biology letters

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Biol. Lett. 2006 **2**, 128-130
doi: 10.1098/rsbl.2005.0386

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doi:10.1098/rsbl.2005.0386
Published online 27 September 2005

Emergence of sperm from female storage sites has egg-influenced and egg-independent phases in *Drosophila melanogaster*

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The coordinated introduction of sperm and eggs is a prerequisite of high fertilization efficiency. In *Drosophila melanogaster*, as in most internally fertilizing animals, mated females store sperm prior to fertilization. Yet the regulation of sperm exit from these storage sites is poorly understood. To test one likely factor that could coordinate gamete availability, we quantified sperm exit from storage in three types of female: genetically matched females that were normal or eggless, and an additional wild-type control. Long-term depletion of sperm stores in normal females and eggless females occurs at similar rates. However, soon after mating, egg presence appears to accelerate the transition from one storage stage to the next. Since male ejaculate components and female factors contribute to sperm depletion, opportunities exist for both cooperation and conflict between the sexes in sperm storage dynamics.

Keywords: female sperm storage; egg; gamete coordination; fertility; sperm use; fertilization

1. INTRODUCTION

Female sperm storage—the residence of sperm within the female reproductive tract preceding fertilization—is essential for reproduction in many animals (Neubaum & Wolfner 1999*b*; Suarez 2002; Bloch Qazi *et al.* 2003). This critical process also influences phenomena involved in post-copulatory sexual selection such as cryptic female choice, sperm competition and male–female antagonistic coevolution (Eberhard 1996; Simmons 2001). Sperm storage encompasses stages including sperm entry, accumulation, and retention within the female storage organs, and sperm depletion from storage (Bloch Qazi *et al.* 2003). Depletion can result from sperm exit for fertilization, female sperm-dumping, passive loss and/or death of stored sperm (Eberhard 1996; Snook & Hosken 2004). Efficient use of sperm for fertilizations requires coordination between sperm exit from storage and egg release from the ovaries. This prevents the loss of viable but unfertilized eggs, and decreases the number of matings required by females to maintain fertility; the latter is important when matings are costly to females (e.g. *Drosophila melanogaster*; Chapman *et al.* 1995).

Drosophila melanogaster is a good model system for studying sperm fate. Approximately 1000 sperm are stored within the female's single seminal receptacle and paired spermathecae (reviewed in Bloch Qazi *et al.* (2003)). Female sperm storage is important for fertility: after a single mating, eggs can be fertilized using stored sperm for up to two weeks (reviewed in Neubaum & Wolfner (1999*b*)). More than 40% of female-stored sperm can fertilize eggs; this contrasts with less than 0.1% sperm use efficiency in some vertebrates (Birkhead & Møller 1993; Neubaum & Wolfner 1999*b*).

Despite their importance, mechanisms of sperm exit from storage remain poorly understood, although both female- and male-derived factors can contribute. In *D. melanogaster*, male-derived seminal proteins (Harshman & Prout 1994), including the ejaculatory duct proteins esterase 6 (Gilbert 1981) and glucose dehydrogenase (Iida & Cavener 2004), promote sperm exit from storage (reviewed in Wolfner (2002) and Bloch Qazi *et al.* (2003)). Gilbert (1981) proposed that sperm motility also aided sperm exit from storage, either independently or in conjunction with seminal protein activity. Female factors promoting sperm exit in *D. melanogaster* are less well understood, but include female-secreted glucose dehydrogenase (Iida & Cavener 2004), and possibly the removal of physical barriers to sperm exit (Filosi & Perotti 1975), changes in abdominal pressure (as occurs in some other insects; reviewed in Bloch Qazi *et al.* (2003)), and the movement of eggs down the reproductive tract affecting the exit of sperm from storage and promoting high fertilization efficiency (Wheeler 1954).

We tested whether female *D. melanogaster* couple egg release with sperm exit from storage by comparing sperm storage between normal and eggless females. If sperm exit from storage is coordinated with egg presence, then sperm depletion should occur more slowly from eggless females. Soon after mating, sperm depletion is influenced by eggs, possibly as a result of accelerating the transition between storage stages. After this initial phase sperm exit continues, but at a rate independent of the presence of eggs in females.

