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Low hatching success in the critically endangered kākāpō is driven by early embryo mortality not infertility

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Abstract

In many endangered species, reproductive failure is a major barrier to recovery. The critically endangered kākāpō *Strigops habroptilus* exemplifies this challenge: 61% of their eggs fail to hatch, and of these 73% show no sign of development. Undeveloped eggs have previously been attributed to male infertility, but recent studies of non-threatened bird species suggest fertilisation failure is rare in the wild. The underlying causes of fertilisation failure and embryo death differ, so distinguishing between them is essential for effective conservation management. Here we show that the majority (72%, $n = 124$) of undeveloped kākāpō eggs are fertilised, and combine this with conservation programme data on natural copulations, artificial inseminations, and paternity of developed eggs, to generate the most precise estimate to date of fertility in a wild population. We also demonstrate, for the first time in a wild bird, that artificial insemination results in greater numbers of sperm reaching the egg.

Introduction

Hatching failure is a common problem for endangered birds, with dramatic consequences for population viability (Jamieson & Ryan, 2000; Heber & Briskie, 2010). Hatching failure can result from either fertilisation failure or embryo mortality, but these have different proximate causes: for example, fertilisation failure may result from poor sperm quality or abnormal mating behaviour (Bakst *et al*., 1994; Lifjeld *et al*., 2007), while embryo mortality is more likely driven by parental genetic incompatibilities/inbreeding (Sittmann, Abplanalp & Fraser, 1966; Hemmings, Slate & Birkhead, 2012a), female condition (Coleman & Siegel, 1966; Lerner *et al*., 1993), and/or environmental factors (Romanoff, 1949; Beissinger, Cook & Arendt, 2005). Distinguishing fertilisation failure from early embryo mortality is therefore crucial for developing effective conservation interventions, but relatively few studies or conservation programmes have attempted to do this; most assume that undeveloped eggs are unfertilised (Assersohn *et al*., 2021).

Distinguishing embryo mortality from infertility is especially challenging if mortality occurs in the first few days of development, which in well-studied domestic poultry is when most embryos die (Christensen, 2001). At this stage, the embryo is a tiny disc of cells, easily mistaken for an unfertilised blastodisc on visual inspection (Kosin, 1944), and hence traditional macroscopic methods of examining eggs can over-estimate fertilisation failure in undeveloped eggs. The few studies that have microscopically inspected unhatched eggs suggest that early embryo death may be the main cause of hatching failure in wild birds (e.g. Hemmings & Evans, 2020), but such studies remain uncommon, particularly in threatened populations where hatching failure is of greatest concern.

The kākāpō *Strigops habroptilus* exemplifies the reproductive challenges faced by many threatened bird species across the world. This large, flightless, nocturnal parrot was once common throughout its endemic New Zealand, but rapidly declined after human settlement due to introduced predators, hunting and land clearance (Powlesland, Merton & Cockrem, 2006), particularly after European colonisation (Bergner *et al*., 2016). Conservation efforts began as early as 1894, but by 1995, their total population had fallen to 51 individuals. Under intensive management kākāpō numbers have since increased, but their irregular breeding seasons and lek mating system, exacerbated by high rates of reproductive failure, pose substantial challenges for management (Elliott *et al*., 2006; Powlesland, Merton & Cockrem, 2006).

Like many other threatened bird species (Briskie & Mackintosh, 2004), kākāpō eggs often fail to hatch. Approximately 61% of kākāpō eggs have failed in the last 4 decades (401/662, 1981–2019; KRT unpublished data), much higher than the average 10%–15% across birds (Koenig, 1982; Spottiswoode & Møller, 2004). Around 73% of failed eggs show no signs of development on visual inspection (293/401) and have previously been assumed to be unfertilised. As a result, management interventions aimed at addressing male infertility, such as the use of artificial insemination, have been proposed as promising solutions to the problem. However, without distinguishing between fertilisation failure and early embryo death, the mechanistic basis of hatching failure in kākāpō remains unclear. Distinguishing between these two causes of hatching failure would help identify individuals, or combinations of parents, that are susceptible to infertility and/or early offspring mortality. The resulting information will have important implications for management decisions, such as which individuals to pair in captivity and which males should be used for artificial insemination.

