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Extraordinary sperm to egg ratios in seabirds

Keywords: sperm storage, fertility, reproductive timing, single-egg clutch, pre-laying exodus, birds

Word count: 4381

Lay Summary

- After mating, female birds store sperm in their reproductive systems for some time before egg production and fertilisation. It is thought that sperm are gradually lost during storage, meaning that later in the storage period, fewer sperm should be available for fertilisation.
- We studied the number of sperm reaching eggs in six seabird species, in which females spend a long time feeding at sea between mating and producing eggs.
- Surprisingly, we found that the number of sperm that reached eggs in these species was much higher than expected, based on a known relationship between egg size and sperm number.
- We suggest that in these species, sperm release from storage is precisely synchronised with ovulation. This may be particularly important in the species we studied, because they all lay a single egg only.
- Our hypothesis implies that female birds have greater control over sperm storage and use than previously thought.

Abstract

Following copulation, females of many seabird species spend a prolonged period of time away from the colony, building up reserves for egg formation and incubation. Here, we report that the number of sperm associated with eggs of single-egg clutch seabirds was almost an order of magnitude greater than predicted from the relationship between ovum size and sperm numbers in multi-egg clutch non-seabirds. Sperm numbers were also several times greater than the estimated number necessary for maximal fertilisation success. Our results are consistent with three unusual features of seabird reproduction: (1) single egg clutches, (2) prolonged sperm storage, and (3) a lag period between the end of yolk formation and ovulation. We hypothesise that sperm release from storage is under precise temporal control in these species, with high sperm numbers acting as an insurance against infertility in single-egg clutches. If true, the lag period may have evolved to provide sufficient time for sperm to be released simultaneously from storage and accumulate at the site of fertilization prior to ovulation.

Introduction

In birds, hundreds or thousands of sperm typically reach the vicinity of the ovum, with several sperm entering the germinal disc in a process known as physiological polyspermy (Snook et al. 2011; Hemmings and Birkhead 2015). This differs considerably from the situation in mammals, where the ratio of sperm to ova at the site of fertilisation is approximately one to one (Cohen 1967), and penetration of the ovum by multiple sperm (polyspermy) is pathological. It has been assumed that large sperm numbers are required to

reach the site of fertilisation in birds because the germinal disc, which contains the female pronucleus, represents a relatively small ‘target’ on the large, yolky ova of birds (Rothschild 1956; Bramwell et al. 1995). The presence of multiple sperm within the germinal disc (Harper 1904) had been assumed to be a non-functional consequence of these large sperm numbers, but recent work has demonstrated that the supernumerary sperm entering the germinal disc of avian eggs are essential for successful early embryo development (Mizushima et al. 2014; Hemmings and Birkhead 2015).

In birds, the window for fertilisation is brief (fifteen minutes immediately following ovulation; Howarth 1974), so in order to maximize the likelihood of fertilization, an adequate supply of sperm must be available at that time. This is achieved by sperm storage – female birds (as well as those of other taxa), store sperm for prolonged periods of time within their reproductive tract, removing the need to synchronise copulation and fertilisation. The duration of sperm storage varies from six to sixty days in different bird species and — with the exception of those species laying a single egg — is positively correlated with both clutch size and the maximum time interval between the last insemination and fertilization (Birkhead and Møller 1992). The main storage sites are the sperm storage tubules located in the utero-vaginal junction, and sperm start to be released a few days before the first ovulation, after which they are transported to the infundibulum where fertilization occurs (Bakst et al. 1994). In all birds studied to date (e.g. Domestic Fowl *Gallus gallus domesticus*, Turkey *Meleagris gallopavo*, Zebra Finch *Taeniopygia guttata*), sperm release from the sperm storage tubules appears to occur at a

constant rate throughout the egg-laying period (Wishart 1987; Colegrave et al. 1995), and the released sperm accumulate in the infundibulum (Hemmings et al. 2015). With each successive ovulation, all sperm are trapped as the perivitelline layer forms, and removed from the infundibulum, which is then repopulated with sperm either from the sperm storage tubules or sometimes from additional inseminations (Bakst et al. 1994). In birds laying multi-egg clutches, successive ova are released from the ovary at intervals of 24h (in some species 48h or more) and hence fertilized individually. The mechanisms controlling the storage and release of sperm from the tubules are poorly understood (Bakst et al. 1994).

