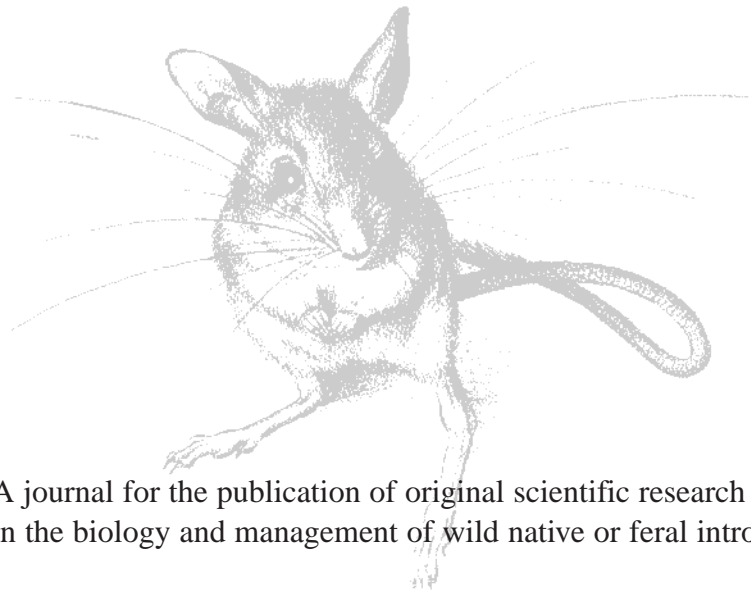

CSIRO PUBLISHING

Wildlife Research

Volume 27, 2000

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A journal for the publication of original scientific research
in the biology and management of wild native or feral introduced vertebrates

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Detecting sperm on the perivitelline membrane of incubated turkey eggs and its implications for research on fertility problems in endangered species

Angus O. Small, Kira Schlusser, Christine J. Ryan and Ian G. Jamieson^A

Department of Zoology, University of Otago, PO Box 56, Dunedin, New Zealand.

^ATo whom all correspondence should be addressed. Email: ian.jamieson@stonebow.otago.ac.nz

Abstract. A method whereby sperm nuclei on the perivitelline membrane are stained and counted under a photomicroscope has been used extensively in the poultry industry to show a correlation between egg infertility and low numbers of spermatozoa near the site of fertilisation. It has been suggested that this method could also be potentially useful for assessing infertility problems in endangered species. For poultry, the staining technique is normally applied to fresh (i.e. unincubated) eggs, but conservation managers normally collect eggs for examination only after they have been incubated for several days and then assessed (by candling) to lack a developing embryo. Whether sperm would persist on the perivitelline membrane of infertile eggs incubated over several days is not known. In relation to our research on egg infertility in the endangered takahe (*Porphyrio hochstetteri*), we examined the above problem by comparing sperm counts on the membrane of fresh versus incubated infertile eggs from artificially inseminated domestic turkey (*Meleagris gallopavo*). We were able to detect sperm in most infertile eggs that had not been incubated, but no sperm at all were detected in infertile eggs that were incubated for seven days, suggesting that warming of the eggs may cause the sperm on the membrane to degrade. The inherent fragility of avian sperm nuclei may limit the usefulness of this technique for assessing infertility in takahe and other species where failed (non-developing) eggs are not removed from the nest until after a short period of incubation.

Introduction

Within minutes of fertilisation in birds, the outer perivitelline membrane is laid down, trapping any spermatozoa that are present between the membrane and the ovum (Bakst and Howarth 1977). The perivitelline membrane acts as a barrier to excessive polyspermy and retains the integrity of the ovum after being penetrated by some spermatozoa (Bakst and Howarth 1977; Howarth 1984). The number of spermatozoa trapped on the outer perivitelline layer reflects the number of spermatozoa inseminated and present near the site of fertilisation (Wishart 1987; Birkhead *et al.* 1994).

A method whereby the sperm nuclei on the membrane are stained with a fluorescent dye and counted under a photomicroscope has been used in the poultry industry to correlate egg infertility with low sperm counts (Wishart 1987). The usefulness of this technique has also been highlighted in studies of sperm competition in birds (Birkhead *et al.* 1994; Birkhead *et al.* 1995; Birkhead 1996), and has recently been suggested as a method to assess possible causes of poor hatching success in wild bird populations (Kempnaers *et al.* 1996). It has also been suggested (T. Birkhead, personal communication) that this method could be used as a non-invasive technique for assessing possible causes of infertility in endangered species.

