower survival or recruitment of the rarer sex (61, 66). However, the exact mechanism behind these differential mortality rates remains unknown (61, 87). The origin of the consistent male bias in the adult sex ratio of hihi is elusive, as the primary (pre-hatch) and secondary (hatchling) sex ratios in this population are practically equal, as shown by this study and previous research which did not include the sexing of early hihi embryos (88). A reasonable explanation is that post-fledging, or adult mortality is higher in females than males in this population, 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 and others have demonstrated that this is a direct cause of male-biased sex ratios (67, 89, 90).

In years when the sex ratio is extremely male biased, we found that the infertility rate of eggs increases. However, hihi have a 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% in 89% of broods; Brekke et al., 2013). Extra-pair copulations frequently take the form of "forced face-to-face copulations", where multiple males chase, attack, and pin down females, who show evasive and defensive behaviour (79, 92, 93). The nature of these forced copulations suggests that they may be stressful and physiologically costly for females, particularly as they occur at a high frequency (up to 16 times an hour) when the sex ratio of the population is extremely male-biased (79). The results of a previous study show that female survival and fledgling production were not reduced in years of high male to female sex ratio (80) suggesting that forced copulations do not have sufficient physiological cost to influence survival rates. However, there may be hidden physiological costs on female reproductive systems resulting in fertilisation failure. In other species, aggressive male behaviour in male-biased population can negatively affect female survival and reproductive output (75, 76). Physiological stress may contribute to elevated levels of reproductive failure via higher maternal corticosterone levels, which have been shown to lead to lower fertility rates of eggs in an experimental study on quail *(Coturnix japonica* (94). Physiological stress can also disrupt the reproductive hormones (95) that control sperm release from storage (96). However, further study is required to identify the physiological mechanisms linking skewed population sex ratio with fertilisation failure.

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 (22). However, the results of our study show that inbreeding coefficient did not negatively affect the outcome of a developing embryo, and that in fact, embryos that failed in the early embryonic stages had significantly lower inbreeding coefficients than those that hatched**.** These results are inconsistent with those of a previous study on hihi, which found that male embryos that died before hatching had a higher microsatellite marker-based inbreeding coefficient than those which survived (albeit with a smaller sample size of later stage failed embryos than our study), and that male embryos that died were significantly more inbred than female embryos that died (22). Other studies which have investigated the effect of embryo inbreeding coefficient (22, 26) or relatedness of parents (56) on survival probability also find negative effects of inbreeding.

The results regarding inbreeding presented in this study are somewhat limited, both by the use of pedigree estimates of inbreeding and the difficulty of genotyping embryos which fail very early in development for inclusion in that pedigree. Genotyping individuals that die early in embryonic development and have undergone post-death incubation presents technical challenges. Resulting samples are smaller and more degraded than blood samples, so are prone to higher genotyping error rates (16). The mean inbreeding coefficient for hihi in this study ($F_{\text{PED}} = 0.023 \pm 0.045$, 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. Using a genetically reconstructed pedigree does not provide estimates as accurate as genomic measures of inbreeding (97), despite often outperforming microsatellite marker-based methods of inbreeding estimation (98). The inbreeding levels of the population of hihi used in this study, on Tiritiri Matangi, have been found previously to be $f = 0.08 \pm 0.009$ (n = 89, (22)) using microsatellite marker based inbreeding coefficients, and are higher again when estimated using runs of homozygosity from genomic data ($F_{\text{ROH}} = 0.29$; (99)). Quantifying the inbreeding coefficients of embryos that die using genomic measures may allow further insight into why early embryonic failure is so prevalent in this population. However, our finding that individuals with within-pair paternity have higher inbreeding coefficients that individuals with extra-pair paternity confirms the results of a previous study in this population, which finds that social mates are more closely related than they would be with random mating (100).

In summary, we have shown that early embryo mortality is the primary cause of hatching failure in this population of hihi (*Notiomystis cincta*), 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 (85, 101). We find that rates of fertilisation failure are lower than previously assumed, yet infertility is still a significant component of individual level hatching failure and is positively associated with population level hatching failure 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. We hope that these results will stimulate further research into fertilisation failure in species on the brink of extinction, with small population sizes and low breeding success. Population demographics are rarely considered in studies of individual-level fertility, and this is the first study to identify a link between small population size, sex ratio bias and reduced fertilisation rates; therefore, more research is needed to establish if this pattern is species-specific or common across species. Identifying the mechanism of fertilisation failure in this species would also be of value; potential mechanisms include stress-induced disruption of female reproductive hormones, or a lower success rate of mating attempts and/or reduced male fertility in populations with high levels of sexual competition. Importantly, we reveal early embryo mortality to be the largest component of hatching failure in this population, but since we do not find a link between variation in embryo mortality rates and inbreeding depression, further research is needed to understand the causes of early embryo death in this species and others. Our results highlight some of the reproductive challenges faced by threatened species with small population sizes and emphasises that embryo failure and egg infertility may have different underlying causes as mechanisms of reproductive failure.

Materials and Methods

At 21 days old, each individual bird in the Tiritiri Matangi population 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 (for details on pedigree construction see (102)). 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 (84) was used to estimate the population size and sex ratio using the sighting data obtained from these biannual surveys and breeding data.

The population of hihi on Tiritiri Matangi 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 3 days after the last egg in a clutch has hatched (14-day incubation period). For this study, all unhatched eggs were collected from hihi nests between the years 2010 and 2020, excluding 2012. The unhatched eggs were opened to inspect the contents; obvious embryos were approximately staged according to Hamburger & Hamilton (1951) based on the size of the embryo and the developmental state of limbs (see also Hemmings & Birkhead (2015) for passerine staging comparison and estimations of development times). The embryos were 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). All macroscopically visible embryos ("Mid Embryo" and "Late Embryo") had tissue samples taken for later DNA extraction.

Between 2010 and 2017 unhatched eggs without signs of embryonic development upon opening of the egg were stored without the shell in 10% formalin. Between 2018 and 2020, unhatched eggs were candled for signs of development and refrigerated (short term) or frozen (long term) in the shell before analysis. Previously, in routine conservation management practice, unhatched eggs without signs of embryonic development and where the yolk remained intact and separate from the albumen were classified as unfertilised, and eggs without signs of embryonic development where the yolk was addled/rotten were classified as having unknown fertilisation status. To determine the accurate fertilisation status of all eggs without macroscopic signs of embryonic development, all formalin-preserved and refrigerated/frozen eggs were dissected to isolate the i) germinal disc; the site of fertilisation and embryonic development, and ii) the perivitelline layer; a membrane surrounding the yolk which sperm must penetrate to fertilise the germinal disc, and in which sperm become trapped during egg formation. The germinal disc and the perivitelline layer were stained with a fluorescent dye targeting DNA and examined using a UV microscope for the presence of embryonic cells and/or sperm cells as sign of fertilisation. These methods are described in more detail in Assersohn et al., 2021 (see associated open access protocols). 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. Eggs were deemed as unfertilised if there was no evidence of embryonic development or sperm penetration.