In 2019, the largest kākāpō breeding season on record allowed us to assess true fertility rates in kākāpō for the first time, using data from two separate breeding islands and multiple clutches per female. We examined the fertility status of undeveloped eggs using methods developed previously for other birds (Hemmings, West & Birkhead, 2012*b*), and combined this with data on kākāpō copulation events in the wild, artificial inseminations, and known parentage of developed embryos to infer patterns of individual fertility and embryo survival across the entire species.

Materials and methods

Study system

The entire kākāpō species (October 2021: 201 individuals) is managed on predator-free offshore islands, the breeding population (107 reproductively mature adults) primarily on Whenua Hou/Codfish Island (lat. −46.77, lon. 167.63) and Anchor Island (lat. −45.76, lon. 166.51). Kākāpō are lek breeders, exhibiting female-only parental care (Eason, Elliott & Merton, 2006) and skewed reproduction among males (Merton, Morris & Atkinson, 1984). All mating events are recorded by individual transmitters (Wildtech New Zealand Ltd) fitted prior to the breeding season. Male transmitters continuously monitor male activity, and activate a receiver to detect nearby female transmitters whenever the preceding ten minutes of activity is ≥96% of the maximum possible activity (i.e. when males are running, fighting or mating). While active, the receiver also records female transmitter activity, allowing non-mating encounters (with low female activity) to be excluded. Every kākāpō nest is monitored: eggs are replaced with decoys and artificially incubated, and chicks returned to nests after hatching (Elliott, Merton & Jansen, 2001).

Kākāpō reproduce only when natural food is abundant (Powlesland & Lloyd, 1994), typically every 2–4 years. Successful breeding depends on the availability of masting tree fruit, particularly podocarps such as rimu *Dacrydium cupressinum* (Powlesland *et al.*, 1992). Masting in 2018/19 was exceptional, with the highest abundance of rimu fruit since records began in 1997. The 2018/19 breeding season was both the largest and earliest on record: 252 eggs were laid, the earliest on 25 December 2018, several weeks earlier than normal. The Kākāpō Recovery Team also induced many females to produce a second clutch by removing and artificially incubating/hand-rearing their first clutch. Twelve females were artificially inseminated during the season.

Egg data collection

During artificial incubation, eggs were candled daily by experienced Kākāpō Recovery Team members and removed from the incubator after 5 days if no embryo was visible. We opened undeveloped eggs, removed the contents, then separated the yolks and preserved them in 5% formalin solution, following Hemmings *et al.* (2012*b*). In consultation with Ngāi Tahu (New Zealand South Island Māori iwi), we then shipped the preserved yolks for analysis at the University of Sheffield, UK (CITES institution GB041). In total, we obtained samples from 128 undeveloped eggs: 98% of all undeveloped eggs laid in 2019. Eggs that failed later in development (i.e. with a visible embryo) were not used in this study, but embryos were staged and DNA-sampled to ascertain paternity as part of the Kākāpō Recovery Team's standard protocols.

Egg fertility and sperm numbers

We removed yolks from formalin and rinsed them with phosphate-buffered saline solution (PBS). We located the germinal disc on the yolk, removed the overlaying perivitelline layer (PVL) using dissecting scissors, and washed the PVL in PBS before mounting on a microscope slide. We also tried to remove the germinal disc, but in many cases, it remained strongly adhered to the PVL so we examined them together. Some yolks disintegrated prior to fixation; in these cases we examined pieces of PVL found within the sample.

We examined the PVL for sperm and germinal disc samples for embryonic cells by staining them with the fluorescent DNA marker Hoechst 33342 (Thermo Fisher Scientific), and inspecting them under a fluorescence microscope with a BP 340-380 excitation filter and LP 425 suppression filter (Birkhead *et al.*, 2008). The number of sperm reaching the avian ovum is related to (a) the number inseminated (Wishart, 1987) and (b) the likelihood of hatching (Eslick & McDaniel, 1992; Hemmings & Birkhead, 2015), so we also counted the number of sperm on known areas of PVL (± 1 mm^2). We classified eggs as either fertilised (embryonic nuclei and sperm present) or unfertilised (no embryonic nuclei and few/no sperm). In total, we established the fertility status of 124/128 (97%) undeveloped eggs; four eggs were too deteriorated to reliably examine.

