

Determinants of sperm precedence in a noctuid moth *Heliothis virescens*: a role for male age

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Abstract. 1. Females of the noctuid moth *Heliothis virescens* F. mate more than once. Thus, sperm from two or more males normally compete for fertilisations within the female reproductive tract. The eggs are typically fertilised by sperm from only one male, either the female's last mate or an earlier mate. Twice-mated females store only one ejaculate's worth of fertilising sperm (eupyrene) but nearly two ejaculates' worth of a nonfertilising sperm morph (apyrene), which is thought to play a role in sperm competition.

2. The mechanism of sperm use in *H. virescens* was investigated by examining factors that vary with paternity, which was assigned based on allozyme variation. The factors included male and female body masses and ages, male genital characters, the size of the sperm package, and the number of sperm stored by the female.

3. One male typically gained sperm precedence; this was nearly twice as likely to be the second male as it was to be the first. Two factors were found to vary significantly with paternity: female mass and male age. The second male to mate was more likely to gain sperm precedence if the female was larger and if the male was older than the female's first mate.

4. The significance of male age and female mass to several hypothetical models of the mechanism of sperm use is discussed.

Key words. Allozyme, apyrene, eupyrene, *Heliothis virescens*, paternity, sperm competition, sperm count.

Introduction

Female multiple mating is a powerful and nearly ubiquitous behaviour that creates the opportunity for post-copulatory sexual selection in the forms of sperm competition and female control of sperm use (Eberhard, 1996; Birkhead & Møller, 1998). In fact, much of the morphological complexity of reproductive organs may have evolved not to enable fertilisation or reduce inter-specific hybridisation but to facilitate post-copulatory sexual selection (Arnqvist, 1998). Knowledge of the mechanisms of sperm use and competition, and how these mechanisms give rise to morphological complexity, is severely lacking in all but a few species (Simmons & Siva-Jothy, 1998). This paucity of information is most probably due to the fact that post-copulatory sexual selection

takes place within the female, where the interactions are, for the most part, not observable.

Recent research has been directed at unravelling the mechanisms of sperm competition in order to understand the male and female influences over sperm use. Many studies have focused on insects (Simmons & Siva-Jothy, 1998) because insects generally mate more than once and have sperm storage organs, spermathecae, in which sperm are stored for use in fertilisation and where sperm may reside for long periods, even for the life of the female. Thus, when female insects remate, sperm competition is a probable outcome (Parker, 1970).

One order of insects that is receiving attention with regard to sperm competition is the Lepidoptera, and some details of the mechanism of sperm use have come to light. Sperm sometimes fail to fertilise eggs because they do not transfer out of the sperm package, or spermatophore (Mann, 1984); they become mired in dead-end chambers (personal observation of *U. ornatrix*) or they are swept from the reproductive tract by passing eggs (Etman & Hooper, 1979). The size of the

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spermatophore has been implicated as a determinant of paternity in some species (LaMunyon & Eisner, 1994; Wedell & Cook, 1998) but the influence of spermatophore size on sperm use is not clear. Across insects, including the Lepidoptera, sperm movement from spermatophore to spermatheca is dependent largely on the female's musculature (Davey, 1958; Tschudi Rein & Benz, 1990; LaMunyon & Eisner, 1993), giving the female the opportunity to influence the use of sperm. Male Lepidoptera may influence sperm competition by producing a nonfertilising, anucleate morph of sperm called apyrene sperm that has been shown to delay female remating in *Pieris* (Cook & Wedell, 1999). Alternatively, the apyrene sperm may interact directly with the rival sperm in a kamikaze role (Silberglied *et al.*, 1984). With all these factors that potentially influence sperm use, it is not surprising that paternity in the Lepidoptera is variable. In lepidopterans where paternity has been studied, one male generally takes sperm precedence, siring most or all of the offspring, but it can either be the last male to mate or an earlier mate (LaMunyon & Eisner, 1993; Bissoondath & Wiklund, 1997; Cook *et al.*, 1997; Wedell & Cook, 1998).