Hatchlings (including those that died before individual colour banding at 21 days) 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; (103, 104), following methods in (102) and (16). Paternity analysis was also performed using microsatellite analysis: DNA samples from hatchlings and embryos were genotyped using 18 autosomal microsatellite markers (83). Paternity assignments were performed in Colony (105), 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 the 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 father and mother of both parents, were present in the pedigree.

All data analysis was carried out in R (version 1.4.1717). More detail on the data included in each model described here as well as the model formulae, model type and family can be found in Table S1 (Supplementary Information). To test if fertilisation failure was a significant component of overall hatching failure, we built a generalised linear mixed model using data on the annual reproductive output of females (the number of eggs laid in a breeding season by individual females which were unfertilised, unhatched, or hatched), including repeated measure of females across years. The model tested the effect of the number of unfertilised eggs on the total number of unhatched vs hatched eggs and used a binomial distribution with a zero-inflation component, due to a large number of zeros in the counts of unhatched eggs and unfertilised eggs in the data. The model was run using the "glmmTMB" package in R (106), with year and female ID as random factors. To test whether there were i) sex biases in embryo mortality, ii) paternity biases in embryo mortality, iii) effects of inbreeding coefficient and iv) sex differences in the effect of inbreeding coefficient on embryo outcome we fitted a model of embryo outcome with sex, extra- versus within-pair paternity, inbreeding coefficient, and an interaction between inbreeding coefficient and sex as independent variables. We ran a cumulative link mixed model using the "ordinal" package in R (107), including mother ID, clutch, and year as random effects, and used a logit link function, selected from available link functions using AIC values. A cumulative link mixed model is designed to deal with ordinal/ordered categorical data, making it suited for the dependent variable of embryo outcome; categorised according to the developmental stage reached and therefore ordered by developmental time. The variation in inbreeding coefficients with paternity was tested in using a generalised linear mixed model with inbreeding coefficient as the dependent variable, paternity (within-pair/extra-pair) as the independent variable and mother ID and year as random effects. The model was run with a tweedie distribution to account for zero inflation in the inbreeding coefficient estimates. The effect of sex ratio and population size on egg infertility probability and hatching probability was tested using four general linear mixed models with the binomial dependent variables of hatched/unhatched and fertilised/unfertilised tested against sex ratio and size of the population as independent variables. The four models all included mother ID, clutch and year as random effects and used a binomial distribution due to the binomial response variable. Population size was scaled (/100) to improve model convergence. The results of the models (estimates, confidence intervals and p values of fixed effects) can be found in Table S2 (Supplementary Information).

Acknowledgements

We acknowledge Ngāti Manuhiri as Mana Whenua and Kaitiaki of Te Hauturu-o-Toi and its taonga, including hihi. We would like to thank hihi conservation officer Mhairi McCready and field assistant Leani Oosthuizen for data collection support as well as volunteers, past students and Department of Conservation staff who have contributed to monitoring the Tiritiri Matangi hihi population and to the Hihi Recovery Group, Department of Conservation, and Supporters of Tiritiri Matangi for maintaining such a long-term vision in monitoring and management of this population. Permissions to conduct the research and collect hihi blood samples were granted by the New Zealand Department of Conservation permit numbers 36186-FAU, AK/13939/RES, 53614-FAU and 66751-FAU. We thank Cristina Ariani, Gemma Clucas, Johanna Nielsen, Kang-Wok Kim, Shuqi Wang and Selina Patel for support with microsatellite genotyping, Lucy Queste for assistance with the life history database and Selina Patel and Katherine Assersohn for ensuring safe passage and arrival of hihi eggs from NZ to the UK during the COVID19 pandemic. We thank Elizabeth Parlato for developing the population models and providing the demographic data.

Funding

This research was financially supported by a Dorothy Hodgkin Fellowship (Royal Society; DH160200) awarded to Nicola Hemmings, Fay Morland's PhD studentship, provided by a Royal Society Research Grant (RGF\R1\180101) and by Research England and British Birds Charitable Trust to Patricia Brekke and The Royal Society of New Zealand Marsden Fund to Anna Santure.

Data Availability

The data and code associated with this analysis will be made publicly available at https://github.com/fmorland/hihipopdemographicsandreprodfailure.git upon acceptance of the manuscript. Hihi are of cultural significance to the Indigenous people of Aotearoa New Zealand, the Māori, and are considered a taonga (treasured) species whose whakapapa (genealogy) is intricately tied to that of Māori. For this reason, the hihi pedigree will not be made available publicly but will be made available by reasonable request on the recommendation of Ngāti Manuhiri, the iwi (tribe) that affiliates as kaitiaki (guardians) for hihi. To obtain contact details for Ngāti Manuhiri, please contact Dr Patricia Brekke at patricia.brekke@ioz.ac.uk. This process is necessary in order to maintain current permit stipulations and is in agreement with the Nagoya Protocol and NZ's treaty of partnership between the British Crown and Māori, Te Tiriti o Waitangi.

References

1. T. Clutton-Brock, Sexual Selection in Males and Females. *Science* **318**, 1882–1885 (2007).

2. A. Hayward, J. F. Gillooly, The Cost of Sex: Quantifying Energetic Investment in Gamete Production by Males and Females. *PLOS ONE* **6**, e16557 (2011).

3. R. Trivers, *Parental investment and sexual selection* (Biological Laboratories, Harvard University Cambridge, 1972).

4. J.-Å. Nilsson, L. Råberg, The resting metabolic cost of egg laying and nestling feeding in great tits. *Oecologia* **128**, 187–192 (2001).

5. B. Silverin, *et al.*, Ambient temperature effects on photo induced gonadal cycles and hormonal secretion patterns in Great Tits from three different breeding latitudes. *Horm. Behav.* **54**, 60–68 (2008).