Statistical methods

Using generalised linear mixed models (GLMMs), we analysed the main effects of clutch (first or second), female multiple mating (one or multiple males), and artificial insemination (yes/no) on (a) egg fertility and (b) the number of PVL sperm. Our dataset had insufficient sample size to effectively explore interaction effects. We specified a binomial distribution and logit link function in the analysis of egg fertility as the response variable was binary (fertile, yes/no), and a Poisson distribution and log link in the analysis of sperm numbers as the response variable was a count (offset = log(PVL area)). We included female identity as a random effect in both models, and excluded data from third clutches because only a single female produced a third clutch. We established the most useful explanatory models of egg fertility and PVL sperm numbers by comparing the seven possible models of our three fixed effects, plus a null model that only included female identity. Models were compared using AICc and AICc weights, and we averaged all supported models (weight > 0.05) to generate a consensus model. Further methodological details, data, and code to reproduce our plots and analyses are available in Supplementary Material S1.

Results

Fertilisation failure and embryo mortality in undeveloped eggs

Of 57 reproductively mature female kākāpō, 49 nested in 2019 on Anchor Island and Whenua Hou/Codfish Island. Nests of 36 females were removed to induce a second clutch, and of these 30 re-nested. Across all clutches, 119/252 eggs were visibly fertile. The rest (133) remained undeveloped, and would previously have been assumed to be unfertilised. A total of 86 eggs hatched and 72 chicks reached juvenile age (150 days) (Fig. 1).

Of 128 undeveloped eggs examined, we found that 90 were fertilised and 34 were unfertilised. Excluding the four eggs that could not be examined due to degradation, 73% (90/124) were fertilised. The true rate of fertilisation failure across all eggs was hence 14% (34/248), considerably lower than 52% (131/252) which would have been assumed
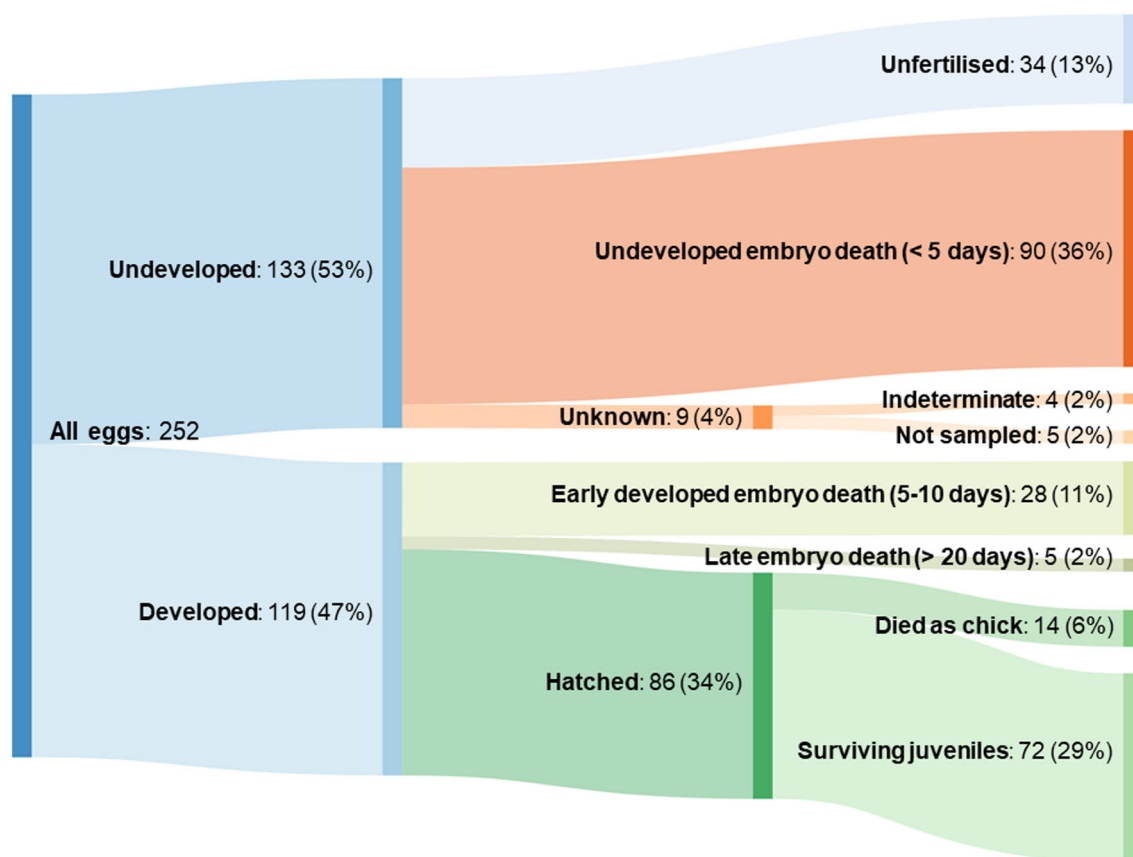
Figure 1 Visualisation of fates of all 252 eggs laid during the 2019 breeding season. Approximately equal numbers of eggs were laid on Anchor Island (123 from 21 females) and Whenua Hou/Codfish Island (129 from 28 females), and there were no differences in egg fertility or embryo death between eggs from different islands. No embryos died during the middle third of the incubation period (10–20 days). Some eggs and chicks were moved between islands for management purposes.

