Received: 12 June 2023 | Accepted: 9 October 2023

DOI: 10.1111/2041-210X.14242

Methods in Ecology and Evolution

RESEARCH ARTICLE

Including the invisible fraction in whole population studies: A guide to the genetic sampling of unhatched bird eggs

Fay Morland[1,2,3] | Selina Patel[4] | Anna W. Santure[4] | Patricia Brekke[2] | Nicola Hemmings[1]

[1]School of Biosciences, The University of Sheffield, Sheffield, UK

[2]Zoological Society of London, Institute of Zoology, London, UK

[3]Department of Anatomy, The University of Otago, Dunedin, New Zealand

[4]School of Biological Sciences, The University of Auckland, Auckland, New Zealand

Correspondence
Fay Morland
Email: fay.morland@otago.ac.uk

Funding information
British Birds Charitable Trust; Marsden Fund; Research England; Royal Society, Grant/Award Number: DH160200 and RGF\R1\180101

Handling Editor: Xingfeng Si

Abstract

1. Early embryo mortality has recently been proven to be a significant component of avian reproductive failure. Due to the difficulty in distinguishing eggs, which have suffered early embryo mortality from unfertilised eggs, this cause of reproductive failure has historically been underestimated and overlooked.
2. We describe methods for recognising and collecting early failed, unhatched eggs from wild bird populations, identifying and isolating embryonic material in unhatched eggs, and efficiently extracting DNA from those samples. We test these methods on unhatched hihi (*Notiomystis cincta*) eggs collected from the field, which have undergone postmortem incubation.
3. We obtained DNA yields from early-stage embryos that are sufficient for a wide range of molecular techniques, including microsatellite genotyping for parentage analysis and sex-typing. The type of tissue sample taken from the egg affected downstream DNA yields and microsatellite amplification rates. Species-specific microsatellite markers had higher amplification success rates than cross-species markers. We make key recommendations for each stage of the sampling and extraction process and suggest potential protocol improvements and modifications.
4. Genetic and possibly genomic analysis of embryos that die early in development has the potential to advance many fields. The methods described here will allow a more in-depth exploration of the previously overlooked causes of early embryo mortality in wild populations of birds, including threatened species.

KEYWORDS
DNA, embryo mortality, hatching failure, invisible fraction, microsatellites, paternity, population genetics

Patricia Brekke and Nicola Hemmings are joint senior authors.

1 | INTRODUCTION

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

In wild bird populations, there is evidence that the invisible fraction may be substantial. An average of almost 14% of wild bird eggs fail to hatch (Marshall et al., 2023) and the predominant cause of hatching failure is embryo mortality rather than fertilisation failure (99.9% in Hemmings & Evans, 2020; 72% in Savage et al., 2022), often very early in development (Hemmings & Evans, 2020; Savage et al., 2022). Despite this, early embryo mortality, defined here as the death of an embryo before it is visible to the naked eye, is often unaccounted for in avian population monitoring. For example, studies that use molecular techniques such as sex determination (Brekke et al., 2010; DuRant et al., 2016; Eiby et al., 2008; English et al., 2014) or parentage analysis (Whittingham & Dunn, 2001) to study embryonic mortality are often missing early failed embryos due to the methods used. The exclusion of this invisible fraction is the result of either nondeveloped eggs not being sampled for molecular analysis or being overlooked completely and assumed to be unfertilised (Birkhead et al., 2008). Eggs are often assumed to be unfertilised due to the absence of macroscopic signs of development during the early stages of development; for example, blood islands are only visible after 40 h of development (Murray et al., 2013) and the rupturing of the perivitelline membrane (Jensen, 1969) and dark eye spot pigmentation (Murray et al., 2013) does not occur until 4.3 days of development in the zebra finch *Taeniopygia guttata*, and these early developmental milestones appear to be highly conservated across species (Cooney et al., 2020).

The inclusion of early-dead embryos in genetic analyses of bird populations is hampered by technical challenges, the most significant of these being degradation. In many field studies, especially of threatened species, unhatched eggs remain in the nest until the end of the incubation period, to reduce the risk of mistakenly removing a viable egg or the intervention leading to nest abandonment. However, the microclimate inside a bird's nest during incubation provides the ideal environment for postmortem DNA degradation via autolysis (Williams et al., 2015). Despite this, DNA samples taken from deceased late-stage embryos, which have been incubated postmortem in the nest, have been successfully used for genetic sex determination in wild, nest-box breeding populations of great tit *Parus major*, blue tit *Cyanistes caeruleus*, collared flycatcher *Ficedula albicollis* (Cichoń et al., 2005), and hihi *Notiomystis cincta* (Brekke et al., 2010). Genetic analysis of early embryos from wild populations has also been demonstrated using samples, which have experienced either a lack of or a limited postmortem incubation period in the nest; in brown-headed cowbird, *Molthrus ater* DNA samples have been taken from live embryos at 1–4 days of development (Strausberger & Ashley, 2001) and in Eurasian tree sparrows *Passer montanus*, DNA samples have been taken from early-failed embryos, which were incubated for up to 2 days of postmortem (Kato et al., 2017). However, the successful extraction and molecular analysis of DNA from embryos in unhatched eggs that have died early and undergone a significant period of postmortem incubation, as is often required by restricted data sampling regimes of wild/protected species, has not been reported.

Here, we describe field methods for collecting and storing fully incubated, unhatched eggs for subsequent dissection. We also describe methods for obtaining cell samples from embryos that died very early in unhatched eggs, and for extracting DNA from those samples. We apply our methods to fully incubated unhatched eggs sampled from a wild population of birds and assess the quantity of DNA obtained and its suitability for microsatellite genotyping, sex determination and paternity analysis, compared with blood samples taken from live individuals from the same species. Although these methods are written for application to field studies, they could also be utilised for the genetic sampling of early failed embryos from captive populations of birds, including laboratory studies, zoo populations and captive breeding populations. The study of these populations could benefit from using the following methods in the experimental study of embryonic failure, or in conservation management.

2 | PROTOCOL RECOMMENDATIONS AND SPECIAL CONSIDERATIONS FOR EARLY EMBRYO SAMPLES

2.1 | Sample collection and storage

When collecting unhatched eggs for analysis from threatened species, it is vital to avoid jeopardising the breeding attempt. We recommend candling eggs no earlier than 4 days after the onset of incubation to identify nondeveloping eggs before removal. The technique of candling involves shining a bright light through the egg to look for signs of development, that is dark eye spots and blood vessels. In protected species, unhatched eggs may need to be left in the nest until after the incubation period. In either instance, to limit DNA degradation after collection, we recommend unhatched eggs are stored at either 1–5°C for short-term storage or −20°C for long-term storage as soon as possible. Samples can be placed in a cool

box when in the field and afterwards transferred to a standard refrigerator or freezer where they should remain until processing. Eggs can be frozen in their shell for long-term storage and thawed before further processing; however, multiple freeze–thaw cycles should be avoided to preserve the integrity of the sample.

2.2 | Sample processing and cell isolation

Unhatched, early-failed eggs are usually found in two distinct states when collected from the field: either the yolk remains intact and separate from the albumen inside the shell (referred to here as separated; Figure 1a), or the contents are addled (yolk broken down and mixed with albumin, often degraded to some degree, Figure 1c). This distinction has been used in the past to falsely categorise eggs as unfertilised when they are separated, or as failed embryos when they are addled (e.g., Cook et al., 2005). However, separated eggs may be fertilised and contain embryonic cells (Assersohn et al., 2021; Birkhead et al., 2008).