6. S. Zhang, *et al.*, Annual variation in the reproductive hormone and behavior rhythm in a population of the Asian short-toed lark: Can spring temperature influence activation of the HPG axis of wild birds? *Horm. Behav.* **95**, 76–84 (2017).

7. P. Monaghan, R. G. Nager, Why don't birds lay more eggs? *Trends Ecol. Evol.* **12**, 270–274 (1997).

8. L. A. Winder, M. J. P. Simons, T. Burke, The optimal clutch size revisited: separating individual quality from the costs of reproduction. 2022.05.30.493969 (2022).

9. A. F. Marshall, F. Balloux, N. Hemmings, P. Brekke, Systematic review of avian hatching failure and implications for conservation. *Biol. Rev.* **98**, 807–832 (2023).

10. I. G. Jamieson, C. J. Ryan, Increased egg infertility associated with translocating inbred takahe (*Porphyrio hochstetteri*) to island refuges in New Zealand. *Biol. Conserv.* **94**, 107–114 (2000).

11. H. Regehr, W. Montevecchi, Interactive effects of food shortage and predation on breeding failure of Black-legged Kittiwakes: indirect effects of fisheries activities and implications for indicator species. *Mar. Ecol.-Prog. Ser. - MAR ECOL-PROGR SER* **155** (1997).

12. K. Assersohn, A. F. Marshall, F. Morland, P. Brekke, N. Hemmings, Why do eggs fail? Causes of hatching failure in threatened populations and consequences for conservation. *Anim. Conserv.* **24**, 540–551 (2021).

13. N. Hemmings, S. Evans, Unhatched eggs represent the invisible fraction in two wild bird populations. *Biol. Lett.* **16**, 20190763 (2020).

14. J. L. Savage, J. M. S. Crane, K. R. Team, N. Hemmings, Low hatching success in the critically endangered kākāpō is driven by early embryo mortality not infertility. *Anim. Conserv.* **25**, 352–360 (2022).

15. K. Assersohn, P. Brekke, N. Hemmings, Physiological factors influencing female fertility in birds. *R. Soc. Open Sci.* **8**, 202274 (2021).

16. F. Morland, S. Patel, A. W. Santure, P. Brekke, N. Hemmings, Including the Invisible Fraction in Whole Population Studies: a Guide to the Genetic Sampling of Unhatched Bird Eggs. 2023.06.13.544703 (2023).

17. P. J. Cordero, J. M. Aparicio, J. P. Veiga, Parental genetic characteristics and hatching success in the spotless starling, *Sturnus unicolor*. *Anim. Behav.* **67**, 637–642 (2004).

18. C. Spottiswoode, A. P. Møller, Genetic similarity and hatching success in birds. *Proc. R. Soc. Lond. B Biol. Sci.* **271**, 267–272 (2004).

19. S. Hooson, I. G. Jamieson, Variation in breeding success among reintroduced island populations of South Island Saddlebacks *Philesturnus carunculatus carunculatus*. *Ibis* **146**, 417–426 (2004).

20. J. Potti, S. Merino, Causes of Hatching Failure in the Pied Flycatcher. *The Condor* **98**, 328–336 (1996).

21. D. C. Seel, Clutch-Size, Incubation and Hatching Success in the House Sparrow and Tree Sparrow *Passer Spp*. at Oxford. *Ibis* **110**, 270–282 (1968).

22. P. Brekke, P. M. Bennett, J. Wang, N. Pettorelli, J. G. Ewen, Sensitive males: inbreeding depression in an endangered bird. *Proc. R. Soc. Lond. B Biol. Sci.*, rspb20101144 (2010).

23. G. Orłowski, *et al.*, The effect of embryonic development on metal and calcium content in eggs and eggshells in a small passerine. *Ibis* **158**, 144–154 (2016).

24. J. H. Wetton, D. T. Parkin, An association between fertility and cuckoldry in the house sparrow, *Passer domesticus*. *Proc. R. Soc. Lond. B Biol. Sci.* **245**, 227–233 (1997).

25. B. Kempenaers, F. Adriaensen, A. J. V. Noordwijk, A. A. Dhondt, Genetic similarity, inbreeding and hatching failure in blue tits: are unhatched eggs infertile? *Proc R Soc Lond B* **263**, 179–185 (1996).

26. Y. Pei (裴一凡), *et al.*, Proximate Causes of Infertility and Embryo Mortality in Captive Zebra Finches. *Am. Nat.* **196**, 577–596 (2020).

27. R. L. Westemeier, *et al.*, Tracking the long-term decline and recovery of an isolated population. *Science* **282**, 1695–1698 (1998).

28. M. E. Brown, C. L. Keefer, N. Songsasen, Factors affecting captive whooping crane egg fertility: A retrospective analysis. *J. Wildl. Manag.* **83**, 1377–1386 (2019).

29. N. Hemmings, T. R. Birkhead, Polyspermy in birds: sperm numbers and embryo survival. *Proc. R. Soc. B Biol. Sci.* **282**, 20151682 (2015).

30. T. Lifjeld, T. Laskemoen, F. Fossøy, A. Johnsen, O. Kleven, Functional infertility among territorial males in two passerine species, the willow warbler *Phylloscopus trochilus* and the bluethroat *Luscinia svecica*. *J. Avian Biol.* **38**, 267–272 (2007).

31. L. B. Rosen, Avian Reproductive Disorders. *J. Exot. Pet Med.* **21**, 124–131 (2012).

32. P. Srinivasan, G. A. Balasubramaniam, T. G. K. Murthy, P. Balachandran, Prevalence and pathology of oviduct impaction in commercial white leghorn layer chicken in Namakkal region of India. *Vet. World* **7**, 553–558 (2014).

33. T. Pizzari, T. R. Birkhead, Female feral fowl eject sperm of subdominant males. *Nature* **405**, 787–789 (2000).

34. J. M. Blanco, D. E. Wildt, U. Höfle, W. Voelker, A. M. Donoghue, Implementing artificial insemination as an effective tool for ex situ conservation of endangered avian species. *Theriogenology* **71**, 200–213 (2009).

35. R. A. Fox, J. R. Millam, Personality traits of pair members predict pair compatibility and reproductive success in a socially monogamous parrot breeding in captivity. *Zoo Biol.* **33**, 166–172 (2014).

36. N. Khan, R. A. Peters, K. Robert, Compensating for a stressful start: maternal corticosterone, offspring survival, and size at fledging in the Zebra Finch, *Taeniopygia guttata*. *Emu - Austral Ornithol.* **116**, 262–272 (2016).