The staining technique developed by Wishart (1987) is normally applied to relatively fresh eggs that are collected and examined soon after they are laid. This may not only be for the practical reason of collecting the eggs as soon as they are available, but also because the membrane and sperm trapped within it would probably degrade if the egg is incubated for too long (Birkhead *et al.* 1995). Conservation managers do not have the luxury of collecting and destroying potentially viable eggs in order to apply the above technique on fresh eggs. However, Birkhead *et al.* (1995) were able to detect sperm on the perivitelline layer of house sparrow (*Passer domesticus*) eggs that had been incubated for three days, which was the minimum period for which fertilisation could be detected by candling. Exactly how long sperm persist on the membrane of incubated eggs is not known.

For the takahe (*Porphyrio hochstetteri*), a large endangered flightless New Zealand rail, lack of development of an embryo can be detected as early as seven days into the incubation period by an experienced candler (C. Ryan, unpublished data). Wild takahe are subjected to high rates of egg infertility (Bunin *et al.* 1997; Jamieson and Ryan 2000), and therefore it would be useful to know whether males are producing adequate numbers of sperm. However, if the perivitelline membrane of an incubated infertile egg was examined

and few or no sperm were detected, it would not be clear whether this was due to few sperm reaching the ovum or sperm breaking down and deteriorating over time as the egg is warmed during incubation.

Therefore, an immediate objective in relation to our research on takahe was to determine whether sperm are still present on the perivitelline membrane of infertile eggs that had been incubated for at least seven days. We examined this problem by comparing sperm counts on the membrane of fresh versus incubated eggs, using artificially inseminated domestic turkey (*Meleagris gallopavo*). We used turkey eggs because they were approximately the same size as takahe eggs and were readily available from two nearby hatcheries. We chose seven days incubation because this was close to the minimum period for which the development status of takahe embryos could be assessed. Our overall aim for doing the study was to determine whether the staining technique could be applied to endangered species such as takahe, where incubation starts after the first egg is laid and fertility cannot be accurately assessed by candling until after several days of incubation.

Methods

Artificially inseminated eggs were obtained from two sources: Cust Turkey Hatchery, Christchurch, and Crozier's Turkey Hatchery, Ashburton. Eggs were opened for examination either fresh (i.e. unincubated but stored for up to three days at 4°C) or after being incubated for seven days. Eggs were opened with scissors and the yolk and albumen separated. Each egg was categorised by eye as fertile or infertile by the appearance of the germinal disk (Kosin 1945). Eggs were categorised into four groups: (1) fresh – infertile, (2) fresh – fertile, (3) incubated – infertile, and (4) incubated – fertile. The fourth group was not included in the analysis because the structure of the perivitelline membrane, and the sperm trapped within it, is known to degrade with development of the embryo (Jensen 1969).

A 2–3-cm square was cut from around the germinal disk area and the membrane removed. The membrane was placed in a Petri dish and rinsed in phosphate-buffered saline (PBS) to remove adherent yolk and albumen, and then placed on a glass microscope slide. The preparation was then stained at room temperature by adding two drops of a 1 µg mL⁻¹ solution of 4',6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co., St Louis, USA) in PBS (Wishart 1987). A coverslip was placed over the entire piece of membrane, excess liquid was removed and the preparation was stored in the freezer until required.

To estimate the numbers of sperm embedded in the membrane the method originally developed by Wishart (1987) and modified by Birkhead *et al.* (1994) was utilised. Slides were removed from the freezer and allowed to thaw. Two parallel lines, were drawn longitudinally 10 mm apart on the coverslip above the membrane with an indelible marker. The blue fluorescing, comma-shaped sperm nuclei were viewed with a Zeiss D-7082 reflected-light fluorescence photomicroscope using a grid square in the eyepiece. Sperm were counted using ×20–40 magnification using the method of Wishart (1987). A defined area of membrane was assessed for the presence of spermatozoa by scanning perpendicularly between the two marker lines, focusing up and down to bring into view spermatozoa at different depths in the membrane. This gave a rectangular area of 8.05 mm². At least four scans were completed on all samples, and an average number of sperm was calculated for the area.

We first analysed sperm counts for fertile versus infertile fresh eggs and then fresh versus incubated infertile eggs. Differences in sperm

counts were analysed statistically using a 2-factor General Linear Model. In the first analysis, farm and fertility status were considered fixed and random factors, respectively, while farm and incubation status were both considered fixed factors in the second analysis. The data were transformed using $Y' = \log(Y + 1)$, as suggested by Zar (1996).

