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Short communication

True fertilisation failure in captivity is rare

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ABSTRACT

Species recovery through captive breeding can be hindered by low reproductive success. However, we know little about the drivers of early reproductive failure in captive populations, due to difficulties distinguishing fertilisation failure from early embryo mortality in most animals. Here, we apply advanced fertility diagnostics on unhatched eggs from 30 avian captive-breeding programs, to assess true rates of fertilisation failure. We find that fertilisation failure is rare across all species, and the main driver of early reproductive failure is early embryo mortality. We also find that macroscopic examination of undeveloped eggs inflates estimates of fertilisation failure rates in breeding programmes. Finally, we find no evidence that fertilisation failure rates are higher in threatened than non-threatened captive birds, providing hope that with careful management, hatching outcomes may be improved in threatened captive populations. Our results show that accurate fertility diagnosis in managed oviparous species provides crucial information on individual reproductive potential, helping the design of more appropriate management interventions to improve recovery.

1. Introduction

Captive breeding of animals is a commonly recommended conservation intervention for species under threat of extinction and plays an important role in supporting biomedical research and global food security (CPSG, 2015; Drazen et al., 2024; Davis and White, 2020). However, reproductive rates of captive animals are often low. Captive birds experience significantly higher rates of hatching failure than wild populations (Marshall et al., 2023), and captive-born individuals are 42 % less likely than their wild counterparts to reproduce successfully (Farquharson et al., 2018). Understanding how and why captivity impacts reproductive success is, therefore, a priority for conservation policy and society more widely.

Captivity may impact reproductive output in several ways. Inbreeding, the accumulation of deleterious mutations, and adaptation

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to captivity (Frankham, 2008) can lead to genetic changes that influence reproductive success, and altered nutrition or social environment may negatively affect reproductive processes. Normal reproductive behaviour (e.g., mechanisms of sexual selection) may be suppressed in captivity (Saint Jalme et al., 1996; Driscoll, 2008) due to a lack of mate choice and competition (Wetton and Parkin, 1991; Zeh and Zeh, 1997; Yuta et al., 2018). Issues with sexual behaviour and/or gamete function that arise due to captivity will most likely manifest as low fertilisation success, whereas genetic issues are more likely to impact embryo development and survival (Assersohn et al., 2021).

Given the different potential drivers of fertilisation failure versus embryo mortality, understanding the consequences of captivity for these two forms of reproductive failure is crucial for conservation management. For example, early identification of individuals or pairs that do not produce fertilised eggs allows for targeted restructuring of breeding regimes and removal of infertile individuals from breeding programs. For many taxa – including mammals – differentiating early embryonic loss (e.g., failure of implantation) from unsuccessful fertilisation can be impossible due to fetal resorption following death in early gestation (Flores et al., 2014). However, in birds and reptiles, methods for determining the fertilisation status of unhatched eggs – which involve microscopically examining failed egg contents to detect sperm that have penetrated the egg and embryonic cells in the germinal disc (Birkhead et al., 2008; Lavigne et al., 2024) – provide an opportunity to answer mechanistic questions about the impact of captivity on fertilisation success.

Identifying whether reproductive failure stems from fertilisation failure or embryo death has broader implications for our understanding of the drivers of hatching failure and its consequences in both wild and captive populations. Hatching failure is a growing concern for threatened bird species; recent estimates place the mean hatching failure rate across birds at approximately 17 %, with threatened populations and those undergoing management experiencing much higher rates (Marshall et al., 2023). Microscopic examination of failed eggs has only been carried out in a small number of wild bird populations (Birkhead et al., 1995; Kempenaers et al., 1999; Hemmings et al., 2012; Kato et al., 2017; Hemmings and Evans, 2020; Morland et al., 2024; Savage et al., 2021). While these consistently suggest that embryo mortality is the main cause of failure, with fertilisation failure being rare, quantification of fertilisation failure rates across a wider range of species and breeding contexts is essential for understanding the generalisability of these results (Evans and Postma, 2025).

In this study, we apply microscopic methods for accurately determining the fertility status of unhatched bird eggs developed by Birkhead et al. (2008), hereafter referred to as 'fluorescence microscopy methods', to undeveloped eggs from captive populations of various bird species in New Zealand and the United Kingdom to (1) identify true rates of fertilization failure among eggs that fail to hatch in captivity, and (2) test whether fertilisation failure rates increase with threat status.