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

38. Y. A. Eiby, J. W. Wilmer, D. T. Booth, Temperature-dependent sex-biased embryo mortality in a bird. *Proc. R. Soc. Lond. B Biol. Sci.* **275**, 2703–2706 (2008).

39. A. Lourens, H. van den Brand, R. Meijerhof, B. Kemp, Effect of eggshell temperature during incubation on embryo development, hatchability, and posthatch development. *Poult. Sci.* **84**, 914–920 (2005).

40. D. Serrano, J. L. Tella, E. Ursúa, Proximate causes and fitness consequences of hatching failure in lesser kestrels *Falco naumanni*. *J. Avian Biol.* **36**, 242–250 (2005).

41. T. E. Wilcoxen, *et al.*, Parental, social and environmental factors associated with hatching failure in Florida Scrub-Jays *Aphelocoma coerulescens*. *Ibis* **153**, 70–77 (2011).

42. J. J. Bruzual, S. D. Peak, J. Brake, E. D. Peebles, Effects of Relative Humidity During Incubation on Hatchability and Body Weight of Broiler Chicks from Young Breeder Flocks. *Poult. Sci.* **79**, 827–830 (2000).

43. M. I. Cook, S. R. Beissinger, G. A. Toranzos, R. A. Rodriguez, W. J. Arendt, Microbial infection affects egg viability and incubation behavior in a tropical passerine. *Behav. Ecol.* **16**, 30–36 (2005).

44. M. I. Cook, S. R. Beissinger, G. A. Toranzos, R. A. Rodriguez, W. J. Arendt, Trans–shell infection by pathogenic micro–organisms reduces the shelf life of non–incubated bird's eggs: a constraint on the onset of incubation? *Proc. R. Soc. Lond. B Biol. Sci.* **270**, 2233–2240 (2003).

45. J. Pinowski, M. Barkowska, A. H. Kruszewicz, A. G. Kruszewicz, The causes of the mortality of eggs and nestlings of Passer sp. *J. Biosci.* **19**, 441–451 (1994).

46. M. I. Cook, S. R. Beissinger, G. A. Toranzos, W. J. Arendt, Incubation reduces microbial growth on eggshells and the opportunity for trans-shell infection. *Ecol. Lett.* **8**, 532–537 (2005).

47. W. Forstmeier, H. Ellegren, Trisomy and triploidy are sources of embryo mortality in the zebra finch. *Proc. R. Soc. Lond. B Biol. Sci.* **277**, 2655–2660 (2010).

48. P. J. Chenoweth, Influence of the male on embryo quality. *Theriogenology* **68**, 308–315 (2007).

49. J. Parinaud, R. Mieusset, G. Vieitez, B. Labal, G. Richoilley, Influence of sperm parameters on embryo quality. *Fertil. Steril.* **60**, 888–892 (1993).

50. R. G. Saacke, S. Nadir, R. L. Nebel, Relationship of semen quality to sperm transport, fertilization, and embryo quality in ruminants. *Theriogenology* **41**, 45–50 (1994).

51. G. R. McDaniel, D. A. Roland, M. A. Coleman, The Effect of Egg Shell Quality on Hatchability and Embryonic Mortality. *Poult. Sci.* **58**, 10–13 (1979).

52. V. G. Narushin, M. N. Romanov, Egg physical characteristics and hatchability. *Worlds Poult. Sci. J.* **58**, 297–303 (2002).

53. D. Fischer, D. Neumann, A. Wehrend, M. Lierz, Comparison of conventional and computer-assisted semen analysis in cockatiels (*Nymphicus hollandicus*) and evaluation of different insemination dosages for artificial insemination. *Theriogenology* **82**, 613–620 (2014).

54. S. J. Schoech, *et al.*, Food supplementation: A tool to increase reproductive output? A case study in the threatened Florida Scrub-Jay. *Biol. Conserv.* **141**, 162–173 (2008).

55. S. Bensch, D. Hasselquist, T. von Schantz, Genetic Similarity Between Parents Predicts Hatching Failure: Nonincestuous Inbreeding in the Great Reed Warbler? *Evolution* **48**, 317–326 (1994).

56. N. L. Hemmings, J. Slate, T. R. Birkhead, Inbreeding causes early death in a passerine bird. *Nat. Commun.* **3**, 863 (2012).

57. L. F. Keller, Inbreeding and Its Fitness Effects in an Insular Population of Song Sparrows (*melospiza Melodia*). *Evolution* **52**, 240–250 (1998).

58. J. V. Briskie, M. Mackintosh, Hatching failure increases with severity of population bottlenecks in birds. *Proc. Natl. Acad. Sci.* **101**, 558–561 (2004).

59. B. Kempenaers, B. Congdon, P. Boag, R. J. Robertson, Extrapair paternity and egg hatchability in tree swallows: evidence for the genetic compatibility hypothesis? *Behav. Ecol.* **10**, 304–311 (1999).

60. A. Charmantier, J. Blondel, P. Perret, M. M. Lambrechts, Do extra-pair paternities provide genetic benefits for female blue tits *Parus caeruleus*? *J. Avian Biol.* **35**, 524–532 (2004).

61. P. F. Donald, Adult sex ratios in wild bird populations. *Ibis* **149**, 671–692 (2007).

62. E. Mayr, The Sex Ratio in Wild Birds. *Am. Nat.* **73**, 156–179 (1939).

63. M. Cichoń, J. Sendecka, L. Gustafsson, Male-biased sex ratio among unhatched eggs in great tit Parus major, blue tit P. caeruleus and collared flycatcher *Ficedula albicollis*. *J. Avian Biol.* **36**, 386–390 (2005).

64. L. A. Whittingham, P. O. Dunn, Survival of extrapair and within-pair young in tree swallows. *Behav. Ecol.* **12**, 496–500 (2001).

65. R. Vega-Trejo, R. A. de Boer, J. L. Fitzpatrick, A. Kotrschal, Sex-specific inbreeding depression: A meta-analysis. *Ecol. Lett.* **25**, 1009–1026 (2022).

66. S. Dale, Female-biased dispersal, low female recruitment, unpaired males, and the extinction of small and isolated bird populations. *Oikos* **92**, 344–356 (2001).

67. J. Gerlach, S. Le Maitre, Sex ratio variation in small island populations of an endangered bird, the Seychelles Magpie Robin, *Copsychus sechellarurm*. *Ostrich* **72**, 114–117 (2001).