2. MATERIAL AND METHODS

(a) *Flies*

Flies were reared on cornmeal–sucrose (Lewis 1960) medium at 25 °C and a 12 : 12 light : dark cycle. Virgins were collected and aged 3–5 days post-eclosion. Sperm exit was compared among three groups of females. Two groups were genetically matched yet differed in their capacity to make eggs. Eggless females (*bw sp tud*^{1}/wt daughters of Oregon R (wild-type, wt) males × *bw sp tud*^{1} homozygous females; Boswell & Mahowald 1985) lacked a female germline due to the maternal-effect *tud*^{1} mutation. Genotype control females (*bw sp tud*^{1}/wt daughters of wt males × *bw sp tud*^{1}/*CyO* females) made eggs. The third group was a wild-type control.

(b) *Quantifying sperm depletion from storage*

Individual females and wild-type males were paired and observed until mating initiated. During mating, females were assigned to post-mating interval groups using a random number table. At 6, 48, 96, 168, 264 or 336 h after the start of mating, individual

The electronic supplementary material is available at http://dx.doi.org/10.1098/rsbl.2005.0386 or via http://www.journals.royalsoc.ac.uk.

Received 1 July 2005
Accepted 12 August 2005

females were dissected and their stored sperm were orcein-stained and counted as in Neubaum & Wolfner (1999*a*). These time points correspond with peak accumulation of sperm in storage (*ca* 6 h) and subsequent depletion of storage organs (*ca* two weeks). In a smaller experimental replicate to test counting consistency (see electronic supplementary material), the coefficient of variation for repeated counts was 6.80%.

(c) *Statistical analysis*

Data were analysed using two-factor analysis of variance (ANOVA), with total number of stored sperm as the dependent variable and both post-mating interval and female genotype as factors. ANOVA at the 6 h and 48 h time points tested for differences in numbers of stored sperm among female genotypes. Bonferroni–Dunn *post hoc* tests tested for differences in mean numbers of stored sperm among the three groups of females. Linear contrasts compared changes in mean numbers of stored sperm between the earliest post-mating intervals (6 h versus 48 h) for each of the three groups of females. Data did not deviate significantly from the assumptions required to perform ANOVA or linear contrasts. Analyses were conducted using StatView software v. 5.0.1 and JMP software (both SAS, Inc., Cary, NC, USA).

3. RESULTS

Sperm were found to exit female storage organs in the absence of eggs. Females' sperm stores were depleted over time (ANOVA, post-mating interval: $F_{5,148}=70.47$, $p<0.0001$; figure 1) and the rate of depletion did not differ significantly among the three types of females (post-mating interval×female genotype: $F_{10,148}=1.46$, $p=0.16$). Rates of sperm exit over the two-week study period were 51.0, 48.6 and 37.3 sperm d^{-1} for wild-type control, genotype control and eggless females, respectively. When rates of sperm depletion are calculated from 96 h after mating, these rates converge to 25.3, 31.9 and 35.0 sperm d^{-1}.

Although egg presence did not correspond with a decline in numbers of sperm stored over time, storage dynamics within the first 48 h after mating did differ among the groups of experimental females. Despite similar numbers of stored sperm 6 h after mating ($F_{2,11}=0.18$, $p=0.84$), differences in sperm storage among the three groups of females were apparent by 48 h after mating ($F_{2,28}=16.31$, $p<0.0001$). Both wild-type and genotype controls showed a significant decline in numbers of stored sperm between 6 and 48 h post-mating (linear contrast: t-ratio$=-6.22$, $p<0.0001$, d.f.$=148$ and t-ratio$=-4.66$, $p=0.0001$, d.f.$=148$, respectively). Eggless females, whose sperm stores had not declined significantly in the intervening period (t-ratio$=-1.50$, $p=0.14$, d.f.$=148$), had significantly more stored sperm than both genotype control (48.7%; Bonferroni–Dunn; mean difference$=-211.64$, $p=0.001$) and Oregon R females (68.8%; mean difference$=263.22$, $p<0.0001$). Sperm storage was similar between control females (mean difference$=51.59$, $p=0.31$).

4. DISCUSSION

Previous research in *D. melanogaster* suggesting that sperm depletion was not tightly coupled to egg production/release included modelling sperm exit and fertility separately over time (Gilbert *et al.* 1981), and observing decreased fertility after a brief dietary suppression of egg deposition (Olivieri *et al.* 1970). We demonstrate directly and under normal physiological conditions that eggs are not required for