The reproductive cycles of many seabirds differ from those of most other birds due to two key features: (1) a single egg clutch, and (2) a “pre-laying exodus” in which the female is absent from the colony, building up reserves for egg formation and incubation. This “exodus” varies from three to fifty days, depending on species; it is most notable in some members of the Procellariiformes (Weidinger 1996; Cuthbert 2004), but shorter absences have been documented in other groups including the Alcids (Wanless and Harris 1986; Hatchwell and Pellatt 1989). Females usually return the day before egg laying. Copulation occurs prior to the exodus (e.g. Hatch 1987). In birds that lay only a single egg, it is not known whether all stored sperm are released simultaneously or, as in multi-egg clutch species, are released from the sperm storage tubules over several days even though there is only a single ovum to be fertilized. The interval between the last insemination and ovulation may be several days or weeks (Hatch 1983; Wanless and Harris

1986; Birkhead and del Nevo 1987; Birkhead and Møller 1992) so it seems biologically implausible that females would 'waste' sperm by releasing them from storage before they could be involved in fertilization. However, simultaneous sperm release would require precise female control over sperm storage and release mechanisms.

Across multi-egg clutch species, in which sperm are released at a constant rate over several days, the number of sperm interacting with the perivitelline layer is positively associated with ovum size (Birkhead et al. 1994). Therefore, if sperm are also released at a constant rate in single-egg clutch seabirds, the number of sperm interacting with the egg should not differ significantly from the number predicted by this relationship, given their ovum size. Here, we examine the numbers of sperm on the perivitelline layer of the eggs of single-egg clutch seabird species and compare them to expected numbers given their ovum size, with the aim of deducing the pattern of sperm release from the storage tubules.

Materials and Methods

We obtained eggs of six single-egg clutch seabird species, from two unrelated taxa, the Alcids (Common Guillemot *Uria aalge* (N = 5), Puffin *Fratercula arctica* (N = 2), and Razorbill *Alca torda* (N = 5)), and the Procellariiformes (Manx Shearwater *Puffinus puffinus* (N = 3), Northern Fulmar *Fulmarus glacialis* (N = 2), and Storm Petrel *Hydrobates pelagicus* (N = 6). All eggs were collected under license (from Natural England, Natural Resources Wales, and Scottish Natural Heritage), on the day they were laid, from Skomer Island, Wales

125 (Guillemot, Manx shearwater, Puffin, and Razorbill), the Faroe Islands,
126 Denmark (Storm Petrel), and Fair Isle, Scotland (Fulmar). Eggs were opened
127 and the yolk was fixed in 5% formalin. Once fixed, the yolk diameter was
128 measured with digital vernier calipers (0.01mm) to estimate mean ovum surface
129 area for each species. Two 0.5 cm^2 pieces of perivitelline layer per egg – one
130 from above the germinal disc and the other from the vegetal pole – were
131 removed from the yolk, cleaned in phosphate buffered saline (PBS) solution,
132 and mounted on a microscope slide for examination. 10µl of the fluorescent
133 DNA stain Hoechst 33342 (0.05mg/ml) was added to each perivitelline layer
134 sample to stain sperm nuclei, as described by Birkhead et al. (2008). The
135 number of sperm trapped in and holes made by sperm in the perivitelline layer
136 and was counted for each perivitelline sample, under a fluorescence
137 microscope with a BP 340-380 excitation filter and a LP 425 suppression filter,
138 dark-field optics, and x 20 objective lens. Our methods for counting sperm and
139 holes were identical to those used by Birkhead et al. (1994), except that we did
140 not correct for a “halo” of holes in the germinal disc region, as we found no
141 evidence for this phenomenon in our samples. Instead, we found that the
142 number of sperm and holes did not differ significantly between the germinal disc
143 and vegetal pole (sperm: $t = -0.01$, d.f. = 22, $p = 0.996$; holes: $t = 1.51$, d.f. =
144 22, $p = 0.145$), so we calculated the mean sperm and hole number per unit area
145 of perivitelline layer, and from this estimated the total number of sperm and
146 holes for each ovum (Table 1).
147
148 To calculate expected sperm numbers for each species, we first re-analysed
149 the relationship between ovum size and both (i) total sperm numbers (sperm