68. J. Nadal, J. Nadal, J. D. Rodriguez-Teijeiro, Red-legged partridge (*Alectoris rufa*) age and sex ratios in declining populations in Huesca (Spain) applied to management. *Rev. Ecol. Terre Vie* **51**, 243–257 (1996).

69. R. Lande, S. Engen, B.-E. Saether, Stochastic population dynamics in ecology and conservation. Oxford University Press. *Oxford* (2003).

70. D. M. Fry, C. K. TooNE, S. M. Speich, R. J. Peard, Sex ratio skew and breeding patterns of gulls: demographic and toxicological considerations. *Stud Avian Biol* **10**, 26–43 (1987).

71. W. C. Allee, Animal Aggregations. *Q. Rev. Biol.* **2**, 367–398 (1927).

72. Y. R. Galimov, A. R. Tukhbatullin, C. R. Haag, A. V. Tchabovsky, Sex ratio effects on reproductive success of male and female Daphnia. *J. Evol. Biol.* **34**, 1817–1826 (2021).

73. G. Ward, G. J. FitzGerald, Effects of sex ratio on male behaviour and reproductive success in a field population of threespine sticklebacks (*Gasterosteus aculeatus*) (*Pisces: Gasterosteidae*). *J. Zool.* **215**, 597–610 (1988).

74. J.-F. Le Galliard, P. S. Fitze, R. Ferrière, J. Clobert, Sex ratio bias, male aggression, and population collapse in lizards. *Proc. Natl. Acad. Sci.* **102**, 18231–18236 (2005).

75. W. G. Gilmartin, L. L. Eberhardt, Status of the Hawaiian monk seal (*Monachus schauinslandi*) population. *Can. J. Zool.* **73**, 1185–1190 (1995).

76. E. Porter, D. Anderson, E. Ferree, Non-breeding Nazca Boobies (*Sula Granti*) Show Social and Sexual Interest in Chicks: Behavioural and Ecological Aspects. *Behaviour* **141**, 959–977 (2004).

77. P. Brekke, P. M. Bennett, A. W. Santure, J. G. Ewen, High genetic diversity in the remnant island population of hihi and the genetic consequences of re-introduction. *Mol. Ecol.* **20**, 29–45 (2011).

78. L. Duntsch, A. Whibley, P. Brekke, J. G. Ewen, A. W. Santure, Genomic data of different resolutions reveal consistent inbreeding estimates but contrasting homozygosity landscapes for the threatened Aotearoa New Zealand hihi. *Mol. Ecol.* **30**, 6006–6020 (2021).

79. J. G. Ewen, D. P. Armstrong, B. Ebert, L. H. Hansen, Extra-pair copulation and paternity defense in the hihi (or stitchbird) *Notiomystis cincta*. *N. Z. J. Ecol.* **28**, 233–240 (2004).

80. J. G. Ewen, R. Thorogood, D. P. Armstrong, Demographic consequences of adult sex ratio in a reintroduced hihi population. *J. Anim. Ecol.* **80**, 448–455 (2011).

81. T. Birkhead, J. Hall, E. Schut, N. Hemmings, Unhatched eggs: methods for discriminating between infertility and early embryo mortality. *Ibis* **150**, 508–517 (2008).

82. V. Hamburger, H. L. Hamilton, A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49–92 (1951).

83. P. Brekke, D. A. Dawson, G. J. Horsburgh, J. G. Ewen, Characterization of microsatellite loci In The hihi *Notiomystis cincta* (*Notiomystidae, Aves*). *Mol. Ecol. Resour.* **9**, 1255–1258 (2009).

84. E. H. Parlato, J. G. Ewen, M. McCready, K. A. Parker, D. P. Armstrong, A modelling framework for integrating reproduction, survival and count data when projecting the fates of threatened populations. *Oecologia* **195**, 627–640 (2021).

85. N. Hemmings, M. West, T. R. Birkhead, Causes of hatching failure in endangered birds. *Biol. Lett.*, rsbl20120655 (2012).

86. T. R. Birkhead, J. P. Veiga, F. Fletcher, Sperm Competition and Unhatched Eggs in the House Sparrow. *J. Avian Biol.* **26**, 343–345 (1995).

87. C. A. Morrison, R. A. Robinson, J. A. Clark, J. A. Gill, Causes and consequences of spatial variation in sex ratios in a declining bird species. *J. Anim. Ecol.* **85**, 1298–1306 (2016).

88. K. J. MacLeod, P. Brekke, W. Tong, J. G. Ewen, R. Thorogood, Do mothers bias offspring sex ratios in carotenoid-rich environments? *Behav. Ecol.* **28**, 131–137 (2017).

89. M. Githiru, L. Lens, Annual Survival and Turnover Rates of an Afrotropical Robin in a Fragmented Forest. *Biodivers. Conserv.* **15**, 3315–3327 (2006).

90. A. Liker, T. Székely, Mortality Costs of Sexual Selection and Parental Care in Natural Populations of Birds. *Evolution* **59**, 890–897 (2005).

91. P. Brekke, P. Cassey, C. Ariani, J. G. Ewen, Evolution of extreme-mating behaviour: patterns of extrapair paternity in a species with forced extrapair copulation. *Behav. Ecol. Sociobiol.* **67**, 963–972 (2013).

92. M. Low, Female resistance and male force: context and patterns of copulation in the New Zealand stitchbird *Notiomystis cincta*. *J. Avian Biol.* **36**, 436–448 (2005).

93. I. Castro, E. O. Minot, R. A. Fordham, T. R. Birkhead, Polygynandry, face-to-face copulation and sperm competition in the Hihi *Notiomystis cincta* (*Aves: Meliphagidae*). *Ibis* **138**, 765–771 (1996).

94. J. B. Schmidt, D. G. Satterlee, S. M. Treese, Maternal corticosterone reduces egg fertility and hatchability and increases the numbers of early dead embryos in eggs laid by quail hens selected for exaggerated adrenocortical stress responsiveness1. *Poult. Sci.* **88**, 1352–1357 (2009).

95. R. P. Novero, M. M. Beck, E. W. Gleaves, A. L. Johnson, J. A. Deshazer, Plasma Progesterone, Luteinizing Hormone Concentrations, and Granulosa Cell Responsiveness in Heat-Stressed Hens1,2. *Poult. Sci.* **70**, 2335–2339 (1991).

96. T. Ito, *et al.*, Progesterone Is a Sperm-Releasing Factor from the Sperm-Storage Tubules in Birds. *Endocrinology* **152**, 3952–3962 (2011).

