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Demographic drivers of reproductive failure in a threatened bird: Insights from a decade of data

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Hatching failure affects up to 77% of eggs laid by threatened bird species, yet the true prevalence and drivers of egg fertilization 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 and 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 fertilization 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 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 unique 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.

infertility | embryo mortality | sex ratio | population demographics | inbreeding

Significance

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: fertilization 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 link between population demographics and fertilization success, with higher fertilization 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.

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 fertilized 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 fertilization failure. Fertilization failure occurs when sperm and ovum fail to fuse, while embryo mortality can occur at any stage of development from fertilization 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 unfertilized by researchers and conservation practitioners (15), 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 fertilization 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 unfertilized (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 fertilization failure or embryo mortality are likely very different, therefore, it is important to distinguish between them to fully

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The authors declare no competing interest.

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understand the causes of reproductive failure and to better direct conservation efforts. There are many possible mechanisms of fertilization 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), and 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 fertilization 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 fertilization 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 fertilization) 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 to 3 d incubation), before macroscopic signs of development are visible, so may over- or underestimate 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 toward 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 behaviors 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 nonpair 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 as 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 fertilization 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 fertilization status of eggs, allowing us to accurately determine whether an egg failed to hatch due to fertilization failure or embryo mortality. This study provides the most accurate estimates of infertility rates in a wild bird population to date and utilizes an undersampled subset of individuals that die very early during embryo development to assess potential causes of embryo mortality at all stages of development.

Results

Fertilization 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 fertilization status and/or developmental stage of 1,437 of these eggs (*SI Appendix*, Table S3 in 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 fertilization failure or embryo death (12, 16, 81). Dead embryos were assessed for their stage of development based on 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 (Fig. 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

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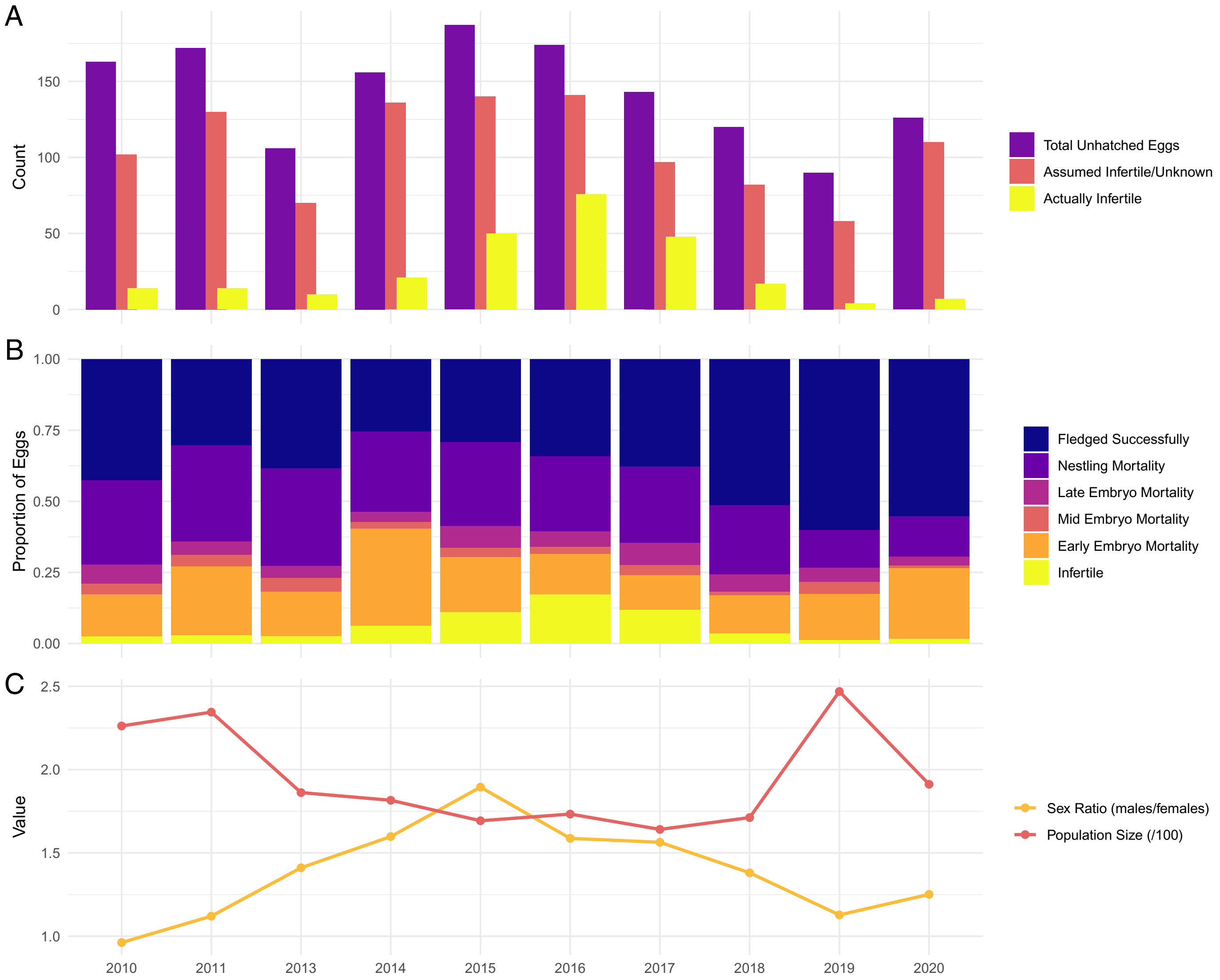