and holes combined) on the perivitelline layer and (ii) hole numbers across species, following Birkhead et al. (1994), using data for 18 species from Birkhead et al. (1994) and an additional two species (Mute Swan *Cygnus olor* and Canada Goose *Branta canadensis*) (Fig 1). All original data was log-transformed, so we log-transformed our own data for comparison. Original data for the Guillemot and Razorbill in Birkhead et al. (1994) was excluded because these two species are under investigation in the current study. Original data for Domestic Fowl was also excluded, since this species is artificially inseminated with excessive numbers of sperm (Taneja & Gowe 1961; our overall results and interpretation do not change if Domestic Fowl is included (Supplemental Material Appendices A & B)). We then used the relationship between ovum size (diameter) and (i) total perivitelline layer sperm or (ii) hole numbers to calculate the number of sperm predicted to (i) reach and (ii) penetrate the ova of each of the single-egg clutch species, given their ovum size. We compared these predicted values with the species mean total sperm and hole counts via paired t-tests. Using our single-egg clutch species data combined with the original multi-egg clutch species data from Birkhead et al. (1994), we also compared generalised linear models of the relationship between ovum size and sperm/hole numbers with and without clutch size (single versus multi) included as an explanatory factor, in order to assess whether clutch size explained a significant amount of variation in sperm numbers reaching and penetrating the perivitelline layer. Details and outputs of models, including versions with and without the data for Domestic Fowl, are provided in the Supplemental Material Appendix B.

In species laying multi-egg clutches, the number of sperm varies across different eggs of the same clutch (Birkhead et al. 1994), so comparing mean values from across the clutch may underestimate the maximum number that can reach a single egg. Therefore, we also compared our mean total sperm counts for single-egg clutch seabirds with the maximum number of sperm recorded on any egg from eight multi-egg clutch species for which data from multiple eggs were available (six from Birkhead et al. (1994), plus our two additional species; Supplemental Material Appendix C), via a two-sample t-test. All analyses were carried out in R Version 3.5.1 (R Core Team 2018).

Results

For single-egg clutch seabirds the total number of sperm reaching and penetrating eggs varied, between ~2,500 and ~1,105,000 (Table 1), and in all cases the numbers of sperm and holes exceeded predicted numbers by an order of magnitude. Overall, the mean number of sperm both reaching (total sperm) and penetrating (holes) ova was significantly higher than predicted, based on species-specific ovum size (total sperm: $t = 12.38$, d.f. = 5, $p < 0.0001$; holes: $t = 10.57$, d.f. = 5, $p < 0.0002$; Fig 1).

Models of the relationship between ovum size and both total sperm numbers and hole numbers had a significantly lower residual deviance and AIC when clutch size (single versus multi) was included as an explanatory variable (total sperm number: reduction in residual deviance = 3.73, reduction in AIC = 15.79, $p < 0.0001$; hole number: reduction in residual deviance = 7.56, reduction in AIC = 19.15, $p < 0.0001$; see full model outputs in the

Supplemental Material Appendix B). This indicates that clutch size explains a significant amount of the variation in sperm numbers, consistent with the idea that sperm numbers are greater in single-egg clutch species.

The mean total number of sperm reaching the ova of single-egg clutch seabirds was also significantly greater than the sum total number of sperm counted across entire clutches of eggs from seven multi-egg clutch non-seabird species for which data was available (Welch's unequal variances test: $t = 3.332$, d.f. = 5.415, $p = 0.018$; Supplemental Material Appendix D, Supplemental Table S1 & Figure S3).

Discussion

Our data reveal that an extraordinarily large number of sperm reach and penetrate the ova of single-egg clutch seabirds. Across the six species studied, observed sperm to egg ratios were consistently over an order of magnitude greater than predicted based on ovum size. This difference was particularly notable for the number of sperm penetrating ova (i.e. the number of holes made by sperm in the inner perivitelline layer).

Two common but not ubiquitous features of seabird biology may explain these large sperm to egg ratios: (i) infrequent and/or unpredictably timed copulation relative to oviposition due to a decline in the female's presence at the colony prior to laying, necessitating prolonged, efficient sperm storage; and (ii) a single egg, which renders any sperm left in storage following ovulation redundant. An important caveat here is that clutch size (one egg versus more

than one egg) and ecological/taxonomic group (seabird versus not seabird) are perfectly confounded in our dataset.

A third peculiarity of seabird reproductive biology may explain how large sperm to egg ratios can be achieved. Many seabirds have a delay or 'lag period' between the end of yolk formation and ovulation, first discovered by researchers using orally administered lipophilic dye to study yolk deposition (Astheimer and Grau 1985; Astheimer 1986). After consumption, lipophilic dye is rapidly deposited into a discrete layer of yolk lipoprotein on the developing follicle, and since each yolk layer takes approximately 24 h to form, the dye layer provides a date marker within the structure of the egg yolk (Grau 1976; Hirsch and Grau 1981; Birkhead and Del Nevo 1987). The lag period can therefore be deduced from the difference between the number of yolk rings following dye deposition and the time between dosing and oviposition. It has been generally assumed that this lag occurs between the end of yolk formation and ovulation (i.e. pre-fertilisation), rather than later in the sequence of egg production (post-fertilisation), since retaining a partially or fully formed (shelled) egg in the oviduct for longer than necessary could be costly to the female. Post-fertilisation egg retention (beyond the ~18 hours required to produce the fully formed shelled egg) has been documented in a few species but is unlikely to explain the delayed onset of laying that occurs in many seabirds. The most notable example of post-fertilization egg retention occurs in avian brood parasites where eggs are internally incubated for a short period of time, conferring a developmental advantage to the embryo relative to host offspring (Birkhead et al. 2010). This phenomenon has also been noted in