97. J. Wang, Pedigrees or markers: Which are better in estimating relatedness and inbreeding coefficient? *Theor. Popul. Biol.* **107**, 4–13 (2016).

98. J. Pemberton, Measuring inbreeding depression in the wild: the old ways are the best. *Trends Ecol. Evol.* **19**, 613–615 (2004).

99. L. Duntsch, *et al.*, Genomic signatures of inbreeding depression for a threatened Aotearoa New Zealand passerine. *Mol. Ecol.* **32**, 1893–1907 (2023).

100. P. Brekke, *et al.*, Postcopulatory mechanisms of inbreeding avoidance in the island endemic hihi (*Notiomystis cincta*). *Behav. Ecol.* **23**, 278–284 (2012).

101. J. L. Savage, J. M. S. Crane, K. R. Team, N. Hemmings, Low hatching success in the critically endangered kākāpō (*Strigops habroptilus*) is driven by early embryo mortality not infertility. 2020.09.14.295949 (2020).

102. P. Brekke, J. G. Ewen, G. Clucas, A. W. Santure, Determinants of male floating behaviour and floater reproduction in a threatened population of the hihi (*Notiomystis cincta*). *Evol. Appl.* **8**, 796–806 (2015).

103. D. A. Dawson, Genomic analysis of passerine birds using conserved microsatellite loci. University of Sheffield, Department of Animal and Plant Sciences. (2007).

104. D. A. Dawson, P. Brekke, N. Dos Remedios, G. J. Horsburgh, A marker suitable for sex-typing birds from degraded samples. *Conserv. Genet. Resour.* **7**, 337–343 (2015).

105. O. R. Jones, J. Wang, COLONY: a program for parentage and sibship inference from multilocus genotype data. *Mol. Ecol. Resour.* **10**, 551–555 (2010).

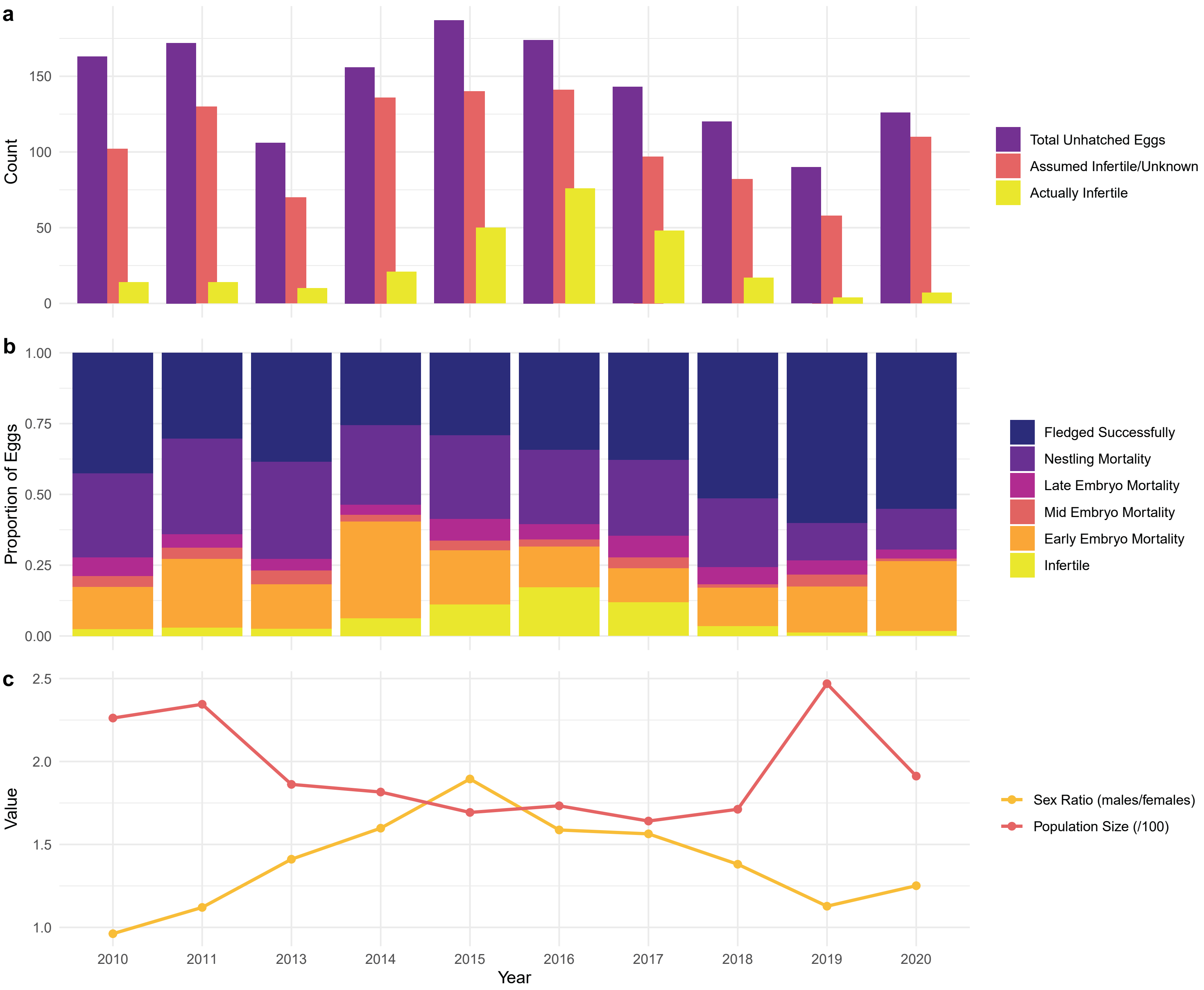
106. M. E. Brooks, *et al.*, glmmTMB Balances Speed and Flexibility Among Packages for Zero-inflated Generalized Linear Mixed Modeling. *R J.* **9**, 378–400 (2017).