Table 1 (a) Comparison of possible GLMMs of fertilization failure in kākāpō, incorporating fixed effects of first/second clutch (Clutch), Artificial Insemination (AI), and female Multiple Mating (MM). All models included female identity as a random effect on intercept. Columns present AICc values, AICc weights, and the ΔAICc compared to the most parsimonious model. (b) Summary of average model; coefficients given are of the natural averages of the five models with weights greater than 0.05.

Model	AICc	ΔAICc	Weight
(a)			
Clutch + AI	179.60	0	0.442
Clutch + AI + MM	180.93	1.33	0.228
Clutch	181.57	1.96	0.166
Clutch + MM	182.84	3.24	0.088
AI	183.84	4.24	0.053
AI + MM	185.79	6.19	0.020
Null (female ID only)	190.04	10.44	0.002
MM	192.09	12.48	0.001

Averaged coefficients	Estimate	SE	*z* value	*P* value
(b)				
Intercept	2.177	0.558	3.887	<0.001
Clutch	1.503	0.619	2.416	0.016
AI	2.208	1.306	1.682	0.093
MM	0.607	0.691	0.874	0.382

without examination of undeveloped eggs. Consequently, the primary driver of hatching failure (and overall reproductive failure) in kākāpō in 2018/19 was very early embryo mortality (<day 5 of development), which occurred in at least 36% (90/252) of all eggs laid (Fig. 1). Almost half of all eggs (118/252; 47%) failed due to embryo death in the first third of the incubation period (days 0–10).

Model selection (Table 1a) indicated that the five best explanatory models of kākāpō fertilisation failure included various combinations of clutch (first/second), female multiple mating, and artificial insemination as predictors. All five models were significant improvements on a null model including no fixed effects (ΔAICc: 10.44, 9.11, 8.48, 7.20, 6.20; P (χ^2) = 0.0007, 0.0015, 0.0012, 0.0035, 0.0041) and had AICc weights greater than 0.05.

Our averaged model of fertilization failure (Table 1b) suggests that it was more common in first than second clutches (estimate = 1.503, z = 2.416, P ($|z|$) = 0.016; Fig. 2). We found no evidence that females that copulated with more than one male had greater egg fertilisation success (estimate = 0.607, z = 0.874, P ($|z|$) = 0.382), but artificially inseminated females may have had increased fertilisation success (estimate = 2.208, z = 1.682, P ($|z|$) = 0.093). Proportions of developed eggs were similar across both islands.