Fig. 1. How egg outcomes vary with population demographics in the Tiritiri Matangi population of hihi across 10 y. (*A*) The total number of eggs which failed to hatch after the full incubation period for every year, compared to the count of eggs previously assumed to be unfertilized/with unknown fertilization status using macroscopic techniques, and the proportion determined to be truly unfertilized 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 y.

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 fertilization failure across the 10 y was 17.8%, with an annual mean of 16.7% ± 13.3% SD (range: 2.2% to 17.6%). These values are significantly lower than estimates of fertilization 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 fertilization status) (Fig. 1*A*; paired Wilcoxon test, $P = 0.003$). To determine whether fertilization failure was a significant component of hatching failure, we tested the relationship between annual fertilization 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 fertilization 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, Fig. 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

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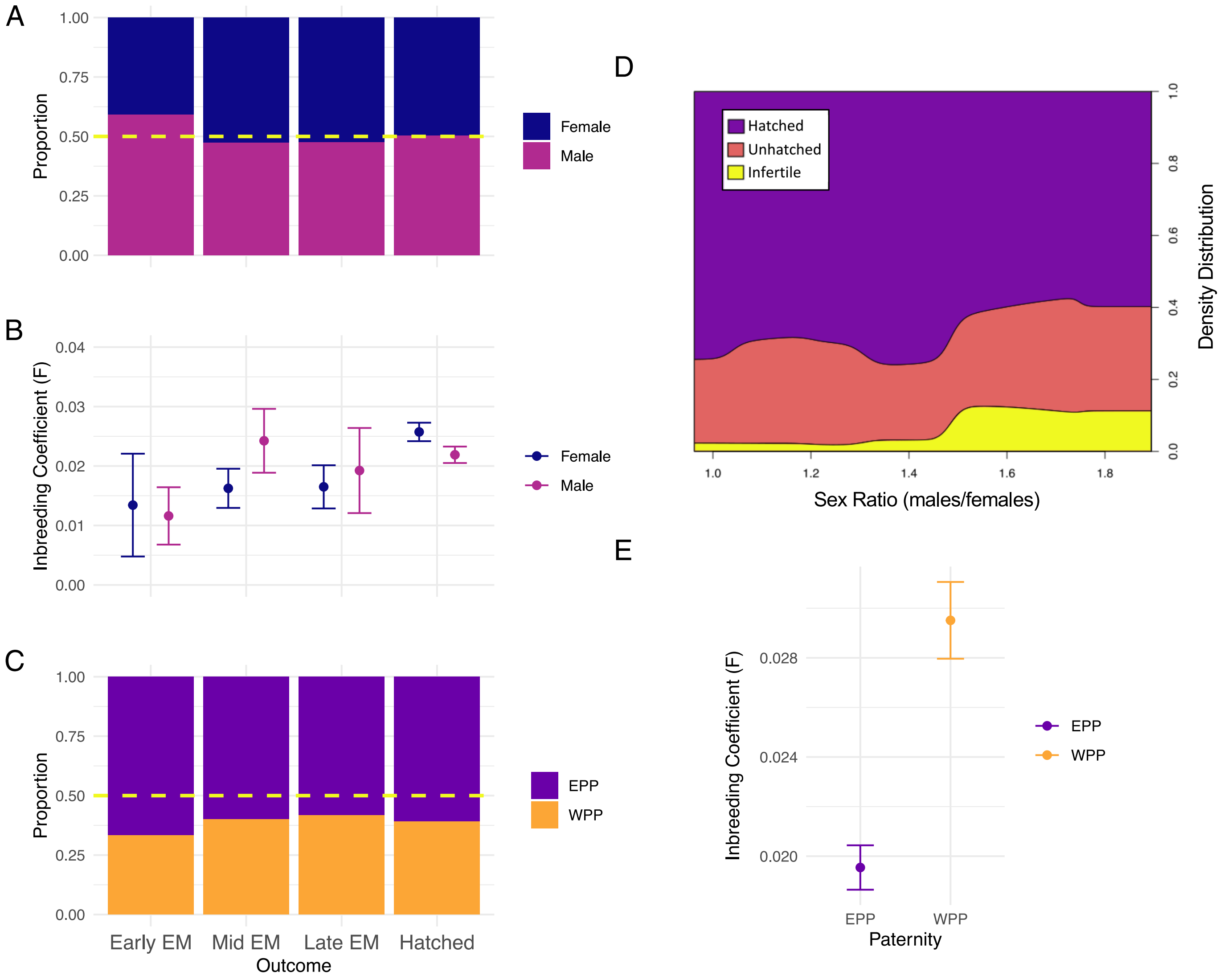

