

Sperm storage and arrangement within females of the arctiid moth *Utetheisa ornatrix*

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Abstract

Female *Utetheisa ornatrix* mate prolifically, a behavior that accrues nuptially transferred gifts of nutrient and defensive alkaloids from males. This behavior also potentially places sperm from numerous males in competition within the female reproductive tract. Here, we investigate sperm interactions within female *U. ornatrix* by exploring the arrangement and numbers of sperm stored within the spermatheca and by examining sperm deposition in the pseudobursa, a presumed digestive organ in the female reproductive tract. Our results show that females store fewer sperm than they receive from their numerous mates, and the data suggest that unwanted sperm is either shunted to the pseudobursa or expelled from the spermatheca. We found no evidence that the apyrene, or non-nucleated, sperm morph common to the Lepidoptera are involved in forming barriers between ejaculates within the spermatheca. Female *U. ornatrix* are thus able to control sperm use, which we argue may contribute to the pattern of paternity observed in this species.

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1. Introduction

Sperm competition is a form of sexual selection that involves the direct interaction between sperm from two or more males within the confines of the female reproductive tract (Parker, 1970). While sperm competition has been intensively studied, the actual interactions among ejaculates and the influence of the female reproductive tract upon those interactions remain largely unknown. Most knowledge of these interactions is inferred either from patterns of offspring paternity (Simmons and Siva-Jothy, 1998), or from the numbers of sperm found within the female as a function of her mating status (Birkhead et al., 1993; Briskie, 1994; LaMunyon, 2000; Lewis and Jutkiewicz, 1998; Watanabe and Hachisuka, 2005). The very fact that sperm are stored within female tissues makes it difficult to study them directly, and this is compounded by the general inability to distinguish sperm from individual males. Only rarely do studies directly observe

sperm in competition (Civetta, 1999; Hosken and Ward, 2000; LaMunyon and Ward, 1998; Price et al., 1999). Such studies are exceedingly important if we are to understand the forces of selection acting on competing sperm. These potential selective forces include sperm numbers, sperm morphology, the sequence of mating, the female reproductive tract, or a combination of these factors. Here, we investigate the factors acting on competing sperm in the arctiid moth *Utetheisa ornatrix*.

The arctiid moth, *U. ornatrix* has been an important model for the study of sexual selection. Female mate choice centers on defensive pyrrolizidine alkaloids that are sequestered from the larval foodplants and retained into adulthood (Eisner and Meinwald, 1995). Female *U. ornatrix* choose mates based on an indirect assessment of their suitors' alkaloidal content (Dussourd et al., 1991). When mating, males pass a portion of their own alkaloid in the spermatophore, and the female transfers this nuptial gift, along with some of her own alkaloid, to the eggs (Dussourd et al., 1988), protecting them from predators (Eisner et al., 2000; Hare and Eisner, 1993; Rossini et al., 2001). In addition to alkaloids, spermatophores contain

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mating containers. All females mated with virgin males; no male mated for a second time. In addition, male mass was determined just prior to their pairing with females (mean mass: 75.4 mg; range: 43.7–109.6 mg). Each female that mated on its first opportunity was paired for mating with a second male, chosen to differ substantially in body mass from the female's first mate (mean difference 33% or 25 mg). The interval between matings was 1–2 days. For 20 females, the second mate was smaller than the first. For another 12 females, the second mate was the larger. Eleven females were allowed to mate only once.

All females were dissected 1.5–5 h after spontaneously disengaging from the males. The reproductive organs, and especially the pseudobursa were examined under phase contrast optics for the presence of sperm. The function of the pseudobursa is unclear, but it always fills with seminal fluids and in some cases receives clumps of sperm (personal observations). Because the clumps of sperm in the pseudobursa appear congealed and perhaps in a state of degradation, we opted to assign a qualitative measure of the amount of sperm in the pseudobursa (none/few, moderate, or full).

To be included in the study, all twice-mated females met two criteria at autopsy: (i) they had sperm in their spermathecae, and (ii) they contained two spermatophores, the second with the exit tube, or collum, aligned properly with the female's seminal duct. The collum is often misaligned after unsuccessful matings in *U. ornatrix* (personal observations, CWL), as has been observed for other insects (Mann, 1984). These two criteria provide some assurance that the female received two normal ejaculates. Three of the initial sample of 35 twice-mated females were excluded for failing to meet these criteria. (One had only one spermatophore, the collum was not aligned properly in another, and the spermatheca was empty in the third.)