For a detailed explanation of the equipment and methodology used to assess the fertility status of unhatched eggs, please see Assersohn et al. (2021) and the open access protocols and videos referred to therein ('Practical resources for identifying the causes of hatching failure in birds' 2021). In brief, to assess an unhatched egg for signs of fertilisation and embryonic cells, first open the egg into PBS (phosphate buffered saline, pH 7.4) in a petri dish (Figure 1). Second, locate the parts of the egg most likely to contain embryonic cells and sperm cells: the germinal disc and the section of perivitelline layer surrounding it. The germinal disc, which contains the female pronucleus, is the site of fertilisation and where embryonic development starts. It appears as a white spot or ring on the surface of the yolk of intact eggs (Figure 1a,b). The perivitelline layer consists initially of a single layer (the 'inner' perivitelline layer), which surrounds the yolk and is penetrated by sperm prior to fertilisation. Shortly after ovulation, a second perivitelline layer (the 'outer' layer) is formed through the secretion of glycoproteins from the oviduct, and sperm that are around the egg at that time are trapped in this outer layer as it forms. Sperm cells can therefore be found in the perivitelline layer, and embryonic cells can be found either in the germinal disc or attached to the overlying perivitelline layer. By locating, isolating, and examining the germinal disc and the perivitelline layer using fluorescence microscopy, following the methods of Assersohn et al. (2021), it is possible to determine whether sperm reached the egg, and whether fertilisation and embryonic development took place.

For separated eggs (Figure 1a), once the germinal disc has been located, cut and remove the surrounding section of perivitelline layer, using surgical scissors and tweezers, and place it into a clean dish of PBS. Remove the germinal disc from the yolk using a pipette or a hair loop (a loop of human hair taped to a pipette tip which creates a delicate tool) and place straight onto a microscope slide. For addled eggs (Figure 1c), search the sample macroscopically or using a stereo microscope to find and remove all pieces

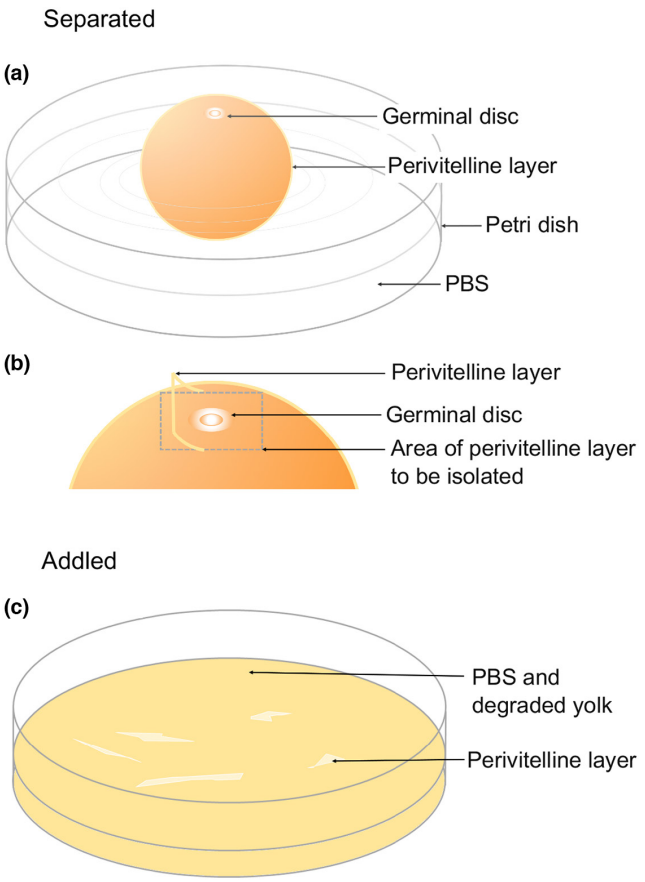

FIGURE 1 (a) A separated unhatched egg removed from the shell and placed in phosphate buffered saline solution (PBS) for dissection and isolation of two key parts: the germinal disc and the perivitelline layer. (b) Isolating the germinal disc and the area of perivitelline layer surrounding the germinal disc will provide the best opportunity of locating embryonic cells and sperm cells. (c) An addled unhatched egg removed from the shell and placed in PBS will create a cloudy mix of yolk, PBS and pieces of degraded perivitelline layer. Locating and isolating pieces of perivitelline layer, on which embryonic cells may be attached and in which sperm cells may be trapped, provides the best opportunity of finding evidence of fertilisation and embryonic development in addled unhatched eggs.

of perivitelline into a clean dish of PBS. For pieces of perivitelline layer isolated from both intact and addled eggs, clean off any excess yolk with a hair loop or by agitating in PBS solution. However, take care not to clean so thoroughly as to potentially dislodge any embryonic cells which may be attached, that is remove large pieces of yellow yolk, which may obscure the perivitelline layer under the microscope, but do not remove all material on the perivitelline layer, as some of it may be embryonic cells. Place the perivitelline layer onto a microscope slide using tweezers and flatten/smooth the pieces out using the hair loop and stain the sample with the fluorescent DNA dye Hoechst 33342. Under a fluorescence microscope, embryonic cells will appear as clustered fluorescent-blue cells (Figure 2). If embryonic cells have been located on the germinal disc or the perivitelline layer, transfer the whole sample into

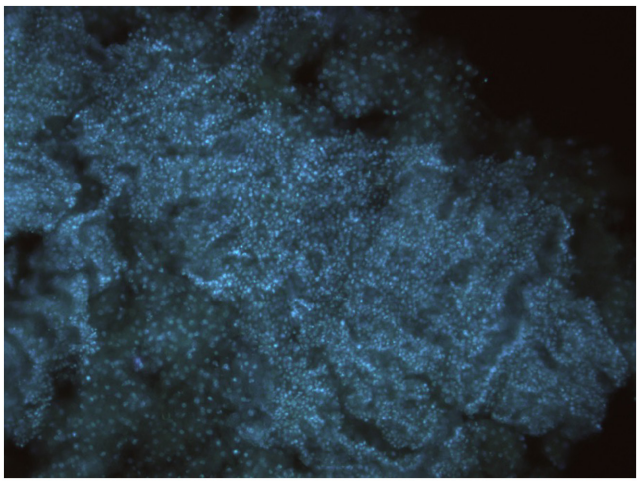

FIGURE 2 Hihi (*Notiomystis cincta*) embryonic cells stained with Hoechst fluorescent dye, as viewed at a magnification of 100× (10× objective lens × 10x eyepiece lens). Embryonic cells are shown as bright blue clusters of cells against a background of unstained yolk/PBS, appearing black.

absolute ethanol to be stored for later DNA extraction. We would recommend using the microscopic techniques described here and in Assersohn et al. (2021) to identify the presence of embryonic cells before attempting to isolate and extract DNA from the germinal disc. The extraction of DNA from an unfertilised egg may yield maternal DNA (Strausberger & Ashley, 2001) and therefore not necessarily indicate the presence of an embryo.