Fig. 2. The probability of an egg being unfertilized is significantly increased in years when the sex ratio of males to females is more biased; however, this does not significantly affect overall hatching failure, which is mostly due to embryo mortality. Embryo mortality, at any stage of development, does not seem to be influenced by 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 unfertilized/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 the infertility rate of eggs. (*E*) Mean (point) and SE (bars) of the inbreeding coefficients of extra-pair (n = 2,029) and within-pair (1,250) hihi offspring from the years 2010 to 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; and d) Inbreeding*Paternity: EPP = 1,396, WPP =888.

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) (Fig. 2 *A*–*C*). Individuals fathered by within-pair males had significantly higher inbreeding coefficients than those fathered by extra-pair males (Fig. 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 (Fig. 2*C*).

Demographic Effects on Fertilization 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 fertilization failure rate. The average primary sex ratio (prehatch 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

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is sex-biased toward 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 (Fig. 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 (Fig. 1*C*, r = −0.76, df = 9, p = 0.007), and therefore, the effect of these demographics on fertilization failure and embryo mortality was tested in separate models.

The probability of an egg being unfertilized significantly increased in years when the size of the population was smaller (Fig. 1, glmm: estimate = 1.21, std. error = 0.32, $P < 0.001$), and the sex ratio of the population was more male biased (Fig. 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, $P = 0.21$) or the adult sex ratio (glmm: estimate = −1.07, std. error = 0.87, $P = 0.21$), despite the appearance of a trend toward higher hatching failure in more male-biased populations (Fig. 2*C*).

Discussion

In this study, we aimed to determine the contribution of fertilization 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 (*N. 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. Fertilization 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 fertilization 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 fertilization failure of eggs is less common in this population than previously thought, fertilization 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 (prehatch) 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 postfledging, 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; (91)]. 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 behavior (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 fertilization failure. In other species, aggressive male behavior 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 fertilization failure.

Although fertilization 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 postdeath 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 [$F_{ROH} = 0.29$; (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 (*N. 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 fertilization 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 fertilization 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, although this study identifies a link between small population size, sex ratio bias and reduced fertilization rates, more research is needed to establish whether this pattern is species-specific or common across species. Identifying the mechanism of fertilization 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 emphasize that embryo failure and egg infertility may have different underlying causes as mechanisms of reproductive failure.

Materials and Methods

At 21 d old, each individual bird in the Tiritiri Matangi population is color 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 prebreeding survey in September and one postbreeding survey in February. An integrated population modeling 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 d after the last egg in a clutch has hatched (14-d 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 (82) based on the size of the embryo and the developmental state of limbs (see also Hemmings & Birkhead (29) for passerine staging comparison and estimations of development times). The embryos were categorized here as "Early Embryo" (Hamburger-Hamilton Stage 1 to 21/day 0 to day 5), "Mid Embryo" (Hamburger-Hamilton Stage 22 to 36/day 5 to day 10), and "Late Embryo" (Hamburger-Hamilton stage 37 – hatching/day 11 to 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 unfertilized, and eggs without signs of embryonic development where the yolk was addled/rotten were classified as having unknown fertilization status. To determine the accurate fertilization 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 fertilization and embryonic development, and ii) the perivitelline layer; a membrane surrounding the yolk which sperm must penetrate to fertilize 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 fertilization. These methods are described in more detail in Assersohn et al., (12) (see associated open access protocols). Following these examinations, unhatched eggs were reclassified as fertilized 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 unfertilized if there was no evidence of embryonic development or sperm penetration.

Hatchlings (including those that died before individual color banding at 21 d) 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 refs. 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 formulas, model type, and family can be found in *SI Appendix*, Table S1 (Supplementary Information), data and code are publicly available (106). To test whether fertilization failure was a significant component of overall hatching failure, we built a generalized 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 unfertilized, unhatched, or hatched), including repeated measure of females across years. The model tested the effect of the number of unfertilized 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 unfertilized eggs in the data. The model was run using the "glmmTMB" package in R (107), 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

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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 (108), 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; categorized according to the developmental stage reached and therefore ordered by developmental time. The variation in inbreeding coefficients with paternity was tested in using a generalized 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 fertilized/unfertilized 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, CI, and *P* values of fixed effects) can be found in *SI Appendix*, Table S2 (Supplementary Information).

Data, Materials, and Software Availability. The data and code associated with this analysis are publicly available at https://github.com/fmorland/hihipop-demographicsandreprodfailure.git (106). 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.

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