2.3. Sperm arrangement in the spermatheca

We observed the arrangement of eupyrene sperm nuclei within the spermathecal sperm masses of laboratory-reared females that had mated ad libitum in culture. The females were dissected and the number of colla counted to determine how many times the females had mated. Eight females contained one spermatophore, and nine females contained more than one spermatophore. The spermathecae were removed and transferred to 200 μ l of Carnoy's fixative for 48 h. After fixation, the spermathecae were placed in a depression slide under PBS, and the walls of the spermathecae were torn away to release the sperm mass. The Carnoy's fixation process crosslinked the sperm to one another so that the released sperm mass remained in its natural arrangement.

To observe sperm arrangement within the spermathecal sperm mass, we labeled the eupyrene nuclei with another DNA fluorophore, Hoechst 33342. After immersion in PBS containing 0.02 mg/ml Hoechst 33342, the sperm masses

were placed on a microscope slide with the PBS-Hoechst solution, and a cover slip was applied. Pressure was applied to the cover slips to flatten the sperm masses before they were observed under epifluorescence with a COHU 4920 Series CCD camera. The masses were too large to capture in a single image, so multiple images were captured for each sperm mass, and the images were overlaid to give a single image for each sperm mass. Approximately, 15 images were captured for each sperm mass.

The composite images were analyzed for eupyrene nuclei distribution using the morphometric analysis program ImageJ (Abramoff et al., 2004). A reference line was drawn down the center of the sperm mass with ImageJ (Fig. 2A and C). The gray value of the pixels was measured along the reference line, and the standard deviation in pixel density recorded. The standard deviation reflects the overall variability in the gray values across the reference line, with higher standard deviation in sperm masses with larger clumps of eupyrene sperm nuclei.

3. Results

3.1. Sperm deposition in the spermatheca

The number of sperm stored within the spermatheca of the 38 females captured in nature varied greatly but was not a function of female mating status. While these females received from 1 to 23 spermatophores, the number of sperm stored within them did not correlate with spermatophore number ($r = 0.029$; $P = 0.863$). Females that contained one spermatophore had an average of 2760 eupyrene sperm within their spermathecae (Fig. 3), an indication of the number received from a single mating. Rather than having double this number, or ca. 5000 sperm, twice-mated females actually contained fewer, with a mean of 2125 sperm, indicating that not all sperm received are stored in the spermatheca. The greatest number of sperm was found in females containing between 3 and 7 spermatophores with an average number of 8868 eupyrene sperm, although the number of sperm in this group of females varied from 500 to 30,000 (Fig. 3). Indeed, four females that contained multiple spermatophores were apparently sterile, containing no sperm in the spermatheca.

3.2. Sperm deposition in the pseudobursa

The pseudobursa was examined as a possible alternative site of sperm deposition. The pseudobursa had no sperm in all but two of the 38 females captured in nature. One of the two females had a large amount of sperm in the pseudobursa, while the other had only a miniscule amount. The pseudobursa appears to have a digestive function in *U. ornatrix*, swelling with seminal fluids immediately after copulation and returning to near its normal size within a day (personal observations). If the pseudobursa is a digestive organ, any sperm deposited there may disappear