Individual fertility

Of 31 males known to have copulated (or used for artificial insemination), 26 (84%) fertilised at least one egg. Most males were therefore not infertile/sterile because at least one of their insemination attempts resulted in successful fertilisation. A total of 18 males produced embryos that reached at

least day five of development (based on KRT data from developed eggs). However, five male kākāpō showed no clear evidence of fertility in 2019 despite copulating with females and/or being used for artificial insemination (four of these males have produced chicks in previous years). Two of these males inseminated females that produced fertilised but undeveloped eggs of unknown paternity, however, the females they mated with also copulated with fertile males and in some cases produced developed eggs of known paternity with other males. It is therefore unlikely that these two males fertilised the eggs laid by these females.

Across all breeding females, only one produced no fertilised eggs in 2019. Of the other 48 females, 10 (21%) produced eggs in which all the embryos died at an early undeveloped stage, and three of these females have produced chicks in previous years. Based on these data, we estimate that individual infertility rates in reproductively active kākāpō in 2018/19 were *c*. 17% in males and 2% in females.

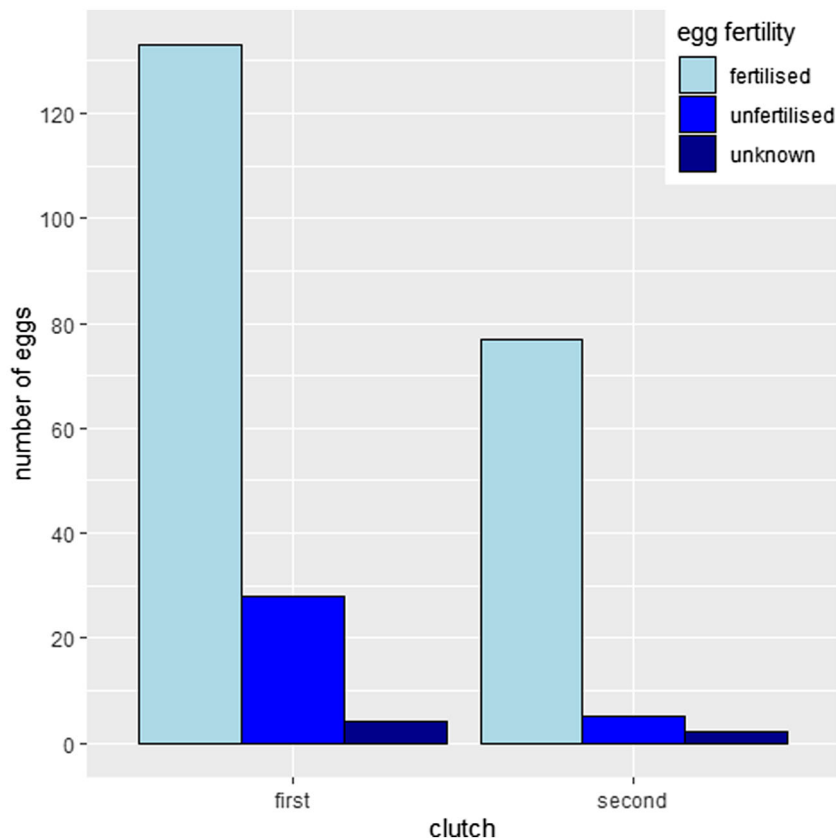### Sperm numbers reaching eggs

We counted the number of sperm on the PVL of 83 fertilised eggs. The number of sperm per cm^2 of PVL ranged from 0 to 82 (median = 15), but these numbers are likely to be conservative because the PVL was often obscured by adhered yolk and/or embryonic tissue (as a result of the sample fixation process). We did not analyse the number of holes made by sperm entering the ovum because the inner PVL was frequently absent or degraded.

When comparing the full set of possible models of PVL sperm numbers (Table 2a), the four best models included all three fixed effects. These models were each significant improvements on a null model including no fixed effects (ΔAICc: 33.14, 31.62, 31.00, 29.51; P (χ^2) all <0.0001) and had AICc weights greater than 0.05.

Our averaged model of sperm numbers (Table 2b) suggests that PVL sperm numbers did not vary between first and second clutches (estimate = 0.018, z = 0.209, P ($|z|$) = 0.834). However, eggs from multiply mated females may have had more sperm (estimate = −0.267, z = −1.883, P ($|z|$) = 0.060), and artificially inseminated females exhibited significantly more sperm (estimate = −1.988, z = 4.871, P ($|z|$) < 0.001) (Fig. 3).