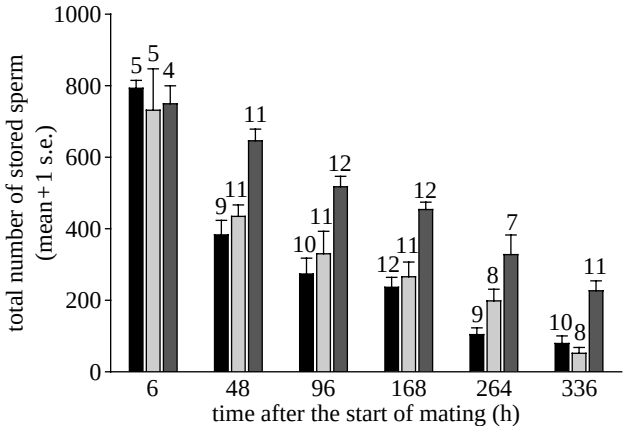

Figure 1. Mean (+1 s.e.) total sperm storage between 6 h and 336 h after the start of mating in wild-type control (Oregon R, black bars), genotype control (light grey bars) and eggless (medium grey bars) females. Sample sizes are shown above error bars.

sperm to exit female storage organs: the long-term decline in number of stored sperm over time does not differ in the presence or absence of eggs over the two-week duration of female fertility. This finding is unlikely to be due to the genetic background of the females used, for two reasons. First, we observed this effect in genetically matched females (*bw sp tud*^{1}/wt) with eggs and without eggs. Second, patterns of sperm exit were similar between females that were phenotypically similar, but from different genetic backgrounds. That is, egg-producing, genotype-control *bw sp tud*^{1}/wt females had similar sperm exit dynamics to wild-type females. Sperm depletion also occurred in both eggless *bw sp tud*^{1}/wt and a different type of eggless female that possessed a germ line (*ovo*^{D}, see electronic supplementary material).

Despite similar long-term rates of sperm exit from storage in the presence or absence of eggs, our results suggest that sperm exit is modulated. First, small variation in the 48–336 h post-mating interval sperm counts indicates that the mechanism(s) of sperm exit may be a tightly controlled aspect of female and/or sperm physiology. Second, the rate of exit is initially high (up to 48 h post-mating) and then decreases (96 h post-mating and after), suggesting that the rate of exit responds to some changing condition(s) in female physiology or to declining numbers of stored sperm (i.e. by feedback) (Gilbert *et al.* 1981). Third, sperm use by females is efficient and prolonged (up to two weeks; Neubaum & Wolfner 1999*b*). Three sperm left storage for every offspring produced, which is comparable to rates reported in other studies (see electronic supplementary material for data and discussion). Potential modulators of sperm release include: sperm motility, male seminal proteins, female contractions, female secreted proteins (reviewed in Bloch Qazi *et al.* (2003)), female nutrition (Roth & Reinhardt 2003) and/or neuropeptides/neurohormones (Heifetz & Wolfner 2004).

Eggs appear to initially influence sperm exit, perhaps by accelerating the transition from one stage of storage (e.g. accumulation) to another (e.g. retention or exit). The difference in sperm retention in eggless females relative to the control groups, which is

most apparent 6–48 h post-mating, may reflect temporal differences in the initiation of sperm exit from storage. Perhaps egg movement through the reproductive tract works in concert with processes terminating sperm accumulation (e.g. eggs forcing out unstored sperm) or initiating sperm release from storage. Lacking this movement, eggless females may accumulate additional stored sperm and/or delay the release of stored sperm until the other processes take effect. Since fertilization success is lower shortly after mating (Prout & Clark 2000), modulation between storage stages could safeguard against excessive gamete wastage as the events leading up to fertilization are initiated. However, once sperm exit has been initiated, the aforementioned factors could modulate the exit rate.

Controlled exit of stored sperm in female *D. melanogaster* can minimize the waste of resource-laden eggs (Fowler & Partridge 1989; Partridge & Fowler 1990), of obvious benefit to the female. It can also prevent sperm waste, an advantage for females since multiple matings incur costs (Fowler & Partridge 1989; Chapman *et al*. 1995) and for males, whose ejaculate can represent a sizable resource investment (e.g. several species of *Drosophila*; Pitnick & Markow 1994). However, the lack of perfect coordination between egg production/release and sperm exit from storage may also be adaptive. If costs of sperm wastage are less than those of egg wastage, then some separation of the two processes may provide a valuable safeguard against egg loss due to a failure to release sperm at the appropriate time (or in the appropriate condition), balancing costs associated with mating to replenish sperm stores. While controlled sperm exit may be adaptive, perfectly coordinated egg and sperm release may not be.

We appreciate the careful and insightful comments provided by Erika Adams, Yael Heifetz, Lisa McGraw, Tanner Miest, Laura Sirot and two anonymous reviewers. We thank Sanjive Qazi for statistical advice. This work was supported by NIH grant HD38921 (M.F.W.) and an American Cancer Society Postdoctoral Fellowship (M.C.B.Q.).

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