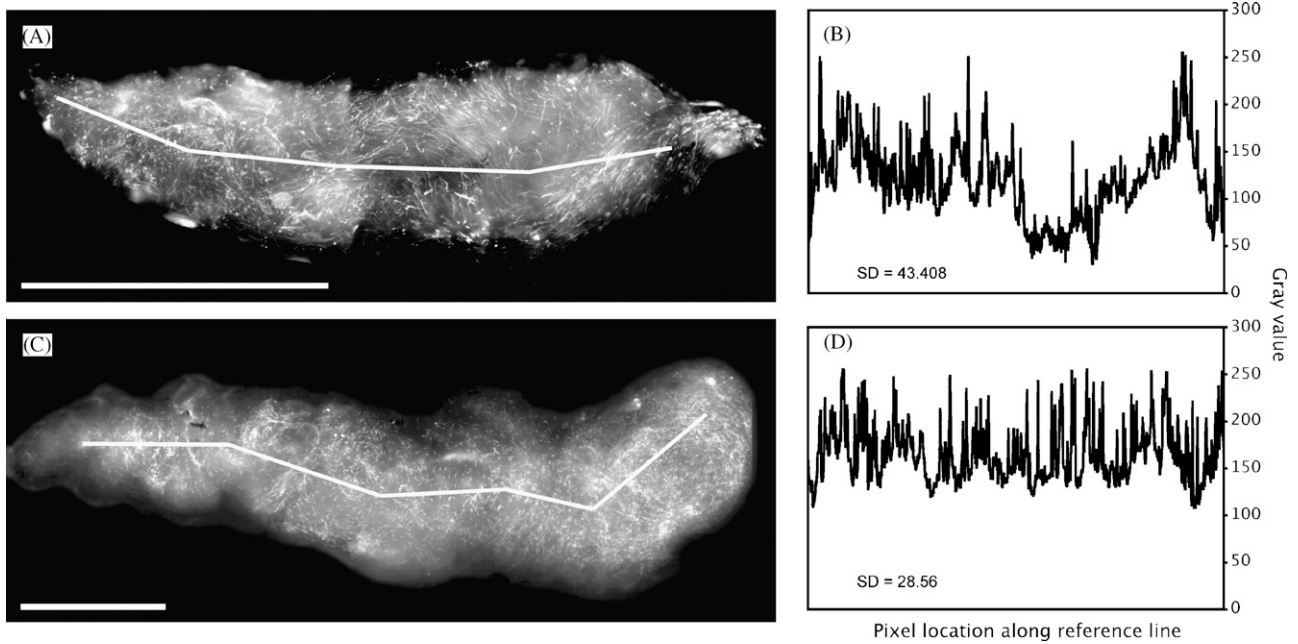


Fig. 2. (A) An epifluorescent image of a spermathecal sperm mass from a once-mated female, and (B) a plot of the gray values encountered along the reference line drawn down the middle of the sperm mass as indicated in (A). (B) and (C) The same as (A) and (B), except that these are for a twice-mated female. Standard deviations for the plots are given. Size bars represent 0.5 mm.

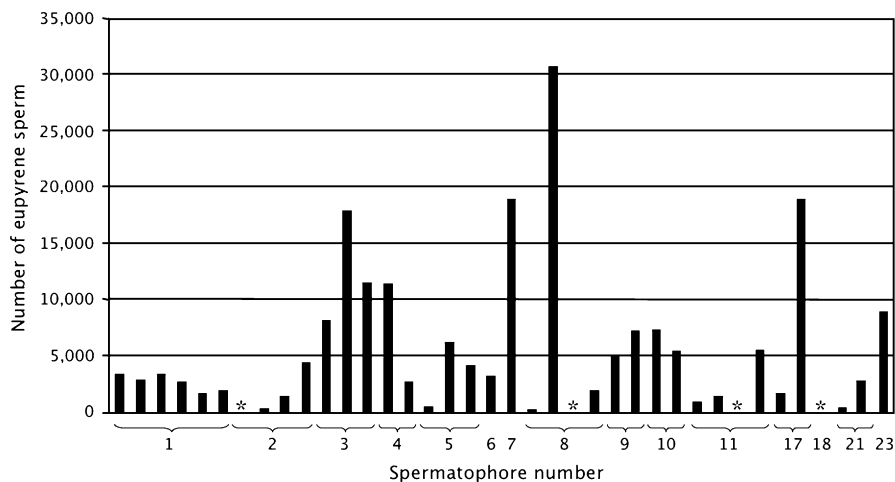


Fig. 3. The number of eupyrene sperm found in the spermathecae of females as a function of the number of spermatophores found within each female. Spermatophore number equates with the number of times each female mated. *Denotes females with empty spermathecae.

rapidly and, therefore, would likely be devoid of sperm in the captured females.