Discussion

Kākāpō are critically endangered, and their recovery is hampered by exceptionally high rates of hatching failure and presumed infertility. Our results show that early embryo mortality is the main driver of kākāpō reproductive failure: almost three-quarters of apparently unfertilised kākāpō eggs are in fact fertilised but die at an early stage of development. True individual infertility rates in kākāpō are hence lower than previously thought, with at least 83% of breeding males being capable of fertilisation and 98% of breeding females producing fertilised eggs. Our results also demonstrate the potential benefits of artificial insemination as a management

Figure 2 The number of fertilised and unfertilised eggs across first and second clutches. Fertilisation failure was more common in first clutches ($z = 2.416$, $P = 0.016$).

Table 2 (a) Comparison of GLMMs of sperm numbers reaching the PVL, incorporating fixed effects of first/second clutch (Clutch), Artificial Insemination (AI), and Multiple Mating (MM). All models included female identity as a random effect on intercept. Columns present AICc values, AICc weights, and the ΔAICc compared to the most parsimonious model. (b) Summary of average model; coefficients given are of the natural averages of the four models with weights greater than 0.05.

Model	AICc	ΔAICc	Weight
(a)			
AI + MM	1815.79	0	0.507
AI	1817.31	1.52	0.237
Clutch + AI + MM	1817.93	2.14	0.174
Clutch + AI	1819.42	3.63	0.083
Null (female ID only)	1848.93	33.14	0
Clutch	1849.49	33.70	0
MM	1850.75	34.96	0
Clutch + MM	1851.49	35.70	0

Averaged coefficients	Estimate	SE	z value	P value
(b)				
Intercept	−1.827	0.250	7.231	<0.001
Clutch	0.018	0.083	0.209	0.834
AI	−1.988	0.404	4.871	<0.001
MM	−0.267	0.141	1.883	0.060

tool in wild populations, as eggs laid by artificially inseminated females had much higher sperm numbers on the perivitelline layer.