2.3 | DNA extraction

Some samples from embryos that died at an early stage of development contain a very small number of cells. It is therefore necessary to take specific steps to maximise the amount of tissue retained for the DNA extraction. We found that the following modifications to the Qiagen DNeasy Blood and Tissue kit spin-column protocol for tissue samples (DNeasy Blood and Tissue Kits, Qiagen) improved DNA yield. Instead of starting step 1 of the protocol by placing tissue in a separate microcentrifuge tube, the ethanol-preserved perivitelline layer and germinal disc samples should be centrifuged in their original sample tubes at maximum speed (13,000 RPM) for 1 min. All ethanol should be removed using a pipette, taking care not to disturb the tissue pellet at the bottom. After ethanol removal, 180 µL Buffer ATL and 40 µL Proteinase K should be added directly to the sample tubes. Sample tubes should then be incubated on a rocking platform at 56°C for 1 h. Only after this step should the samples be transferred into new 1.5-mL Eppendorf tubes, followed by incubation in a rotating oven at 56°C overnight or until the samples are completely lysed. The method of lysis in the original sample tube was adopted to ensure that no cells (e.g. those potentially attached to the walls of the sample tube), were lost through transferring the sample into a new tube for extraction. Following this modification, the Qiagen Blood and Tissue kit protocol can be followed without further modifications from Steps 3 to 7. Following extraction, the concentration of the DNA should be tested and samples concentrated/diluted to a standard concentration required for amplification and genotyping. While we used the Qiagen DNeasy Blood and Tissue kit, other extraction kits or protocols are likely to also prove successful with similar modifications to reduce sample loss.

3 | METHODS: CASE STUDY—*NOTIOMYSTIS CINCTA*

3.1 | Study system

The eggs we used to test our methods were collected from a managed-wild population of hihi, *Notiomystis cincta*, on Tiritiri Mātangi Island, Hauraki Gulf, Aotearoa (New Zealand). The hihi is a threatened species (Vulnerable on the IUCN Red List), which is taxonomically distinct, being the only member of the endemic New Zealand bird family; *Notiomystidae*. The study population was reintroduced from the single remaining remnant population and has high levels of inbreeding due to this history of genetic bottlenecks (mean $F_{ROH} \sim 0.29$, Duntsch et al., 2023). Hihi in this population nest in nest boxes, allowing monitoring during the breeding season. Fledglings are colour banded and blood sampled, allowing for individual identification and the construction of a long-term genetic pedigree using microsatellite genotyping (Brekke et al., 2009, 2015). Due to the high rates of extra-pair paternity (68%) in this species, genotyping is required for accurate parentage assignment (Brekke et al., 2013; Ewen et al., 2004). Hihi lay an average of 1.6 clutches each breeding season, containing an average of 4 eggs incubated for 14 days, 30% of which fail to hatch. For the purposes of this study, unhatched eggs with no sign of embryonic development were collected from this population across two breeding seasons spanning 2019–2021, screened for signs of embryonic development, and sampled for DNA. Hihi have a 14-day incubation period, and unhatched eggs were collected on Day 13, to limit disturbance to natural incubation behaviour and comply with permits. Permissions to collect failed hihi eggs were granted by the New Zealand Department of Conservation permit numbers 36186-FAU, AK/13939/RES, 53614-FAU and 66751-FAU. Before collection, eggs were candled in situ at the nest box with a long torch to ensure there was no embryonic development visible. Unhatched eggs were stored in small, sealable sample bags and placed inside plastic 50-mL centrifuge tubes, buffering the egg from movement and impact during transportation to the field station/laboratory. The centrifuge tubes were placed into a cool box with ice blocks for transportation from the field site to a fridge or freezer to minimise tissue and DNA degradation as much as possible. The refrigerated samples were stored for a maximum of 19 days and frozen eggs were stored between 361 and 463 days and thawed before microscopic analysis and DNA sampling using the microscopic methods described in the Protocol

Recommendations (Section 3.2). A total of 145 tissue samples were taken from eggs lacking macroscopic signs of development but that were found to contain embryonic cells (49 from 2019 to 2020, 96 from 2020 to 2021). The tissue samples were stored in ethanol before DNA extraction using the methods described in the Protocol Recommendations (Section 3.3). The blood samples used as a comparison were taken before fledging, from the 21 mothers of the embryos included in this study, between the years of 2013 and 2018.

3.2 | Microsatellite analysis

DNA was quantified using a Qubit fluorometer and diluted if over 25 ng/μL or concentrated if under 5 ng/μL using a DNA vacuum concentrator, depending on the initial concentration, and volume of the DNA. The target concentration for this microsatellite analysis method when using blood samples is 20 ng/μL, however due to the low concentration and volume of some samples, and to avoid excessive drying and re-diluting which can cause DNA damage and salt accumulation which can inhibit PCR, we aimed for a sample concentration between 5 and 25 ng/μL. The average initial concentration was 20.5 ng/μL. DNA samples from embryos that died early in development and remained in the nest for the full incubation period were genotyped using 20 microsatellite markers, comprising of two sex-typing markers (Z002a and Z037b; Dawson, 2007; Dawson et al., 2015), 15 species-specific markers, and three markers developed for other passerines (Brekke et al., 2009, two primers listed in this paper were not used: Nci014 and MSLP4).

Microsatellite regions were amplified using PCR before genotyping, with each 6.75 μL PCR containing 2 μL of genomic DNA (at the average concentration 20.5 ng/μL this resulted in each PCR containing an average of 41 ng of DNA), 3 μL of Qiagen PCR Kit Mix and 1.75 μL of 1 of 4 multiplex primer mixes. Each multiplex primer mix contained 4–6 primers at 0.2–0.6 μM concentration. Primers were grouped into multiplex mixes based on annealing temperature and allele size range (Brekke et al., 2009). The PCR followed a thermal cycle of 9°C for 15 min, followed by 30 cycles of 94°C for 30 s, annealing temperature of 56°C or 64°C (depending on primer mix, see Brekke et al., 2009) for 30 s, 72°C for 90 s, and a final step of 72°C for 10 min. Microsatellites were genotyped using an Applied Biosystems 3130 Genetic Analyser and output used to assign microsatellite genotypes for each individual and locus using the GeneMapper 5 software. The same microsatellite analysis protocol is used for genotyping fledglings using DNA obtained from blood samples, although Geneious software with the Microsatellite plugin is sometimes used for genotyping. Due to COVID-19 travel restrictions, the genotyping of the 2020–2021 season was performed in a different laboratory and on a different sequencer to the other samples included in this study and to the laboratory and sequencer that was used to characterise the population's alleles.

3.3 | Parentage analysis

To test the ability of the resultant microsatellite genotypes to accurately infer known maternal links, the sample genotypes were used to carry out parentage analysis in the Colony software (Jones & Wang, 2010). Colony is provided with the genotypes of the offspring (here, egg samples as well as other fledglings from the same sampling year) and of all candidate mothers and fathers. In addition, information can be provided about locus error rates. Information can also be provided about the paternal and/or maternal sibship of the offspring and known mothers and fathers. In the case of hihi, paternal sibship and paternity cannot be inferred due to the high rates of extra pair paternity (Brekke et al., 2013). However, maternal sibship (individuals from the same nest) and maternity can be accurately inferred from field data due to the individual identification of nesting individuals from their unique colour band combinations.