When the pseudobursae of the experimental females were examined, we did find sperm. These females were dissected soon after the end of copula and early in the digestion process. The amount of sperm in the pseudobursa of these females was a function of their mating history. In once-mated females, the pseudobursa was either empty or contained small to moderate amounts of sperm, but it was never full of sperm (Fig. 4). The pattern was similar for twice-mated females that mated with a small male first and a larger male second, except that two of the 12 females had

pseudobursae full of sperm. The pattern changed for females mated to a large male first and a smaller male second: their pseudobursae were significantly more likely to be full of sperm compared to the other twice-mated females (Fig. 4; $G = 7.942$, $P < 0.025$). These results thus indicate that a male's sperm may be deposited in the pseudobursa, especially when he is smaller than a female's previous mate.

3.3. Sperm arrangement in the spermatheca

Examination of the 17 spermathecal sperm masses revealed that the eupyrene nuclei were distributed

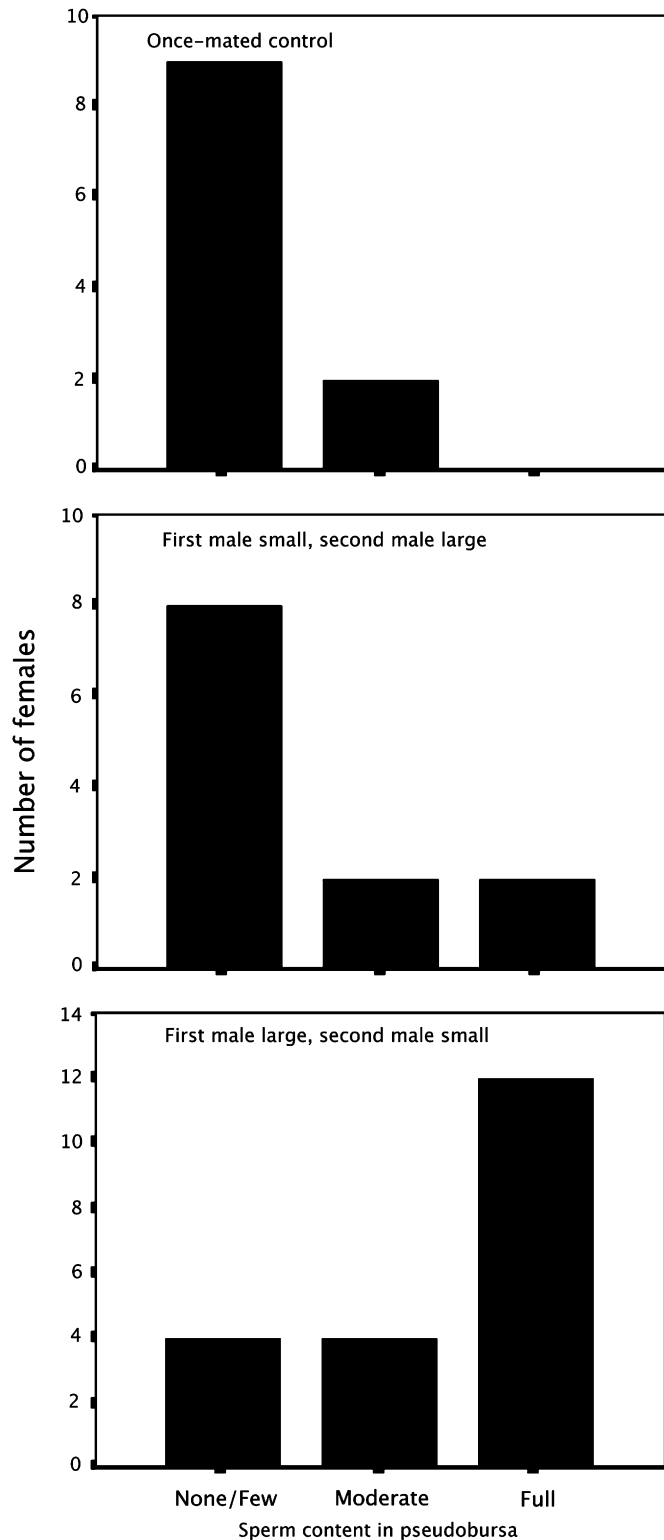


Fig. 4. Sperm content of the pseudobursa of experimental female moths that mated once or twice.

both singly mated and multiply mated females (Fig. 2A and C). There were no obvious regions in the sperm masses from any of the multiply mated females that lacked eupyrene nuclei, indicating an absence of layers of apyrene sperm, which do not fluoresce under these conditions. The variability in the pattern of eupyrene nuclear localization along the reference lines drawn down the middle of each sperm mass image was not significantly different for singly mated females (37.09) compared to multiply mated females (33.98). We compared the mean standard deviations by *t*-test ($t = 0.867$, $P = 0.399$), but because of small sample size ($n = 17$), we also compared the results with a Mann–Whitney *U* test ($U = 29.00$, $P = 0.501$) out of concerns about deviation from normality. Thus, there is no apparent sperm structuring within the spermatheca.