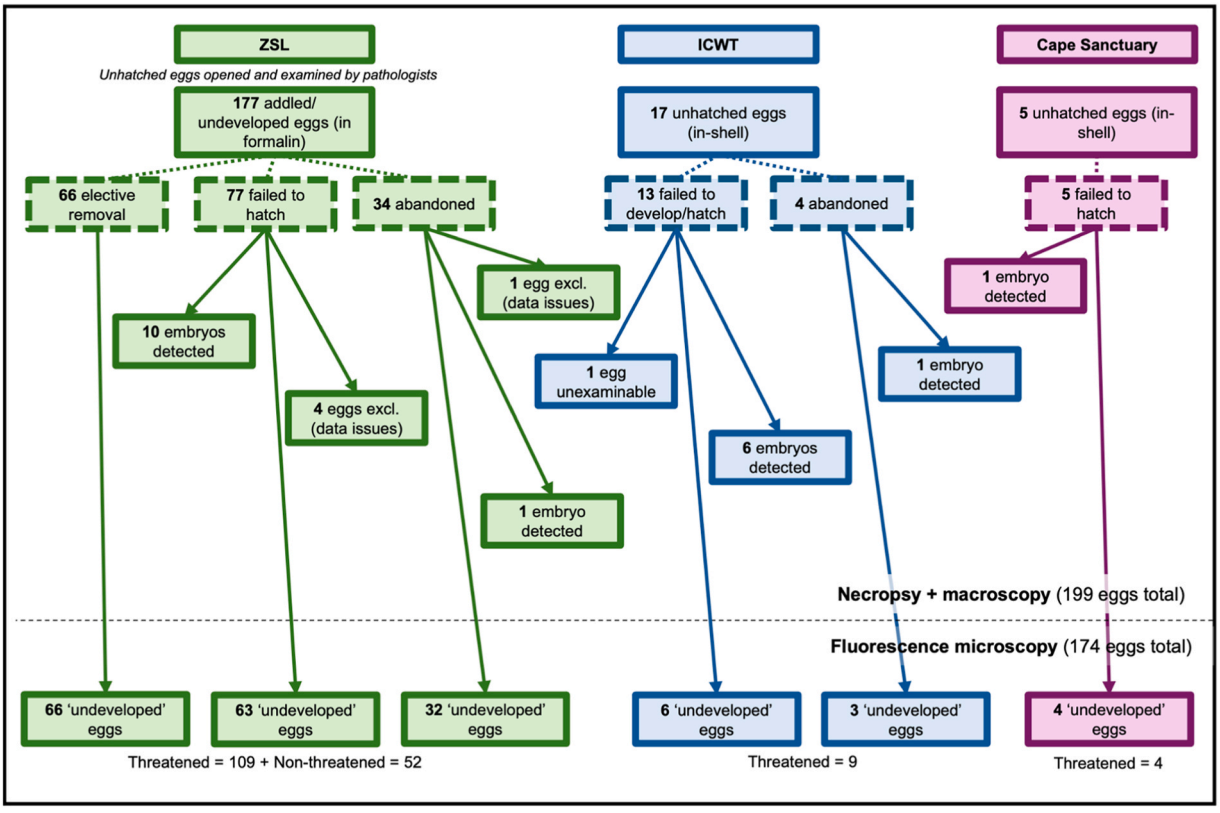

Fig. 1. Failed eggs processing flowchart from necropsy to microscopy with sample sizes/preservation for each step. Each institution is highlighted in a different colour. Dashed boxes show the underlying reasons for egg loss at each institution.

2. Materials and methods

2.1. Study populations

2.1.1. UK ZSL living bird collections

UK samples were collected from the Zoological Society of London (ZSL) Living Bird collection, which holds ~150 bird species across two sites: London Zoo and Whipsnade Zoo. Almost all eggs that fail to hatch are collected and examined macroscopically as part of a necropsy by ZSL veterinary pathologists. Each egg is examined externally for shell damage or excess mineralization, then opened, at the blunt end to check for a mature embryo (Rideout, 2012). The egg contents are then emptied and the embryo (if present), internal membranes, and fluid sacs, are examined. Visible embryos are fixed in 10 % buffered formalin and archived. If no embryo is found, egg contents are checked for the presence of an embryonic (germinal) disc and blood vessel formation, and if no other evidence of embryo development is found, eggs are ultimately identified as 'lacking in gross evidence of fertility'.