In an initial Colony analysis with maternal sibship and known maternal ID included, it became clear that there were high rates of nonconcordance between the failed embryo and the maternal genotype, that is, the offspring genotype shared no alleles with the maternal genotype at a given locus. Due to the absence of intraspecific nest parasitism in this species (Brekke et al., 2012; Castro et al., 2004; Ewen et al., 1999), mismatching between mother and embryo in this case is indicative of mistyping or allelic drop-out. Further investigation revealed that this was likely a result of miscalibration of allele sizes across different sequencing laboratories. Sequencing outputs were manually rechecked with peak size assignment adjusted and genotypes recalled before estimating locus specific error rates (due to e.g. mutation or allele dropout) based on persistent nonconcordance with known maternal genotypes. An error rate of 0.01 (1%) for all microsatellite markers is used when performing parentage analysis on fledgling blood samples (Brekke et al., 2015); however, we expected higher error rates here given that these samples are likely to be degraded. A study on the effectiveness of including markers with high error rates in paternity analysis found that markers with error rates up to 70% are still informative, provided the correct error rates are provided to Colony (Wang, 2019).

Colony was then run with the following parameters: a 'full-likelihood' analysis method, 'high' likelihood precision, 'weak' sibship priors and with locus specific genotyping error rates (range 3%–16%). We ran Colony both with (i) maternal sibship included but no maternal ID and (ii) with maternal sibship and maternal ID included, to (i) simulate the application of this method to field sampling where all eggs or offspring in a nest are sampled but parents may not be sampled or known and (ii) to utilise the known mother to estimate mother–offspring genotype concordance, and hence estimate genotyping error in the degraded egg sample. Colony outputs include the reconstructed pedigree and the probabilities associated with each of the reconstructed parent–offspring links. Before running Colony, we also checked for genotype duplicates between an egg sample and a parent, which may indicate parental DNA contamination of the egg sample.

4 | RESULTS

4.1 | DNA quantity

A total of 142 of the 145 unhatched, early-failed egg samples that were found to contain microscopically visible embryonic cells, yielded enough DNA following extraction using the methods described in the Protocol Recommendations (Section 4.3) for detection using a high sensitivity Qubit assay. A total of three samples had DNA concentrations too low to be read (and are included in mean estimates as a concentration of 0); however, upon genotyping only two of these samples failed to amplify at any microsatellite, suggesting that only 1 of the 145 samples yielded no DNA. The mean total DNA quantity obtained per egg from the samples was 2620 ng ($n = 145$), but values for individual samples ranged from 6.6 ng to 12,400 ng. For comparison, the expected yield of DNA from nucleated bird blood when using the Qiagen DNeasy Blood and Tissue kit is 9000–40,000 ng ('DNeasy Blood & Tissue Kits', Accessed: 19-09-2022). The yield of DNA obtained varied between embryo stages and sample type (Table 1). As expected, the quantity of DNA obtained was higher for embryos that died at a later stage of development, that is, those that did not reveal development on candling but an embryo was obvious via macroscopic inspection once the egg was opened. For very early embryonic samples (≤HH stage 7), where no development was visible on macroscopic examination, samples of perivitelline layer with the germinal disc attached provided the highest yields of DNA on average. The results of an analysis of variance including 93 very early embryonic embryo samples (≤HH stage 7) with both sample type and DNA yield recorded show that sample type significantly impacts the DNA yield ($F = 6.3$, $p = <0.05$, $df = 2$), and a post hoc Tukey's test revealed that samples of the germinal disc yield significantly more DNA than samples of perivitelline layer (diff = 2.926 ng, $p = <0.01$).

Samples were either kept in the refrigerator before microscopic analysis and DNA sampling for between 2 and 19 days, or frozen on the day of removal from the nest and frozen for between 361 and 463 days before microscopic analysis and DNA sampling. For refrigerated samples, we did not find a relationship between processing latency (i.e. the time between egg collection and dissection/storage of sample in ethanol) and DNA quantity (cor = 0.06, $p = 0.7$, $df = 40$) or the number of microsatellites amplified in downstream genotyping (cor = 0.012, $p = 0.9$, $df = 42$). We did not find a significant difference in DNA quantity ($F = 0.008$, $p = 0.93$, $df = 1$), or the number of microsatellites amplified in downstream genotyping ($F = 1.67$, $p = 0.2$, $df = 1$) between samples which were frozen or refrigerated before sampling.

4.2 | Microsatellite analysis

Of all samples processed, 50 out of 145 samples amplified across the entire microsatellite panel and the majority (135 samples) showed at least some amplification. Mean microsatellite amplification rates were similar for pre- and post-HH stage 8 embryos (Table 1) and very poor amplification rates were found only for perivitelline layer samples categorised as having very few embryonic cells found during microscopic examination (average success rate = 10%, $n = 6$). DNA extracted from the germinal disc/embryonic material provided the provided the highest yields of DNA and the highest success rate in microsatellite analysis. These results suggest that prioritising samples in which the embryo or germinal disc can be found, and discounting samples for which very few embryonic cells are found during microscopic examination, will prove the most efficient approach if resources and/or time are limited.

The average microsatellite success rate was 77% across samples, representing a higher failure rate in microsatellite amplification than blood samples taken from fledglings from the same population, for

TABLE 1 Mean yield of DNA and microsatellite amplification rates from germinal disc, perivitelline layer (PVL), and embryos of different developmental stages (HH Stage refers to Hamburger-Hamilton developmental stages [Hamburger and Hamilton, 1951]).

HH stage of embryo	Sample type [sample size]	Mean (std error) yield of DNA (ng)	Mean (std error) concentration (ng/μL)[a]	Percent of markers amplified [samples that showed no amplification | samples that showed amplification of >2 alleles at 1 or more loci][b]
≤7	Germinal Disc [$n = 41$]	3542 (689)	37 (7)	81% [1|0]
≤7	PVL [$n = 29$]	616 (204)	6 (2.03)	72% [5|3]
≤7	Germinal Disc + PVL [$n = 23$]	1799 (727)	18 (7.3)	73% [2|1]
≥8	Germinal Disc/Embryo [$n = 37$]	3806 (660)	39 (6.5)	79% [1|2]
≥8	PVL [$n = 3$]	8 (4.3)	0.1 (0.05)	62% [1|0]
≥8	Germinal Disc + PVL [$n = 12$]	2884 (1291)	30 (12.8)	79% [0|0]

[a]Dilution volume was adjusted according to estimated number of cells/embryo size and is therefore not constant.

[b]A total of 10 samples did not yield a genotype at any microsatellite marker. Amplification rates of markers are calculated including these failed samples.

which the average microsatellite amplification success rate 97.4% (Figure 2). The DNA amplification showed some of the highest rates of failure with cross-species microsatellite primers; two of the four highest failure rate of the microsatellite markers were two of the three cross-species markers; BMC04 (37.5%) and Tgu-Gga (35.8%). This suggests that genotyping efforts for early embryonic samples may be more productive when using species-specific molecular markers. The length of the target allele was not associated with microsatellite failure rate when including (cor = −0.004, $p = 0.9$, df = 18), or excluding (cor = −0.18, $p = 0.5$, df = 15) cross-species primers.

4.3 | Parentage analysis and sex typing

There were six potential cases of DNA contamination from parental DNA, detected as individuals amplifying for three or more alleles at a single locus. The contamination may have originated from sperm trapped in the perivitelline layer (Carter et al., 2000), maternal and/or paternal DNA from other egg components such as egg shell fragments (Martín-Gálvez et al., 2011; Strausberger & Ashley, 2001) or cross-contamination during sampling or extraction. The six samples with potential contamination were not included in Parentage analysis.