domestic poultry where oviposition can be delayed as a result of stress (Reynard and Savory 1999). However, the duration of these post-fertilisation lags in brood parasites and in poultry is typically short (<24 hrs excluding the time taken to produce the egg). Moreover, none of the eggs used in the current study (obtained on the same day they were laid) showed evidence of particularly advanced development (Supplemental Material Appendix E). Thus, post-fertilisation delays/internal incubation are unlikely to contribute significantly to the lag period between yolk formation and oviposition in these species.

The length of the lag period between yolk formation and oviposition varies, from three to four days in the Common Guillemot (Birkhead and del Nevo 1987), to four to six days in penguins (Grau 1984; Astheimer and Grau 1985), and ten days in some albatrosses (Grau 1984), but no such delay occurs in either the Domestic Fowl, or passerines whose reproductive biology has been studied (Grau 1984). To our knowledge, the only non-seabird species for which an extended lag period has been documented is the Emu (*Dromaius novaehollandiae*), reported to have an average lag period of 10 days (Hirsch and Grau 1981).

The function of the lag period is unknown. We propose that in species with prolonged sperm storage, the lag period may have evolved to provide sufficient time for sperm to accumulate in the infundibulum (Fig 2). This in turn provides an explanation for the relatively large numbers of sperm on the perivitelline layer of these species: it is possible that females release their entire store of sperm from the sperm storage tubules simultaneously over a

just few days, stimulated – crucially – by the end of yolk deposition (i.e. the start of the lag period), thereby giving sperm sufficient time to reach and accumulate in the infundibulum (Fig 2). A lag period allowing time for sperm to accumulate at the site of fertilisation would also explain the particularly high numbers of sperm that penetrate the ovum (i.e. hole numbers; see Table 1 & Fig 1). The exceptionally high sperm penetration rate observed in our single-egg clutch seabird species suggests that most sperm were present in the infundibulum at the time of ovulation, ready to penetrate the perivitelline layer. If, in contrast, the majority of sperm was in the process of reaching/entering the infundibulum at the time of ovulation, we would expect a greater proportion of them to have been trapped by the glycoprotein matrix of the outer perivitelline layer, preventing penetration. If our hypothesis is true, variation in the duration of the lag period across species may be related to differences in the time it takes for sperm to reach the infundibulum, perhaps due to differences in the length of the female reproductive tract.

Consistent with the hypothesis that time is required for stored sperm to accumulate in the infundibulum, Hemmings et al. (2015) showed that in domestic fowl, sperm start to be released from the sperm storage tubules about eight days before the first ovulation and accumulate in the infundibulum. The sperm release strategy we propose here for single-egg clutch species (Fig 2) predicts that very few sperm will remain in storage after the single ovum is fertilized. Although no empirical data on this are available, one previous study noted that the sperm storage tubules of three Fulmars examined on the day of egg laying were completely empty (Hatch 1983).

Ensuring sufficient sperm are available for fertilisation may be particularly important in single-egg clutch species, to avoid wasting an entire breeding season by incubating an unfertilized egg. For ethical and practical reasons, we were unable to obtain eggs from any single-egg clutch non-seabird species (most of which are threatened), nor from any seabirds with multi-egg clutches, but comparing the number of sperm reaching the egg in these species would provide an interesting test of our model – particularly since single-egg clutch non-seabird species are unlikely to require an 'exodus' or gap between copulation and egg-laying, as seabirds do. Interestingly, the numbers of sperm found per unit area of perivitelline layer of our six seabird species (Table 1) are all multiple times greater than the number necessary for the maximum likelihood of fertility in domestic fowl (approximately 36 sperm per $1cm^2$ perivitelline layer; Wishart 1987), suggesting this is not simply an adaptation to avoid infertility. Since the ratio of sperm to eggs is relatively high in all birds (Birkhead et al. 1994), and can be extremely high in Domestic Fowl artificially inseminated with large numbers of sperm (Taneja & Gowe 1961), birds are clearly able to tolerate a considerable number of 'excess' sperm reaching and penetrating the ovum. How bird eggs prevent excessive numbers of sperm entering the germinal disc remains to be discovered; presumably there is an upper limit to how many supernumerary sperm can be involved in physiological polyspermy.