Between January 2019 andJune 2021, 177 unhatched eggs examined by the ZSL pathology team, from 27 different species, were found to be either (a) addled (decomposed) or (b) lacking in embryo or other clear sign of development (Fig. 1). All were preserved in 10 % formalin solution. Sixty-six eggs had been electively removed early in incubation (generally within 24–48 h of oviposition) as part of management protocols. Electively removed eggs received little or no parental incubation and were humanely euthanized via isoflurane gas exposure. Electively removed eggs cannot be considered as truly unhatched (failed) eggs, so were not included in our estimations of natural fertilisation and embryo death rates. However, they were included in our comparison of gross macroscopic inspection versus fluorescence microscopy for fertility diagnosis.

Seventy-seven eggs had undergone full incubation and failed to hatch, and 34 were known or suspected to have been abandoned by the parents at an unknown point during the incubation period. Abandoned eggs were also excluded from our estimations of natural fertilisation and embryo death rates and included in the fertility diagnosis accuracy test only, because abandoned eggs would not typically be included in our pre-defined estimations of hatching failure (the proportion of eggs present at the end of the incubation period that fail to hatch, excluding eggs lost due to predation, desertion, accident, extreme weather, or disappearance). Of the 77 eggs that failed for unknown reasons, three contained visible embryos that were detected during the necropsy exam and mistakenly sent for further investigation, and two didn't receive a macroscopic assessment during the necropsy so comparison was not possible. These 5 eggs were excluded from all analyses (Fig. 1).

2.2. Aotearoa New Zealand conservation captive breeding programs

Aotearoa New Zealand samples were taken from the Isaac Conservation and Wildlife Trust (ICWT) in Christchurch, South Island, and Cape Sanctuary in Hawke's Bay, North Island. Both facilities are involved in the conservation of several native New Zealand species which are managed through recovery programs in conjunction with the Department of Conservation. All successful and unsuccessful breeding attempts are recorded. Eggs are typically left with parents to be hatched and reared, and eggs that fail to hatch are left in the nest well beyond the predicted incubation period before collection to reduce the risk of removing a viable egg or disturbing hatched chicks. At ICWT, some eggs are removed from parents for artificial incubation to encourage replacement clutches (e.g., in kakī) or to protect eggs from harm (e.g. from aggressive breeding pairs). Artificially incubated eggs are monitored throughout incubation using candling methods and may be removed before the predicted hatch date if they show no signs of development. Between October 2019 andJanuary 2020, in-shell necropsies were conducted followed by fluorescence microscopy examination on 22 eggs, across three species, that appeared undeveloped from external examination. 18 of these had failed to hatch by the end of the estimated incubation period or had not progressed during artificial incubation, and four were abandoned before or during incubation (Fig. 1).

2.3. Egg examinations

Formalin-fixed egg contents from the ZSL Living Bird Collection were examined at the University of Sheffield. Egg contents were poured into petri dishes and checked for any small and/or degraded embryos that may have been previously missed. If no embryo was found, fertility status was determined by staining the germinal disc and the perivitelline layer of the ovum with Hoechst 33342 fluorescent DNA stain (0.05 mg ml^{-1}) and examining these using a Leica DMLB fluorescence microscope with a BP 340–380 excitation filter, LP 425 suppression filter, and darkfield filter, to detect: (1) nuclei of embryonic cells in the germinal disc, (2) sperm in the perivitelline layer, and (3) penetration holes in the perivitelline layer made by sperm entering the ovum (following Birkhead et al., 2008).

Following Hemmings and Evans (2020), fertilisation success was determined based on the presence of nuclei from embryonic cells (demonstrating that an embryo had started to develop). The presence of perivitelline sperm or penetration holes (demonstrating the sperm had reached and entered the egg, respectively) was used as supporting evidence only. Eggs were conservatively classified as unfertilised only if the germinal disc was located and both embryonic and sperm cells were absent, to minimize the risk of incorrect diagnoses. If the germinal disc was not confidently located, fertility status was designated as 'inconclusive', since embryonic cells may have been missed. If the egg contents had degraded to the point where no examinable material remained, the fertility status was classified as 'unknown'. For analysis purposes, these two categories were combined into a single 'undetermined' category. A full description of the fertility diagnosis criteria is provided in Table S1.