Despite attempting to resolve calibration errors between sequencing machines before running Colony analyses, there were also still cases of non-concordance between the offspring and the mothers, with 8.46% of alleles non-concordant with maternal genotypes. Of these nonconcordant alleles, 55% were homozygous, suggestive of allelic drop-out; however, the cause of the remaining 45% is unclear. Nonhomozygous alleles which are non-concordant with maternal genotyping could result from technical issues such as laboratory temperature differences (Davison & Chiba, 2003) or electrophoresis artefacts (Fernando et al., 2001), a lack of DNA quality and/or quantity (Goossens et al., 1998) or human error at any stage of the process (Bonin et al., 2004), including human error in field data recording, microsatellite peak assignment or PCR and/or sequencing protocol. In this case, it may also be possible that not all calibration errors between sequencing machines were fully resolved.

Despite the variable genotyping rates across loci and across samples, and the presence of non-concordant genotypes between samples and their known mothers, Colony successfully inferred maternity in 69% of cases where maternal sibship, but not maternal identity, was given and in 74% of cases where both maternal sibship and maternal identity were given.

Most samples (84%) could be sexed from the two sex-typing markers used in microsatellite analysis. The sex ratio of all failed early embryos was 1.5 (males: females), which may be due to sex-biased mortality at this stage, or allelic drop-out during microsatellite genotyping leading to incorrect sex assignment. However, the latter explanation is unlikely since although the sex bias was largest in the early stages of embryonic failure (≤HH stage 7, 1.96 males: females, $n = 77$) compared to the later stages of embryonic failure (≥HH stage 8, 1.09 males:females, $n = 48$), the rate of identified mistypes was not higher in these early stage samples (≤HH stage 7, mean number of mistyped alleles = 9) compared to the later stages (≥HH stage 8, mean number of mistyped alleles = 10). In addition, the use of two separate sex markers may help to protect against sex-typing errors due to allelic drop-out.

5 | DISCUSSION

The results of our study demonstrate that it is possible to harvest tissue from avian embryos that die early in development and use this for DNA extraction and downstream molecular analyses, even when unhatched eggs are incubated for prolonged periods in natural nests prior to sampling. Our findings suggest this approach may be highly applicable to a wide range of wild bird population studies across multiple fields. With particular care given to sample collection, microscopic fertility examination techniques, and DNA extraction protocols, it is possible to obtain high yields of genomic DNA, even with very early embryos (≤HH stage 7/less than 2 days of development). The DNA we extracted from early embryo samples was of relatively high molecular yield despite small quantities of often degraded tissue available, and it was found to be suitable for several molecular techniques including microsatellite genotyping for sex typing and parentage assignment.

Figure 4 highlights the steps that are important for the sampling, processing, DNA extraction and microsatellite sequencing of failed embryos in unhatched, early-failed eggs.

In our study, all but one sample extracted in this study yielded at least some DNA detectable through a high sensitivity Qubit assay. This is a higher success rate than a previous attempt to extract DNA from bird faeces, which had extraction success rates of 34%–80% depending on the method used (Alda et al., 2007). In addition, the mean amount of DNA obtained from early-dead embryos in our study was 2122 ng, which is ample for several molecular techniques. For example, a minimum of 10 ng of DNA per is recommended per reaction for multiplex microsatellite analysis (Brekke et al., 2010; Narina et al., 2011; Neff et al., 2000), 2.5 ng of DNA per sample is required for RAD-seq (Etter et al., 2011), 20–1000 ng for Illumina Sequencing (Sample Requirements), depending on the precise method, and a maximum of 200 ng is required for Sanger sequencing (Sample Requirements).

An advantage of microsatellite analysis is that it can be applied to degraded DNA due to the short sequence length of microsatellite target regions (Queller et al., 1993). We demonstrate here that DNA samples taken from embryos that died early in development and were then left in the nest for up to 2 weeks had an average microsatellite amplification success rate of 77%. Although this is substantially lower than the average amplification success rate of blood samples stored in ethanol immediately after sampling (97%—Figure 3), it does at least allow some degree of genotyping and downstream analysis, for example parentage analysis and sex-typing. It has been demonstrated that short microsatellite markers are more effective than longer microsatellite markers for

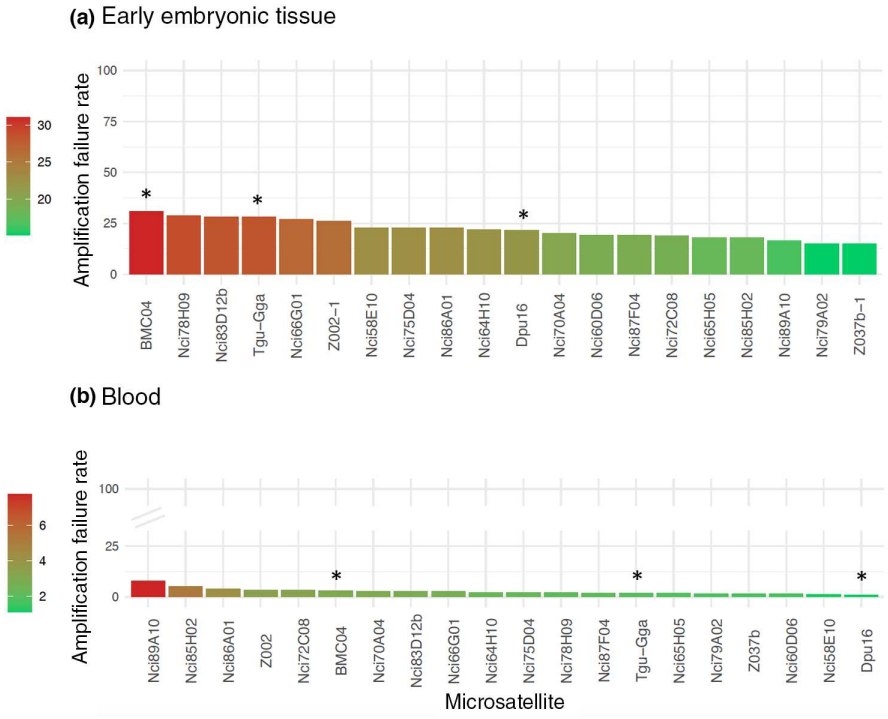

FIGURE 3 Microsatellite amplification failure rates for DNA obtained from (a) cell samples taken from embryos that died early ($n = 150$) and (b) blood samples taken from nestlings ($n = 3162$). The green to red colour gradient indicates the markers which failed the least (green) to markers which failed the most (red), relative to the failure rates for each sample type; note the different scales of the colour gradients. Cross-species markers are indicated by *. The two sex-linked markers are Z002 and Z037b.

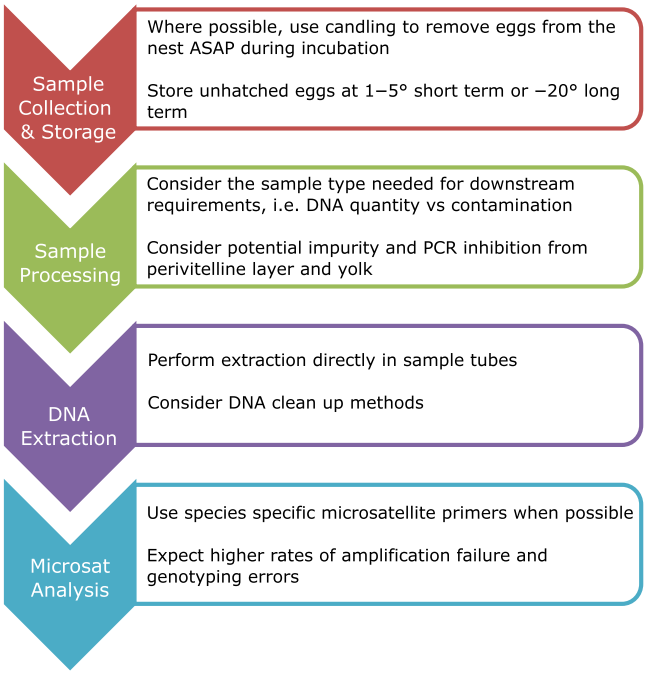

FIGURE 4 Summary of the important steps recommended during sample collection, storage, processing, DNA extraction and microsatellite analysis.