4. Discussion

Multiply mated female *U. ornatrix* store many fewer sperm than they receive. Singly mated females averaged nearly 3000 eupyrene sperm in their spermathecae. If a full complement of sperm from all matings were stored in the spermatheca, then each mating subsequent to the first should increase the number of sperm stored by approximately 3000. Our twice-mated females actually stored fewer sperm on average than our once-mated females. Perhaps the spermatheca has a limited capacity, and it cannot accommodate all sperm received. However, several females contained large numbers of eupyrene sperm. One female in particular had over 30,000 eupyrene sperm in her spermatheca, having likely stored all the sperm delivered in the eight spermatophores she received. This indicates that the *U. ornatrix* spermatheca is elastic enough to house ten times the number of sperm received in a single mating. Therefore, other processes must be responsible for the missing sperm in the majority of multiply mated females.

The appearance of sperm in the pseudobursa suggests that some sperm never enter the spermatheca. While the pseudobursa may receive small amounts of sperm after a female's first mating, our data show that it is never full of sperm from that mating. After the second mating, however, we found that the pseudobursa may fill with sperm, supporting the hypothesis that the female shunts unwanted sperm to that organ. An alternative explanation for the appearance of sperm in the pseudobursa is that it acts as a "staging" organ, storing sperm temporarily on its transit to the spermatheca. However, the pseudobursa was never full in our once-mated females, suggesting that normal sperm transit does not require pseudobursal involvement. Another alternative is that the pseudobursa receives excess sperm that will not fit in the spermatheca, but the spermatheca can accommodate much more than two ejaculates' worth of sperm. The fact that the pseudobursa was more likely to fill when a female remated to a smaller male, supports earlier paternity data showing that sperm from smaller males generally fail to fertilize eggs (LaMunyon and Eisner, 1993). Taken together, these data suggest

throughout the spermatheca. We did observe some clumping of eupyrene nuclei, as if the sperm heads had entered the spermatheca in an ordered fashion and remained associated, but these associations occurred in

that at remating, smaller males' sperm are likely to be shunted to the pseudobursa where they are doomed.

Our data also suggest that the female can remove sperm from spermatheca. Nine of our sample of 38 females had 1000 or fewer sperm in the spermatheca, and all 9 were multiply mated. Four of these females had empty spermathecae. While we cannot rule out the possibility that some of these females had impaired sperm transport, the most likely explanation is that the female emptied sperm from their spermathecae. Sperm ejection appears to be a common feature in insects as it has been demonstrated in dipterans (Snook and Hosken, 2004), odonates (Cordoba-Aguilar, 2006), orthopterans (Reinhardt and Meister, 2000), and even other lepidopterans (Etman and Hooper, 1979). Thus, female *U. ornatix* can shunt incoming sperm to the pseudobursa and empty previously stored sperm from the spermatheca. Such control of sperm use (Eberhard, 1996) allows female *U. ornatix* to use sperm from only their preferred mates, although they collect nutrient and alkaloid laden ejaculates from all their mates.

Female *U. ornatix* therefore limit the interactions of sperm within their reproductive tracts, but sperm interactions are not eliminated. Spermathecae may contain sperm from more than one ejaculate, but those sperm are not noticeably layered. If the apyrene sperm formed a barrier between preexisting sperm and the incoming eupyrene sperm, then we should have observed a region within the spermathecal sperm masses where there was an absence of eupyrene nuclei, but no such stratification existed. Sperm from individual ejaculates may still be layered in the spermatheca, but our data show that the eupyrene and apyrene sperm are interspersed within the spermatheca and not layered. In other species, the apyrene sperm serve to delay remating, protecting the eupyrene sperm from immediate competitors (Cook and Wedell, 1999), but female *U. ornatix* have no refractory period and will mate on consecutive nights. Therefore, the function of the apyrene sperm in *U. ornatix* is as yet unclear.

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