Eggs from ICWT and Cape Sanctuary were opened using small scissors or blunt forceps and emptied into petri dishes containing sterile phosphate-buffered saline (PBS) solution. Visible embryos were isolated. If no embryo was found, the egg contents were fixed in

5 % formalin solution prior to fluorescence microscopy examination as previously described, with the exception that Aotearoa eggs were examined at the University of Auckland using a Leica DMR fluorescence microscope fitted with a BP 340–380 excitation filter, LP 425 suppression filter, and darkfield filter.

The proportion of fertilised and unfertilised eggs in our study sample that came from threatened (Critically Endangered, Endangered, and Vulnerable) and non-threatened (Near Threatened and Least Concern) species were also compared, classified according to the IUCN Red List (www.iucnredlist.org; Table S2) across all institutions in both countries.

Results were statistically analysed using pairwise chi-squared tests of independence with a Monte Carlo simulation using the 'chisq. test' function from the package *stats* v4.2.1 (R Core Team, 2022), and the alpha level used for statistical significance was 0.05.

3. Results

3.1. True rates of infertility in captive birds

Across all unhatched egg samples (n = 199), 19 were excluded because microscopy revealed visible embryos (11 from ZSL, 7 from ICWT, 1 from Cape Sanctuary), five were excluded due to data issues, and one sample from ICWT was excluded because it had no examinable material. This left 174 samples that were examined using fluorescence microscopy and included in our estimates of fertility diagnosis accuracy and fertilisation/embryo death rates.

Initial fertility diagnoses based on macroscopic necropsy examination suggested that just 5.2 % (9/174) of all undeveloped eggs were fertilised, while 75.9 % (132/174) were found to have no evidence of fertilisation. 19.0 % (33/174) were classified as 'undetermined'. However, fluorescence microscopy examination revealed that 65.5 % (114/174) of these eggs were fertilised, and just 8.6 % (15/174) were unfertilised, with 25.9 % (45/174) classed as 'undetermined' (Fig. 2; Table S3). For eggs where both necropsy and microscopy methods were successfully applied (n = 103, see Fig. 2), there was a significant difference in fertility diagnosis (X^2 = 143.6, *d.f.* = 1, *p-value* < 0.001). Considering addled eggs only, necropsy examination diagnosed 5.6 % (2/36) as fertilised, found no evidence of fertilisation in 86.1 % (31/36), leaving 8.3 % (3/36) undetermined. In contrast, fluorescence microscopy diagnosed 52.8 % (19/36) as fertilised and 5.6 % (2/36) as unfertilised, but the fertility status of the remaining 41.7 % (15/36) could not be confidently determined. In those addled eggs where both methods were successfully applied (n = 19), there was a significant difference in fertility status (X^2 = 23.7, *d.f.* = 1, *p-value* < 0.001). Therefore, necropsy examination frequently led to mistaken diagnoses of fertilisation failure (Fig. 2).

When limited to eggs that failed for unknown reasons (i.e., excluding abandoned or electively removed eggs), necropsies found no evidence of fertilisation in 86.3 % (63/73) of eggs, diagnosed 6.8 % (5/73) as fertilised, and 6.8 % (5/73) remained undetermined. In contrast, fluorescence microscopy methods diagnosed just 4.1 % (3/73) as unfertilised, 61.6 % (45/73) as fertilised, and 34.2 % (25/73) as undetermined. For those eggs that failed naturally, where both methods could be applied (n = 44) there was a significant difference in the fertility diagnoses (X^2 = 59.0, *d.f.* = 1, *p-value* < 0.001). The proportion of undeveloped eggs from captive populations that experienced fertilisation failure was therefore in the range of 4.1–38.4 % (depending on the true fertility status of undetermined