degraded DNA, such as that from museum specimens (Nakahama & Isagi, 2017). Our results here suggest that limited DNA degradation may not be an issue in early-dead embryo samples, as we see no relationship between the length of the microsatellite marker and the amplification failure rate. Microsatellite amplification failures may instead be due to the presence of proteins and fats (Acharya et al., 2017), which are known PCR inhibitors (Schrader et al., 2012) and make up a large component of the egg yolk (Kowalska et al., 2021). Another category of known PCR inhibitors are proteases (Schrader et al., 2012), which are also present in egg yolk (Shbailat et al., 2016). A total of 412 proteins, including glycoproteins, have also been identified in the chicken perivitelline layer (Brégeon et al., 2022), which may also act as inhibitors. Samples containing perivitelline layer had the worst rates of microsatellite amplification in this study, which may be a consequence of the large number of proteins present in those samples. An improvement on the methods presented here could involve a DNA purification step, such as ethanol precipitation, silica columns or magnetic bead cleaning, to remove protein and lipid contaminants.

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

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

secondary sex ratio in Eurasian tree sparrow *Passer montanus* was due to differential mortality in very early embryos (≤HH stage 5), with 97% of early mortalities being males (Kato et al., 2017). Since females are the heterogametic sex (ZW) in birds, it has also been suggested that females may adjust the sex ratio of their ova based on environmental conditions. The methods outlined here will help to build a complete picture of the sex ratio of all ova, including those previously overlooked due to early embryo mortality. These methods will also help identify and further investigate the poorly understood causes of early embryo mortality in hihi *Notiomystis cincta* and other threatened bird species, such as the expression of deleterious alleles early in embryonic development due to inbreeding, or adverse environmental conditions. We have shown that molecular information can be relatively easily gleaned from the 'invisible fraction' (Grafen, 1988) of bird embryos that die early, and this information is likely to have important implications for ecology, evolution and conservation. Specifically, data on the invisible fraction will provide vital information on levels of selection in wild populations, as well as improving our understanding of life-history trade-offs (e.g., sex allocation), reproductive biology (e.g. levels of extra-pair paternity) and inbreeding depression. Genetic and potentially genomic analysis of embryos that die early in development has the potential to inform these and many other areas of study. We hope that the methods described in this paper will be widely adopted, allowing a more in-depth exploration of the nature of the invisible fraction and the causes of early embryo mortality.

AUTHOR CONTRIBUTIONS

Fay Morland wrote the manuscript with support from all other authors. Nicola Hemmings conceptualised the study. Sample collection, microscopy and data analysis was carried out by Fay Morland. Nicola Hemmings, Patricia Brekke, Fay Morland and Selina Patel developed the methods used in the study with support from Anna W. Santure. Fay Morland and Selina Patel carried out the DNA extraction and sequencing with support and supervision from Nicola Hemmings, Patricia Brekke and Anna Santure. All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

We acknowledge Ngāti Manuhiri as Kaitiaki of hihi and the significance of Tiritiri Matangi to iwi whose rohe encompasses the Hauraki Gulf|Tīkapa Moana. We would like to thank hihi conservation officer Mhairi McCready and field assistants Leani Oosthuizen and Emma Grey 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 failed hihi eggs 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 and Shuqi Wang for support with microseatellite genotyping. Many thanks to Dada Gottelli and Kevin Hopkins of the Institute of Zoology and Adrian Turner of The University of Auckland for support with microscopy and genotyping which formed the basis of this manuscript. 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 Grant to Patricia Brekke and The Royal Society of New Zealand Marsden Fund to Anna Santure.

FUNDING INFORMATION

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.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/2041-210X.14242.

DATA AVAILABILITY STATEMENT

The code used to generate the analysis and the data regarding the fertility status of unhatched eggs will be made publicly available at https://github.com/fmorland/unhatchedeggs.git and https://zenodo.org/records/10011626 (Morland, 2023) upon acceptance of the manuscript. The sequences of the microsatellite primers used in this study are available in Brekke et al. (2009). 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 sequencing data and pedigree for hihi are not 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.

STATEMENT ON INCLUSION

Our study was carried out both in the United Kingdom and Aotearoa New Zealand and brings together authors from both of those countries who were all involved in the study from an early stage. The

case study utilises samples collected from an endemic Aotearoa New Zealand species, the hihi, which is considered a taonga (treasured) species by the indigenous people of Aotearoa New Zealand, the Māori. Unfortunately, our study fails to include authors of Māori descent and suffers from a lack of input from the perspective of Māori researchers. However, the approval, wishes and rights of the kaitiaki (guardians) of hihi, the Ngāti Manuhiri iwi (tribe) were sought and respected at every stage of the study design, from permit application to data availability. Efforts by the hihi charitable trust are ongoing to engage with and disseminate research to the relevant iwi in Aotearoa New Zealand, which will hopefully lead to fruitful future collaborations.

ORCID

Fay Morland https://orcid.org/0000-0002-3037-2118
Selina Patel https://orcid.org/0009-0007-4695-5127
Anna W. Santure https://orcid.org/0000-0001-8965-1042
Patricia Brekke https://orcid.org/0000-0001-6298-3194
Nicola Hemmings https://orcid.org/0000-0003-2418-3625

REFERENCES

Acharya, K. R., Dhand, N. K., Whittington, R. J., & Plain, K. M. (2017). PCR inhibition of a quantitative PCR for detection of Mycobacterium avium subspecies paratuberculosis DNA in feces: Diagnostic implications and potential solutions. *Frontiers in Microbiology*, *8*, 115. https://www.frontiersin.org/articles/10.3389/fmicb.2017.00115

Alda, F., Rey, I., & Doadrio, I. (2007). An improved method of extracting degraded DNA samples from birds and other species. *Ardeola*, *54*(2), 331–334.

Assersohn, K., Marshall, A. F., Morland, F., Brekke, P., & Hemmings, N. (2021). Why do eggs fail? Causes of hatching failure in threatened populations and consequences for conservation. *Animal Conservation*, *24*(4), 540–551. https://doi.org/10.1111/acv.12674

Birkhead, T., Hall, J., Schut, E., & Hemmings, N. (2008). Unhatched eggs: Methods for discriminating between infertility and early embryo mortality. *Ibis*, *150*(3), 508–517.