Embryo mortality as a driver of reproductive failure in threatened birds

Like many other threatened bird species, kākāpō have been through a severe genetic bottleneck that is likely to have ongoing consequences for their viability. Only 51 kākāpō existed in 1995, and all but one of this founder population originated from a relatively small and potentially inbred population from Rakiura/Stewart Island (Bergner, Jamieson & Robertson, 2014). The risk of inbreeding after this bottleneck is exacerbated by the species' lek breeding system, with local paternity dominated by few males. A previous study found no link between male heterozygosity and fertility in male kākāpō (White *et al.*, 2015), however, that study assumed all undeveloped eggs were unfertilised. Moreover, early embryo death is more likely to be related to heterozygosity of the embryo itself than that of either parent and is an expected consequence of inbreeding as inbred individuals express more lethal recessives during development (Charlesworth & Willis, 2009). In contrast, the evidence that
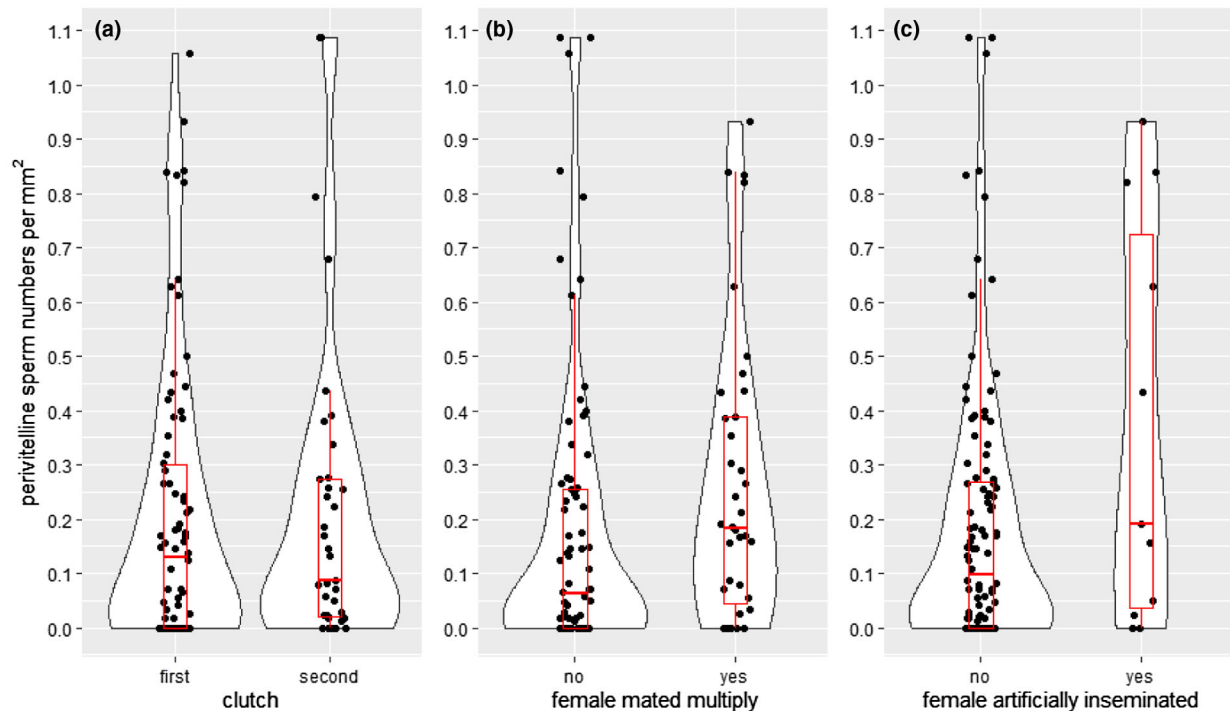
Figure 3 The number of sperm (per mm^2) counted on the perivitelline layer (PVL) of undeveloped eggs, compared between (a) first and second clutches; (b) females that didn't (no) or did (yes) mate with multiple males; and (c) females that weren't (no) or were (yes) artificially inseminated. Boxplots (red) show median values and quartiles. The violin plot illustrates the kernel probability distribution; the width of the white area represents the proportion of the data located there. There were no significant differences in sperm numbers between first and second clutches ($z = 0.209$, $P = 0.834$), but eggs from multiply-mated females may have had more sperm ($z = 1.883$, $P = 0.060$) and eggs from artificially inseminated females had significantly more sperm ($z = 4.871$, $P < 0.0001$).

inbreeding depresses adult sperm function and individual fertility in wild populations is equivocal (Losdat, Chang & Reid, 2014; Losdat *et al.*, 2018). It therefore seems likely that early embryo mortality in kākāpō is largely driven by inbreeding depression on early survival. This result adds to the growing body of evidence that fertilisation failure is relatively uncommon in wild/free-living bird populations (Hemmings & Evans, 2020). However, it is important to note that true infertility may be a more significant problem for birds in captive breeding programmes (Hemmings *et al.*, 2012*b*) or populations exposed to environmental contaminants (Fry, 1995). Conservation managers should therefore seek to identify whether infertility or embryo mortality is driving hatching failure within their own focal population(s), and adopt targeted interventions based on this information (Assersohn *et al.*, 2021).

Identification of infertile individuals and consequences for breeding management

In wild populations, it is often impossible to identify infertile individuals, as copulations are rarely monitored and fertilisation failure is largely misidentified (Hemmings & Evans, 2020). Most long-term population studies, for example, only collect breeding information on individuals that provide care and/or produce offspring; identifying males who copulate but

fail to father or care for offspring is practically impossible since extrapair copulations are typically brief and cryptic (Birkhead, 1987). The management regime of kākāpō, with copulations remotely recorded and eggs artificially incubated, hence represents a unique opportunity to accurately assess individual fertility across an entire species.

Our results indicate that individual fertility rates in kākāpō are higher than previously assumed. Although we identified five males that exhibited no evidence of fertilisation success despite copulation attempts (representing 17% of breeding kākāpō males in 2019), four of these produced offspring in previous breeding seasons, and are therefore not totally sterile. However, individual fertility is likely to change with age and condition, with potentially important consequences for long-lived species like kākāpō. Investigating how fertility changes through time, and whether this is the result of physiological (e.g. sperm production), behavioural, and/or genetic factors will be a valuable step towards understanding how the reproductive success of the population may change in the future.