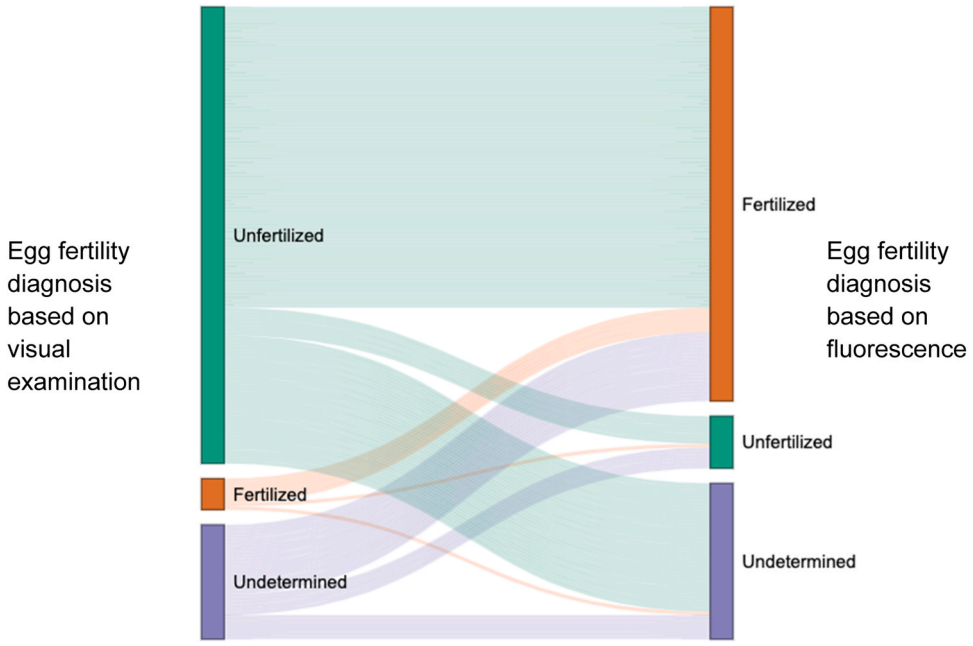

Fig. 2. Suspected fertility status of eggs from the ZSL living bird collection, ICWT, and cape sanctuary captive-breeding programs based on visual examination (left) and fluorescence microscopy examination (right). Each line represents one egg (n = 174).

eggs; minimum range value assumes all undetermined eggs were fertilised; maximum value assumes all undetermined eggs were unfertilised). Of the unhatched eggs originally deemed as showing no evidence of fertilisation during necropsy, only 4.8 % (3/63) were confirmed as unfertilised using fluorescence microscopy, while 57.1 % (36/63) were found to be fertilised and 38.1 % (24/63) were undetermined.

3.2. Impact of species threat status on fertilisation rates

In threatened (Critically Endangered, Endangered, or Vulnerable) species, fluorescence microscopy revealed 66.4 % (81/122) undeveloped eggs to be fertilised, 9.8 % (12/122) unfertilised, and 23.8 % (29/122) undetermined. In non-threatened (Near Threatened and Least Concern) species, 63.5 % (33/52) of undeveloped eggs were fertilised, 5.8 % (3/52) were unfertilised, and 30.8 % (16/52) were undetermined. There was no significant difference in the fertility status of eggs from threatened and non-threatened species based on successful fluorescence microscopy (X^2 ($n = 129$) $= 0.5$, *d.f.* $= 1$, *p-value* $= 0.47$). The proportion of all undeveloped eggs that experienced fertilisation failure ranged from 9.8–33.6 % for threatened species, while for non-threatened species it ranged from 5.8–36.5 %.

When limited to eggs that failed for unknown reasons (i.e., excluding those abandoned or electively removed for management purposes), fluorescence microscopy revealed 66.0 % (33/50) of undeveloped eggs from threatened species to be fertilised, 6.0 % (3/50) unfertilised, and 28.0 % (14/50) undetermined, while 52.2 % (12/23) of undeveloped eggs from non-threatened species were fertilised, 47.8 % (11/23) were undetermined, and no eggs were confirmed as unfertilised. The difference in fertility status between threatened and non-threatened species' eggs was not significant (X^2 ($n = 48$) $= 1.1$, *d.f.* $= 1$, *p-value* $= 0.3$). Therefore, the proportion of undeveloped eggs that experienced fertilisation failure ranged from 6.0–34.0 % for threatened species, and 0.0–47.8 % for non-threatened species.

4. Discussion

Our results support the hypothesis that rates of true fertilisation failure (i.e., the proportion of unfertilised eggs) are overestimated in captive populations across a wide range of bird species. Most eggs for which existing management protocols found no evidence of fertilisation were ultimately found to be fertilised, and levels of true fertilisation failure appeared to be similar across threatened and non-threatened captive species. Taken together, our results suggest that egg necropsy examinations undertaken as part of captive management programmes are likely to inflate estimates of fertilisation failure and underestimate the incidence of early embryo mortality, which could have consequences for management decisions.

4.1. Rates of fertilisation failure in captivity

We found true fertilisation failure to be rare and overestimated in captive bird populations. Over two thirds of eggs assessed as showing no sign of fertilisation during necropsy were found to be fertilised when examined microscopically, dramatically changing estimated rates of fertilisation success in these populations. Considering the large proportion of eggs that either fail during late-stage embryo development or hatch (neither of which is included in this study), this suggests that fertilisation failure plays a relatively minor role in determining reproductive outcomes in captive bird populations.