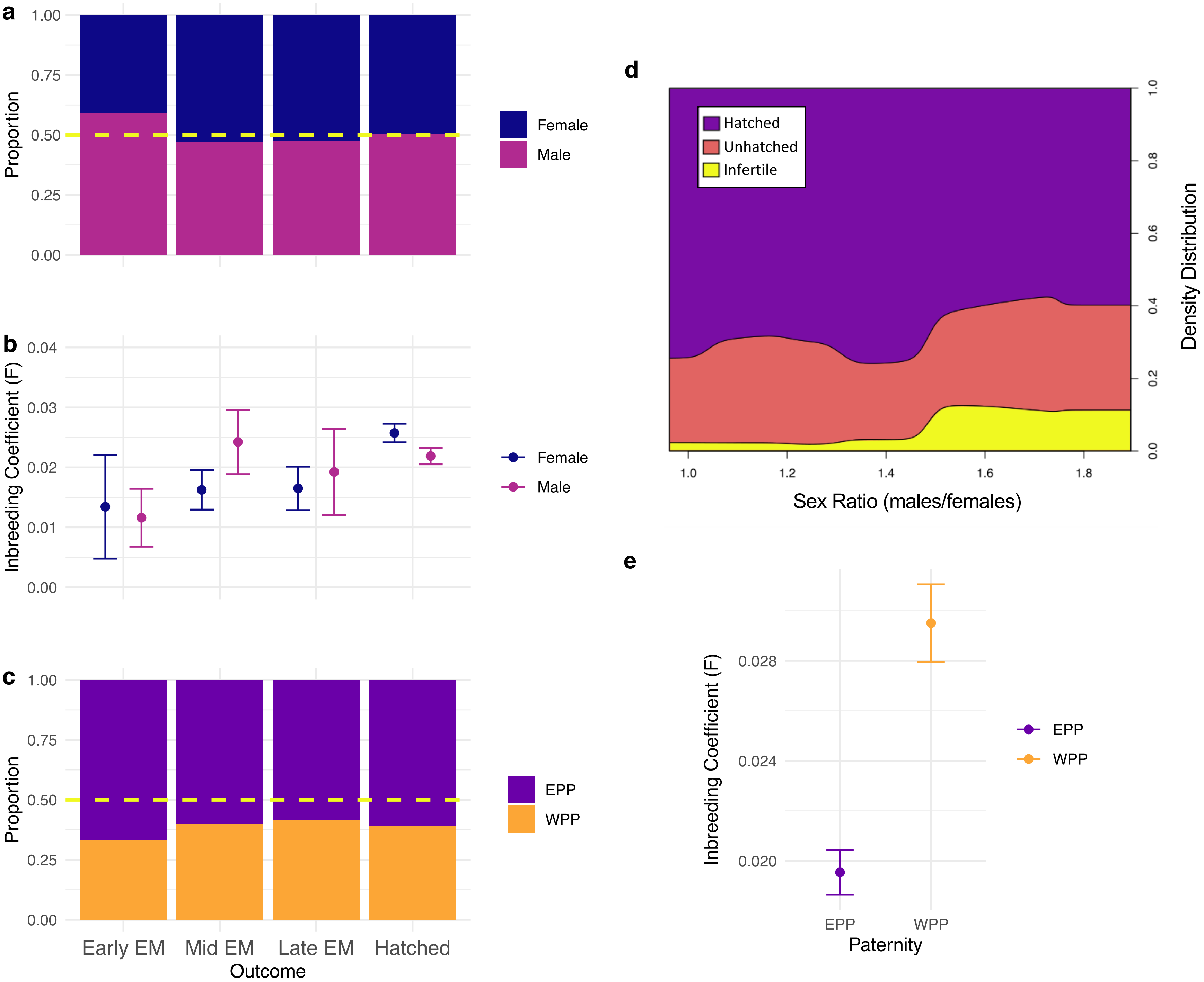
107. R. Haubo, B. Christensen, Cumulative Link Models for Ordinal Regression with the R Package ordinal in (2018) (January 19, 2024).

Figure Captions

Figure 1. How egg outcomes vary with population demographics in the Tiritiri Matangi 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 to the count of eggs previously assumed to be unfertilised / with unknown fertilisation status using macroscopic techniques, and the proportion determined to be truly unfertilised through microscopic analysis. 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 morality 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.

Figure 2. The probability of an egg being unfertilised is significantly increased in years when the sex ratio of males to females is more biased; however, this does not significantly affect overall hatching failure, which is mostly due to embryo mortality. Embryo mortality, at any stage of development, does not seem to be influenced by the sex, paternity of inbreeding level of the embryo. 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. d) The density distribution of the probability that a hihi egg will be unfertilised/infertile, unhatched, or hatched, 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. e) 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. Plotted sample sizes are a) Sex: Early EM = 115, Mid EM =103, Late EM = 218, Hatched = 2,856; b) Inbreeding*Sex: Early EM = 11, Mid EM = 74, Late EM = 164, Hatched = 1,618); c) Paternity: Early EM = 68, Mid EM = 107, Late EM = 220, Hatched = 2,833; e) Inbreeding*Paternity: EPP = 1,396, WPP =888.


a
Count
0
50
100
150
Total Unhatched Eggs
Assumed Infertile/Unknown
Actually Infertile
b
Proportion of Eggs
0.00
0.25
0.50
0.75
1.00
Fledged Successfully
Nestling Mortality
Late Embryo Mortality
Mid Embryo Mortality
Early Embryo Mortality
Infertile
c
Value
1.0
1.5
2.0
2.5
Sex Ratio (males/females)
Population Size (/100)
2010
2011
2013
2014
2015
2016
2017
2018
2019
2020
Year


a
1.00
0.75
0.50
0.25
0.00
Proportion
Female
Male
b
0.04
0.03
0.02
0.01
0.00
Inbreeding Coefficient (F)
Female
Male
c
1.00
0.75
0.50
0.25
0.00
Proportion
EPP
WPP
Early EM
Mid EM
Late EM
Hatched
Outcome
d
Hatched
Unhatched
Infertile
1.0
1.2
1.4
1.6
1.8
Sex Ratio (males/females)
1.0
0.8
0.6
0.4
0.2
0.0
Density Distribution
e
0.028
0.024
0.020
Inbreeding Coefficient (F)
EPP
WPP
EPP
WPP
Paternity

PNAS

Supporting Information for

Demographic Drivers of Reproductive Failure in a Threatened Bird: Insights from a Decade of Data

Fay Morland*[1,2,3], John G. Ewen[2], Anna W. Santure[4], Patricia Brekke†[2], Nicola Hemmings†[1]

1. Department of Biosciences, University of Sheffield, 2. Institute of Zoology, Zoological Society of London, 3. Department of Anatomy, University of Otago, 4. School of Biological Sciences, University of Auckland
†Joint senior authors

Paste Fay Morland
Email: fay.morland@otago.ac.nz

This PDF file includes:

Tables S1 to S3
Supporting text

Table S1. Information on the statistical models performed which have results presented in the main manuscript including: the question that the model aimed to address, information on the dependent variables and fixed effects included in the models, the model formulae including the random effects (denoted by (1|random effect)) and the type of model used.