This research was approved by the University of Otago Animal Ethics Committee (AEC # 75–96).

Results

For fresh eggs, sperm counts between fertile and infertile eggs were highly significantly different ($F_{1,51} = 20.7$, $P < 0.001$). Fresh fertile eggs from both farms had more sperm than fresh infertile eggs (Table 1). There was no significant effect of farm ($F_{1,1} = 10.8$, $P = 0.19$), but the interaction between farm and fertility showed a trend toward significance ($F_{1,51} = 3.4$, $P = 0.07$); mean number of sperm was much higher for fertile eggs from Cust's hatchery than from Crozier's hatchery. However, mean sperm counts between fresh and incubated infertile eggs were also significantly different ($F_{1,37} = 13.4$, $P < 0.001$), with no sperm at all detected on the incubated eggs (Table 1). In this case, there was a significant effect of farm ($F_{1,1} = 6.6$, $P < 0.05$), and a significant interaction between farm and incubation status ($F_{1,1} = 6.6$, $P < 0.05$), although the number of infertile eggs obtained from one of the farms (Cust) was relatively small.

Table 1. Number of sperm (mean ± s.e.) per unit area (8.05 mm²) on the perivitelline membrane of turkey eggs varying in fertility, incubation status and origin

Egg status	Crozier's farm		Cust's farm	
Fresh, fertile	2.3 ± 0.8	n = 21	30.5 ± 9.5	n = 10
Fresh, infertile	0.2 ± 0.8	n = 19	2.1 ± 1.5	n = 4
Incubated, infertile	0 ± 0	n = 15	0 ± 0	n = 2

Discussion

The technique to detect sperm on the perivitelline membrane appeared to work well for fresh unincubated turkey eggs. For example, we found that eggs from Cust's Hatchery had higher fertility rates and tended to have greater numbers of sperm than those from Crozier's Hatchery, which may have been due to seminal fluid being diluted twice as much for artificial inseminations at Crozier's Hatchery than at Cust's Hatchery (S. Shaw and P. Crozier, personal communications).

We also found that fresh infertile eggs from both farms had fewer numbers of sperm on the perivitelline membranes than did fresh fertile eggs, consistent with Wishart's (1987) findings with chickens. However, no sperm at all were detected for incubated infertile eggs, suggesting that warming of the eggs over a period of time may cause any sperm trapped on the perivitelline membrane to degrade.

Wishart (1997), working with fresh eggs, found turkey sperm to be more prone to degeneration on the perivitelline membrane than chicken sperm. It is possible that turkey eggs

are unusual in that all sperm trapped between the perivitelline layers degenerates after seven days of incubation, whereas sperm numbers in other species would go unchanged or be less affected, but this seems unlikely. Degradation over time (i.e. several days) is probably due to avian nuclei in general lacking cystein-rich protamines that give eutherian sperm nuclei their long-term stability (Bedford and Calvin 1974).

This inherent fragility may be important because it limits the usefulness of the technique for assessing infertility in takahe and other species where failed (non-developing) eggs are not removed from the nest until after a short period of incubation. We did examine three takahe eggs that had been incubated for 10 days (the normal period when eggs are candled, and their development status assessed), but the perivitelline membranes were no longer intact. The results with the turkey eggs indicate that, for takahe, we either need to sacrifice one fresh egg (from a normal clutch of two) from several breeding pairs to assess whether infertile eggs are correlated with low sperm counts or use more invasive techniques such as obtaining ejaculate samples directly from males.

Acknowledgments

We thank Sandy Shaw, Manager at the Cust Turkey Hatchery, and Philip and Judith Crozier, owners and operators of Croziers Turkey Hatchery, for advice and for supplying turkey eggs for the study. Access to a reflected-light fluorescence photomicroscope as well as technical advice was provided by Dave Wharton. Alison Cree provided lab space and Corey Bradshaw helped with statistical analyses. We particularly thank Graham Wishart for helpful comments on the manuscript. Funding for our research on takahe has been provided by the New Zealand Department of Conservation, Science and Research Division (Contract no. 2139), the University of Otago, the Miss E. L. Hellaby Indigenous Grassland Research Trust and the Flight Centre (corporate sponsors of the Takahe Recovery Programme).

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Manuscript received 24 August 1999; accepted 22 February 2000