The mechanism by which sperm are released from the sperm storage tubules in birds is unknown. It has been suggested that in Domestic Fowl, the timing

and release of sperm from the storage is determined by sperm motility (Froman 2003); sperm swim against a current within the sperm storage tubules, and when their ability to maintain their position ends, because the energy supply has declined, they are swept out of the tubules and into the reproductive tract. However, there is little empirical evidence that sperm are motile inside the sperm storage tubules, and recent studies demonstrate that sperm release is under the influence of the female hormone progesterone (Ito et al. 2011; Hemmings et al. 2015). Our hypothesis that sperm release from the sperm storage tubules must be primarily under female control therefore seems more intuitive. In order to confirm or refute the model of sperm storage and release we have presented, we ultimately need data on the sperm storage capacity of females and ejaculate size of males, both of which are unfortunately lacking for seabird species in general.

In conclusion, we suggest that: (i) the relatively high numbers of sperm reaching and penetrating the eggs of single-egg clutch seabirds is a consequence of females releasing the contents of their sperm storage tubules simultaneously, and (ii) the lag period has evolved to allow sufficient time for the released sperm to accumulate in the infundibulum in readiness for fertilization when ovulation occurs. Our hypothesis provides an adaptive explanation for the otherwise puzzling phenomenon of the lag period and the large numbers of sperm on the ova of birds laying a single egg.

Author contributions

Both authors jointly conceived the study and wrote the manuscript. The first author collected the data and conducted the analyses.

Conflicts of interest

The authors declare no conflicts of interest.

Permits

Eggs were collected under licenses from Natural England, Natural Resources Wales, and Scottish Natural Heritage and the Faeroese Museum of Natural History.

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Fig 1. The relationship between ovum size and (a) the total number of sperm on the perivitelline layer ($\log_{10}[s] = 3.13 \times \log_{10}[d] - 0.58$, where s = total sperm number and d = ovum diameter) and (b) the number of holes left by sperm that have penetrated the perivitelline layer ($\log_{10}[h] = 2.75 \times \log_{10}[d] - 0.73$, where h = the number of holes). Regression lines are based on open circle data only, which are for 18 multi-egg clutch species derived from Birkhead et al. (1994) plus two additional species collected by the authors. Regression lines apply where $0.92 \leq \log_{10}[d] \geq 1.94$. Filled circles show the mean total sperm and hole numbers (± s.e.m.) on the six single-egg clutch seabird species sampled in this study. Numbering corresponds to species listed in the key table (the phylogenetic distribution of these species is presented in Supplemental Material Appendix C, Figure S2).

Fig 2. Schematic diagram showing: (A) a simple model of the pattern of sperm accumulation and release from sperm storage tubules (black line) and in infundibulum (pale grey inset) over time (t) in multi-egg clutch species, and (B) our hypothesized pattern of sperm accumulation and release in seabirds with a single-egg clutch. In multi-egg clutch birds (A), copulations typically start several days before clutch initiation, peaking in the days just prior to egg-laying, and declining markedly after the first egg is laid (Birkhead et al. 1987; Birkhead et al. 1989). Assuming a simple pre-laying insemination schedule where several copulations occur just prior to egg-laying, but none after, the number of sperm in the storage tubules increases during the days just prior to egg-laying and sperm are passively released at a constant rate, resulting in a gradual decline in stored sperm numbers over the course of egg-laying.

Released sperm accumulate in the infundibulum but are removed with each successive ovulation until clutch completion. In contrast, single-egg clutch seabirds (B) copulate a relatively long time before egg-laying and store sperm for a prolonged period. Our model suggests that rather than passively releasing sperm over this time, which would result in a gradual decline in stored sperm numbers and therefore relatively low numbers of sperm remaining by the time the ovum is ovulated, single-egg clutch seabirds simultaneously release all sperm just prior to ovulation. Stored sperm numbers therefore remain relatively constant after the last insemination, until their release is triggered by the beginning of the lag period (when yolk deposition is complete), resulting in rapid loss of all sperm from the tubules. Over the next few days, all sperm accumulate in the infundibulum, ready to fertilise the single ovum. This model predicts that following ovulation all sperm should be removed from the female reproductive tracttract.