Bonin, A., Bellemain, E., Bronken Eidesen, P., Pompanon, F., Brochmann, C., & Taberlet, P. (2004). How to track and assess genotyping errors in population genetics studies. *Molecular Ecology*, *13*(11), 3261–3273. https://doi.org/10.1111/j.1365-294X.2004.02346.x

Brégeon, M., Tomas, D., Bernay, B., Zatylny-Gaudin, C., Georgeault, S., Labas, V., Réhault-Godbert, S., & Guyot, N. (2022). Multifaceted roles of the egg perivitelline layer in avian reproduction: Functional insights from the proteomes of chicken egg inner and outer sublayers. *Journal of Proteomics*, *258*, 104489. https://doi.org/10.1016/j.jprot.2022.104489

Brekke, P., Bennett, P. M., Wang, J., Pettorelli, N., & Ewen, J. G. (2010). Sensitive males: Inbreeding depression in an endangered bird. *Proceedings of the Royal Society of London B: Biological Sciences*, *277*, rspb20101144. https://doi.org/10.1098/rspb.2010.1144

Brekke, P., Cassey, P., Ariani, C., & Ewen, J. G. (2013). Evolution of extreme-mating behaviour: Patterns of extrapair paternity in a species with forced extrapair copulation. *Behavioral Ecology and Sociobiology*, *67*(6), 963–972. https://doi.org/10.1007/s00265-013-1522-9

Brekke, P., Dawson, D. A., Horsburgh, G. J., & Ewen, J. G. (2009). Characterization of microsatellite loci in the hihi *Notiomystis cincta* (Notiomystidae, Aves). *Molecular Ecology Resources*, *9*(4), 1255–1258. https://doi.org/10.1111/j.1755-0998.2009.02626.x

Brekke, P., Ewen, J. G., Clucas, G., & Santure, A. W. (2015). Determinants of male floating behaviour and floater reproduction in a threatened population of the hihi (*Notiomystis cincta*). *Evolutionary Applications*, *8*(8), 796–806. https://doi.org/10.1111/eva.12287

Brekke, P., Wang, J., Bennett, P. M., Cassey, P., Dawson, D. A., Horsburgh, G. J., & Ewen, J. G. (2012). Postcopulatory mechanisms of inbreeding avoidance in the Island endemic hihi (*Notiomystis cincta*). *Behavioral Ecology*, *23*(2), 278–284. https://doi.org/10.1093/beheco/arr183

Carter, K. L., Robertson, B. C., & Kempenaers, B. (2000). A differential DNA extraction method for sperm on the perivitelline membrane of avian eggs. *Molecular Ecology*, *9*(12), 2149–2150. https://doi.org/10.1046/j.1365-294x.2000.01114.x

Castro, I., Mason, K. M., Armstrong, D. P., & Lambert, D. M. (2004). Effect of extra-pair paternity on effective population size in a reintroduced population of the endangered Hihi, and potential for behavioural management. *Conservation Genetics*, *5*(3), 381–393. https://doi.org/10.1023/B:COGE.0000031146.51681.b0

Cichoń, M., Sendecka, J., & Gustafsson, L. (2005). Male-biased sex ratio among unhatched eggs in great tit *Parus major*, blue tit *P. caeruleus* and collared flycatcher *Ficedula albicollis*. *Journal of Avian Biology*, *36*(5), 386–390. https://doi.org/10.1111/j.0908-8857.2005.03589.x

Clutton-Brock, T., & Sheldon, B. C. (2010). Individuals and populations: The role of long-term, individual-based studies of animals in ecology and evolutionary biology. *Trends in Ecology & Evolution*, *25*(10), 562–573. https://doi.org/10.1016/j.tree.2010.08.002

Cook, M. I., Beissinger, S. R., Toranzos, G. A., Rodriguez, R. A., & Arendt, W. J. (2005). Microbial infection affects egg viability and incubation behavior in a tropical passerine. *Behavioral Ecology*, *16*(1), 30–36. https://doi.org/10.1093/beheco/arh131

Cooney, C. R., Sheard, C., Clark, A. D., Healy, S. D., Liker, A., Street, S. E., Troisi, C. A., Thomas, G. H., Székely, T., Hemmings, N., & Wright, A. E. (2020). Ecology and allometry predict the evolution of avian developmental durations. *Nature Communications*, *11*(1), Article 1. https://doi.org/10.1038/s41467-020-16257-x

Davison, A., & Chiba, S. (2003). Laboratory temperature variation is a previously unrecognized source of genotyping error during capillary electrophoresis. *Molecular Ecology Notes*, *3*(2), 321–323. https://doi.org/10.1046/j.1471-8286.2003.00418.x

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

Dawson, D. A., Brekke, P., Dos Remedios, N., & Horsburgh, G. J. (2015). A marker suitable for sex-typing birds from degraded samples. *Conservation Genetics Resources*, *7*(2), 337–343. https://doi.org/10.1007/s12686-015-0429-3

Dawson, D. A., Horsburgh, G. J., Küpper, C., Stewart, I. R. K., Ball, A. D., Durrant, K. L., Hansson, B., Bacon, I., Bird, S., Klein, Á., Krupa, A. P., Lee, J.-W., Martín-Gálvez, D., Simeoni, M., Smith, G., Spurgin, L. G., & Burke, T. (2010). New methods to identify conserved microsatellite loci and develop primer sets of high cross-species utility—as demonstrated for birds. *Molecular Ecology Resources*, *10*(3), 475–494. https://doi.org/10.1111/j.1755-0998.2009.02775.x

DNeasy Blood & Tissue Kits. (n.d.). Retrieved from https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/dneasy-blood-and-tissue-kit/

Duntsch, L., Whibley, A., de Villemereuil, P., Brekke, P., Bailey, S., Ewen, J. G., & Santure, A. W. (2023). Genomic signatures of inbreeding depression for a threatened Aotearoa New Zealand passerine. *Molecular Ecology*, *32*(8), 1893–1907. https://doi.org/10.1111/mec.16855

DuRant, S. E., Hopkins, W. A., Carter, A. W., Kirkpatrick, L. T., Navara, K. J., & Hawley, D. M. (2016). Incubation temperature causes skewed sex ratios in a precocial bird. *Journal of Experimental Biology*, *219*(13), 1961–1964. https://doi.org/10.1242/jeb.138263

Eiby, Y. A., Wilmer, J. W., & Booth, D. T. (2008). Temperature-dependent sex-biased embryo mortality in a bird. *Proceedings of the Royal*

Society of London B: Biological Sciences, *275*(1652), 2703–2706. https://doi.org/10.1098/rspb.2008.0954

English, W. B., Schamel, D., Tracy, D. M., Westneat, D. F., & Lank, D. B. (2014). Sex ratio varies with egg investment in the red-necked phalarope (*Phalaropus lobatus*). *Behavioral Ecology and Sociobiology*, *68*(12), 1939–1949. https://doi.org/10.1007/s00265-014-1800-1

Etter, P. D., Bassham, S., Hohenlohe, P. A., Johnson, E. A., & Cresko, W. A. (2011). SNP discovery and genotyping for evolutionary genetics using RAD sequencing. In V. Orgogozo & M. V. Rockman (Eds.), *Molecular methods for evolutionary genetics* (pp. 157–178). Humana Press. https://doi.org/10.1007/978-1-61779-228-1_9

Ewen, J. G., Armstrong, D. P., Ebert, B., & Hansen, L. H. (2004). Extra-pair copulation and paternity defense in the hihi (or stitchbird) *Notiomystis cincta*. *New Zealand Journal of Ecology*, *28*(2), 233–240.

Ewen, J. G., Armstrong, D. P., & Lambert, D. M. (1999). Floater males gain reproductive success through extrapair fertilizations in the stitchbird. *Animal Behaviour*, *58*(2), 321–328. https://doi.org/10.1006/anbe.1999.1150

Fernando, P., Evans, B. J., Morales, J. C., & Melnick, D. J. (2001). Electrophoresis artefacts—A previously unrecognized cause of error in microsatellite analysis. *Molecular Ecology Notes*, *1*(4), 325–328. https://doi.org/10.1046/j.1471-8278.2001.00083.x

Goossens, B., Waits, L. P., & Taberlet, P. (1998). Plucked hair samples as a source of DNA: Reliability of dinucleotide microsatellite genotyping. *Molecular Ecology*, *7*(9), 1237–1241. https://doi.org/10.1046/j.1365-294x.1998.00407.x

Grafen, A. (1988). On the uses of data on lifetime reproductive success. In *Reproductive success* (pp. 454–485). University of Chicago Press.