Question	Dependent Variable	Fixed Effects	Model Formula	Model type
What is the effect of sex, paternity and inbreeding on embryo outcome and does the effect of inbreeding differ between the sexes?	Embryo outcome, ordered categorical variable with the following levels: “failed in early embryo development”, “failed in mid embryo development”, “failed in late embryo development”, “hatched”	Inbreeding coefficient Sex Paternity Interaction between Inbreeding Coefficient and Sex	Embryo outcome ~ inbreeding coefficient * sex + paternity + (1|maternal ID) +(1|year) + (1|clutch)	Cumulative link mixed model for ordered, categorical data
Is fertilisation failure a significant component of hatching failure?	Repeated annual measures of female hatching success, i.e. count of number of eggs hatched by each female each year combined with (cbind function) the count of number of eggs laid but unhatched.	Repeated annual counts of the number of unfertilised eggs laid by each female in each year.	cbind(hatched,unhatched) ~ unfertilised + (1| maternal ID) + (1|year) + zi=~unfertilised	Generalised linear mixed model with zero inflation component, binomial distribtuion
What is the effect of parernity on inbreeding coefficient?	Inbreeding coefficient as determined through pedigree analysis	Paternity; within pair vs extra pair	Inbreeding coefficient ~ paternity + (1| maternal ID) + (1|year)	Generalised linear mixed model with tweedie distribution
What is the effect of population demographics on hatching failure probability?	Binary TRUE/FALSE of whether an egg hatched or not.	Sex ratio of population	Hatched(true/false) ~ sex ratio of populations + (1| maternal ID) + (1|year) + (1|clutch)	Generalised linear mixed model with binomial distribution
		Population size	Hatched(true/false) ~ size of populations + (1| maternal ID) + (1|year) + (1|clutch)	Generalised linear mixed model with binomial distribution
What is the effect of population demographics on fertilisation failure probability?	Binary TRUE/FALSE of whether an egg was fertilised or not	Sex ratio of population	Fertilised(true/false) ~ sex ratio of populations + (1| maternal ID) + (1|year) + (1|clutch)	Generalised linear mixed model with binomial distribution
		Population size	Fertilised(true/false) ~ sex ratio of populations + (1| maternal ID) + (1|year) + (1|clutch)	Generalised linear mixed model with binomial distribution

Table S2. The output of the models presented in Table S1, including the estimates and 95% confidence intervals on the estimates of all fixed effects included in the models and the significance (p value) of those fixed effects.

Model Formula	Effect	Estimate	95% Confidence Intervals on Estimate (+/-)	p
Embryo outcome ~ inbreeding coefficient * sex + paternity + (1|maternal ID) +(1|year) + (1|clutch)	Inbreeding	6.58	0.44, 12.72	0.03
	Sex	0.13	-0.19, 0.44	0.44
	Paternity	-0.04	-0.34, 0.26	0.79
	Inbreeding*sex	-4.86	-12.37, 2.65	0.204
cbind(hatched,unhatched) ~ unfertilised + (1| maternal ID) + (1|year) + zi=~unfertilised	Conditional model: unfertilised	-0.507	-0.58, -0.44	<0.001
	Zero-inflation model: unfertilised	0.2	0.025, 0.42	0.027
Inbreeding coefficient ~ paternity + (1| maternal ID) + (1|year)	Paternity	0.31	0.19, 0.43	<0.001
Hatched(true/false) ~ sex ratio of populations + (1| maternal ID) + (1|year) + (1|clutch)	Sex ratio	-1.07	-2.78, 0.63	0.22
Hatched(true/false) ~ size of populations + (1| maternal ID) + (1|year) + (1|clutch)	Population size	0.29	-0.16, 0.73	0.2
Fertilised(true/false) ~ sex ratio of populations + (1| maternal ID) + (1|year) + (1|clutch)	Sex ratio	-3.58	-6.25, -0.91	0.0085
Fertilised(true/false) ~ size of populations + (1| maternal ID) + (1|year) + (1|clutch)	Population size	1.19	0.56, 1.82	<0.001

Table S3. An account of all eggs laid across the study period and whether sex, paternity and inbreeding level was determined. This is to aid understanding of the sample sizes presented in the main text.

		Data Available for:		
	Total	Sex	Paternity	Inbreeding
Hatched	2,901	2,856	2,835	2,082
Failed in late embryo development	204	218	220	179
Failed in mid embryo development	133	103	108	78
Failed in early embryo development	803	115	72	28
Unfertilised eggs	261			
Abandoned Eggs	12			
Developmental/fertilisation stage unknown due to:				
Sample dried out	4			
Egg laid without yolk	7			
Records missing	10			
Total Unhatched	*1,470*			
Grand Total	***4,371***			

Accounting for "unknown" eggs

To test the consequence of the 33 eggs with undetermined developmental outcome (Table S3), we repeated some key analyses twice: 1) with the 12 abandoned eggs removed and all other 21 eggs classified as infertile, 2) with the 12 abandoned eggs removed and all other 21 eggs classified as failing in early embryo development.

1) When the 12 abandoned eggs removed and all other 21 eggs classified as infertile:
    a. The results of the model of how individual level hatching success is related to individual level fertilisation failure rate did not change in direction or significance but did show a decreased estimate. (glmm conditional model (number of failed eggs): estimate = -0.0091, std. error = 0.00006, p = <0.001, zero-inflation model (probability of zero hatched eggs): estimate = 0.00054, std. error = 0.00022, p = 0.014).
    b. The results of the model of fertilisation rate varying with population sex ratio did not change, in neither the direction of the effect, the significance nor the estimate. (glmm: estimate = -3.16, std error = 1.06, p = 0.003)

2) When the 12 abandoned eggs are removed and all other 21 eggs classified as early embryo mortality:
    a. The results of the model of how individual level hatching success is related to individual level fertilisation failure rate did not change in direction or significance but did show a decreased estimate. (glmm conditional model (number of failed eggs): estimate = -0.0015, std. error = -0.000017, p = <0.001, zero-inflation model (probability of zero hatched eggs): estimate = 0.00053, std. error = 0.00022, p = 0.014).
    b. The results of the model of fertilisation rate varying with population sex ratio did not change, in neither the direction of the effect, the significance nor the estimate. (glmm: estimate = -3.55, std error = 1.3, p = 0.0089)