A considerable proportion of undeveloped eggs in this study could not be conclusively assigned a fertility status using microscopic methods, due to the deterioration of the egg contents. It is unlikely that this subset of eggs is more likely to have experienced fertilisation failure; in fact, eggs that have undergone some degree of early embryo development tend to degrade more quickly than those that have not (Morland et al., 2024). However, under the implausible assumption that all undiagnosed eggs were unfertilised, the overall rate of fertilisation failure in our sample would be 37 %, which while relatively high, is similar to previous estimates for wild house sparrow (*Passer domesticus*) (Birkhead et al., 1995), wild managed kākāpō (*Strigops habroptilus*) (Savage et al., 2021), and captive helmeted honeyeater (*Lichenostomus melanops cassidix*) (Hemmings et al., 2012), and lower than that found for captive orange-bellied parrot (*Neophema chrysogaster*), and captive Spix's macaw (*Cyanopsitta spixii*) (Hemmings et al., 2012). Alternatively, if most undetermined eggs were fertilised, the rate of fertilisation failure misdiagnosis would be even higher than we have reported.

Our results do not support the hypothesis that captive populations are more likely to experience fertilisation failure than wild populations (Hemmings et al., 2012), and instead suggest that fertilisation failure rates across birds are generally overestimated, regardless of breeding or management context. However, some species or populations may have greater fertility issues than others; we were unable to compare between species due to sampling constraints, but future work considering intra- and inter-specific variation in fertilisation rates of wild and captive-laid eggs would be valuable.

4.2. Do fertilisation rates differ between threatened and non-threatened captive birds?

We found no evidence that fertility rates differed between threatened and non-threatened bird species, adding to the mounting evidence that hatching failure is mainly driven by early embryo mortality and not low fertility in birds (Morland et al., 2024). These findings have important implications for captive breeding management. Without accurate information on egg fertilisation rates, pairs producing a high rate of undeveloped eggs may be split and paired with new mates, under the assumption that they have fertility issues. However, this may be detrimental, as several studies show that reproductive success, particularly fertilisation success - - improves the longer pairs are kept together (Brosset, 1981; Olsen and Olsen, 1981; Wiemeyer, 1981; Fournier et al., 2007). In extreme

cases, individuals may be removed from the breeding pool altogether, which is particularly concerning for endangered species, where removal of individuals could greatly impact genetic health and population recovery (Williams and Hoffman, 2009). Early embryo survival, on the other hand, may be influenced by external factors such as incubation conditions, which can potentially be improved via management interventions. Accurately distinguishing early embryo mortality from fertilisation failure can therefore increase the efficiency and effectiveness of management strategies, allowing more targeted interventions and improving breeding outcomes.

In summary, key information on fertilisation rates in captive populations will be missed if unhatched eggs are only examined macroscopically, as they currently are under most management protocols. Our findings are relevant to all oviparous taxa, given the recent application of fluorescence microscopy methods to turtle, tortoise, and crocodile eggs (Croyle et al., 2016; Lavigne et al., 2024; Augustine, 2017). Improving our understanding of the causes of hatching failure in threatened populations will allow us to develop more effective mitigation strategies, improving hatching success and increasing population numbers.

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CRediT authorship contribution statement

Patricia Brekke: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ashleigh Marshall:** Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Anne Richardson:** Writing – review & editing, Resources, Project administration. **Edmund Flach:** Writing – review & editing, Resources, Project administration, Methodology, Data curation. **Liz Brown:** Writing – review & editing, Supervision, Resources, Project administration, Data curation. **François Balloux:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Nicola Hemmings:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Gary Ward:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Simon Spiro:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation. **Tammy E. Steeves:** Writing – review & editing, Supervision, Project administration, Methodology, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics approval

Ethics approval for dissecting UK eggs was issued through the Zoological Society of London Ethics Committee (UK). New Zealand egg samples were collected during routine management checks by the New Zealand Department of Conservation (DOC) at the captive breeding facilities in Twizel (DOC) and Christchurch (Isaac Conservation and Wildlife Trust), New Zealand, by approval of the DOC Animal Ethics Committee (AEC #283).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.gecco.2025.e03687.

Data availability

Data are available in the Supplementary Material

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