Hemmings, N., & Evans, S. (2020). Unhatched eggs represent the invisible fraction in two wild bird populations. *Biology Letters*, *16*(1), 20190763. https://doi.org/10.1098/rsbl.2019.0763

Jensen, C. (1969). Ultrastructural changes in the avian vitelline membrane during embryonic development. *Development*, *21*(3), 467–484. https://doi.org/10.1242/dev.21.3.467

Jones, O. R., & Wang, J. (2010). COLONY: A program for parentage and sibship inference from multilocus genotype data. *Molecular Ecology Resources*, *10*(3), 551–555. https://doi.org/10.1111/j.1755-0998.2009.02787.x

Kato, T., Matsui, S., Terai, Y., Tanabe, H., Hashima, S., Kasahara, S., Morimoto, G., Mikami, O. K., Ueda, K., & Kutsukake, N. (2017). Male-specific mortality biases secondary sex ratio in Eurasian tree sparrows Passer montanus. *Ecology and Evolution*, *7*(24), 10675. https://doi.org/10.1002/ece3.3575

Kowalska, E., Kucharska-Gaca, J., Kuźniacka, J., Lewko, L., Gornowicz, E., Biesek, J., & Adamski, M. (2021). Egg quality depending on the diet with different sources of protein and age of the hens. *Scientific Reports*, *11*(1), Article 1. https://doi.org/10.1038/s41598-021-82313-1

Margalida, A. (2017). Importance of long-term studies to conservation practice: The case of the bearded vulture in the Pyrenees. In J. Catalan, J. M. Ninot, & M. M. Aniz (Eds.), *High mountain conservation in a changing world* (pp. 343–383). Springer International Publishing. https://doi.org/10.1007/978-3-319-55982-7_15

Marshall, A. F., Balloux, F., Hemmings, N., & Brekke, P. (2023). Systematic review of avian hatching failure and implications for conservation. *Biological Reviews*, *98*(3), 807–832. https://doi.org/10.1111/brv.12931

Martín-Gálvez, D., Peralta-Sánchez, J. M., Dawson, D. A., Martín-Platero, A. M., Martínez-Bueno, M., Burke, T., & Soler, J. J. (2011). DNA sampling from eggshell swabbing is widely applicable in wild bird populations as demonstrated in 23 species. *Molecular Ecology Resources*, *11*(3), 481–493. https://doi.org/10.1111/j.1755-0998.2010.02953.x

Morland, F. (2023). *Early embryo DNA data and code*. https://doi.org/10.5281/zenodo.10011626

Murray, J. R., Varian-Ramos, C. W., Welch, Z. S., & Saha, M. S. (2013). Embryological staging of the Zebra Finch, *Taeniopygia guttata*. *Journal of Morphology*, *274*(10), 1090–1110. https://doi.org/10.1002/jmor.20165

Nakahama, N., & Isagi, Y. (2017). Availability of short microsatellite markers from butterfly museums and private specimens. *Entomological Science*, *20*(1), 3–6. https://doi.org/10.1111/ens.12215

Narina, S. S., d'Orgeix, C. A., & Sayre, B. L. (2011). Optimization of PCR conditions to amplify microsatellite loci in the bunchgrass lizard (*Sceloporus slevini*) genomic DNA. *BMC Research Notes*, *4*, 26. https://doi.org/10.1186/1756-0500-4-26

Neff, B. D., Fu, P., & Gross, M. R. (2000). Microsatellite multiplexing in fish. *Transactions of the American Fisheries Society*, *129*(2), 584–593. https://doi.org/10.1577/1548-8659(2000)129<0584:MMIF>2.0.CO;2

Pemberton, J. M. (2008). Wild pedigrees: The way forward. *Proceedings of the Royal Society of London B: Biological Sciences*, *275*(1635), 613–621. https://doi.org/10.1098/rspb.2007.1531

Queller, D. C., Strassmann, J. E., & Hughes, C. R. (1993). Microsatellites and kinship. *Trends in Ecology & Evolution*, *8*(8), 285–288. https://doi.org/10.1016/0169-5347(93)90256-O

Sample Requirements. (n.d.). Retrieved from https://rtsf.natsci.msu.edu/genomics/sample-requirements/

Savage, J. L., Crane, J. M. S., Team, K. R., & Hemmings, N. (2022). Low hatching success in the critically endangered kākāpō is driven by early embryo mortality not infertility. *Animal Conservation*, *25*(3), 352–360. https://doi.org/10.1111/acv.12746

Schrader, C., Schielke, A., Ellerbroek, L., & Johne, R. (2012). PCR inhibitors – Occurrence, properties and removal. *Journal of Applied Microbiology*, *113*(5), 1014–1026. https://doi.org/10.1111/j.1365-2672.2012.05384.x

Shbailat, S. J., Qanadilo, S., & Al-Soubani, F. A. (2016). Protease activity in the egg yolk during the development of *Meleagris gallopavo* (Galliformes: Phasianidae) embryos. *Italian Journal of Zoology*, *83*(3), 291–297. https://doi.org/10.1080/11250003.2016.1206976

Strausberger, B. M., & Ashley, M. V. (2001). Eggs yield nuclear DNA from egg-laying female cowbirds, their embryos and offspring. *Conservation Genetics*, *2*(4), 385–390. https://doi.org/10.1023/A:1012526315617

Taig-Johnston, M., Strom, M. K., Calhoun, K., Nowak, K., Ebensperger, L. A., & Hayes, L. (2017). The ecological value of long-term studies of birds and mammals in Central America, South America and Antarctica. *Revista Chilena de Historia Natural*, *90*(1), 7. https://doi.org/10.1186/s40693-017-0070-5

Wang, J. (2019). Pedigree reconstruction from poor quality genotype data. *Heredity*, *122*(6), 719–728. https://doi.org/10.1038/s41437-018-0178-7

Whittingham, L. A., & Dunn, P. O. (2001). Survival of extrapair and within-pair young in tree swallows. *Behavioral Ecology*, *12*(4), 496–500. https://doi.org/10.1093/beheco/12.4.496

Williams, T., Soni, S., White, J., Can, G., & Javan, G. T. (2015). Evaluation of DNA degradation using flow cytometry: Promising tool for post-mortem interval determination. *The American Journal of Forensic Medicine and Pathology*, *36*(2), 104–110. https://doi.org/10.1097/PAF.0000000000000146

Wultsch, C., Waits, L. P., & Kelly, M. J. (2014). Noninvasive individual and species identification of jaguars (*Panthera onca*), pumas (*Puma concolor*) and ocelots (*Leopardus pardalis*) in Belize, Central America using cross-species microsatellites and faecal DNA. *Molecular Ecology Resources*, *14*(6), 1171–1182. https://doi.org/10.1111/1755-0998.12266

How to cite this article: Morland, F., Patel, S., Santure, A. W., Brekke, P., & Hemmings, N. (2023). Including the invisible fraction in whole population studies: A guide to the genetic sampling of unhatched bird eggs. *Methods in Ecology and Evolution*, *00*, 1–11. https://doi.org/10.1111/2041-210X.14242