While infertility was generally low, we identified a significant number of individuals (male and female) whose offspring never developed beyond the earliest stages of embryo development (<day 5 of development). Since early embryo death is likely to be associated with inbreeding depression, an important next step will be to explore whether parental

genetic similarity is related to the likelihood of early embryo survival, to inform the selection of individuals for artificial insemination and/or translocation.

In addition to analysing the prevalence of natural fertility rates, we also used data from a relatively small number of females that were artificially inseminated during the breeding season to assess whether artificial insemination had the intended positive effect on fertilisation success. We found promising evidence that more sperm may reach eggs after artificial insemination (Fig. 3c), as well as some (albeit weak) evidence that a higher proportion of artificially inseminated females' eggs were fertilised. Although this is encouraging, these results are based on a small sample of just 12 artificially inseminated females and should therefore be interpreted cautiously. It is also important to note that procedure effectiveness probably improved through the course of the breeding season (D. Eason & A. Digby, pers. comm.). However, our findings certainly suggest that further trials of artificial insemination will be valuable: if our preliminary results are confirmed with larger sample size, this relatively new conservation management tool could help maximise future reproductive success and genetic diversity in threatened species like the kākāpō.

It should be noted that revealing this degree of individual-specific information on fertility across an entire wild population was only possible because of the extensive data on kākāpō behaviour, reproductive success and life-history collected through their conservation management. Beyond the direct benefits to ecosystem recovery and persistence, long-term programmes such as Kākāpō Recovery generate datasets on parentage, mating behaviour, movement ecology, habitat preferences, and reproductive investment (among other topics) comparable to those of long-term research-focused studies, which are invaluable to our understanding of endangered species management and behavioural ecology more generally (Ewen, Adams & Renwick, 2013), As long-term individual-based studies are disproportionately valuable but rare because of a dearth in long-term funding (Clutton-Brock & Sheldon, 2010; Hughes *et al.*, 2017), and conservation organisations can seldom dedicate staff to full-time research, effective collaboration between conservation professionals and researchers can greatly benefit both parties.

Timing of breeding and consequences for fertility

The early 2018/19 breeding season allowed the Kākāpō Recovery Team to induce second clutches, almost doubling the reproductive capacity of the population. We found that fewer eggs were fertilised from first than from second clutches, indicating that individual fertility was lower earlier in the season; perhaps because females were unable to obtain sufficient sperm, or because males had not reached peak sperm production capacity. In many species the environmental and social cues that trigger reproduction are complex, and responses to these cues may differ between males and females (Ball & Ketterson, 2008). This raises the concerning possibility that as species shift their timing of breeding in

response to global warming (e.g. Hällfors *et al.*, 2020), infertility might increase through sex-specific physiological constraints and resulting differences in reproductive timing.

Conclusions

Our study has demonstrated the need for avian conservation programmes to identify the underlying reproductive problems leading to high levels of hatching failure in the threatened birds they manage. This can be achieved by using the methods adopted here – and outlined in more detail in Assersohn *et al.* (2021) – to gain detailed individual-specific information on fertility. Such data will enable a rapid and targeted response to reproductive failure, while also improving the selection efficiency of individuals for pairing (in captive breeding programmes), males for artificial insemination, and founder populations for translocation.

Author contributions

Conceptualization: JC, NH and JS; Data curation: JS, NH and KRT; Funding acquisition: JS, NH and JC; Investigation: NH, JC, JS and KRT; Methodology: NH and JS; Project administration: JS and NH; Resources: NH, KRT; Visualization: JS, NH; Writing – original draft: JS and NH; Writing – review & editing: NH, JS, JC and KRT. All authors gave final approval for publication and agree to be held accountable for the work performed therein.

Conflict of interests

The authors declare no competing interests.

Ethics approval

The project was conducted as part of the New Zealand Department of Conservation Kākāpō Recovery Programme's conservation management activities, with approval from Ngāi Tahu.

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Supporting information

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

Supplementary Material S1. Data source and R code.