In an effort to understand the mechanisms that give rise to the variable patterns of paternity in the Lepidoptera, the work reported here investigated the noctuid moth *Heliothis virescens*. Females of this economically important species mate with up to seven different males, averaging 2.6 matings each (Raulston *et al.*, 1975). Offspring paternity in *H. virescens* is characteristic of the pattern found in other Lepidoptera where one male gains sperm precedence, but it can be either the female's last mate or a previous mate (Flint & Kressin, 1968; Pair *et al.*, 1977). In an earlier study of *H. virescens*, twice-mated females stored only one ejaculate's worth of fertilising (eupyrene) sperm ($\approx 15\,000$) but nearly two ejaculates' worth of apyrene sperm ($\approx 300\,000$; LaMunyon, 2000). Variation in the numbers of eupyrene sperm stored was explained by female pupal mass and male age (LaMunyon, 2000). In the work reported here, paternity in twice-mated female *H. virescens* was investigated to determine whether paternity is influenced by a number of factors including body mass, age interval between matings, male fluctuating asymmetry, the numbers of sperm stored by females, and spermatophore size.

Materials and methods

Moths used in this study were provided by the Western Cotton Research Laboratory (U.S.D.A.), Phoenix, Arizona, through the Department of Entomology at the University of Arizona. As they were received, the pupae were sexed, weighed, and placed individually in containers in an incubator at 22 °C with a LD 14:10h photoperiod. Male pupae averaged 211 mg (range: 154–272); female pupae averaged 206 mg (range: 141–268). On eclosion, the moths were given cotton wicks wetted with sugar water (10% sucrose).

Moths were paired for matings in 0.25-litre cylindrical containers. At their first matings, females were 1–6 days post-eclosion (mean: 2.8 days) and their mates were 1–7 days old

(mean: 2.5 days). Two to 4 days after their first mating, the females were mated with a second male. All twice-mated females included in the experiment laid fertile eggs between matings and contained a properly placed second spermatophore at autopsy (tip of the spermatophore exit tube aligned with the opening of the seminal duct). Thus, each female received a fertile first mating, and proper spermatophore placement gave reasonable assurance that the second mating was also fertile (LaMunyon, 2000). Twenty-four females met these criteria and were included in the experiment. Eggs were collected from each female daily. Twelve females were frozen 4 days after their second mating; the other twelve females were allowed to lay eggs until they died. Four days after they were laid, the eggs were placed on artificial diet (BioServe® #F9915B, Frenchtown, New Jersey). Emergent larvae were allowed to grow until they were ≈ 1 cm in length, when they were frozen at -80 °C for paternity analysis. Survivorship of offspring from egg to harvest was recorded.

Paternity of offspring was assigned on the basis of variation at the glucose phosphate isomerase or phosphoglucosmutase allozyme loci. Allozyme phenotypes were determined following standard procedures for cellulose acetate gel electrophoresis (Hebert & Beaton, 1989). For each female, at least 42 offspring were analysed, representing 15–20% of their total offspring. To assess changes in paternity over time, approximately half the offspring analysed came from eggs laid on the first day of collection, the rest from eggs laid on the third or later days of collection.

Moths were selected for pairing on the basis of their allozyme phenotypes, which were ascertained beforehand. The left mesothoracic leg of each moth was removed and analysed (the amputation had no noticeable effect on the moths' subsequent mating performance). With four of the females, the two male partners were homozygous for different alleles at one of the two allozyme loci, allowing unambiguous assignment of paternity to all progeny. For the other 20 females, one male partner was heterozygous and shared one allele with the homozygous male, so that the offspring bearing the shared allele could not be assigned unambiguously to either sire. In these cases, paternity was estimated using offspring bearing the unshared allele. The heterozygous male was assigned twice the fraction of the progeny that bore the unshared allele because only half its progeny (on average) would have received that allele.

Females were subjected to autopsy and their sperm storage organs were removed for sperm counts. The main sperm storage organ, the spermatheca, was taken together with the spermathecal duct, after removal of the spermathecal gland. The spermatophore receptacle, or bursa copulatrix, was also removed to check for proper placement of the second spermatophore. The size of the second spermatophore was estimated by measuring the diameter of its exit tube, or collum. Because the second spermatophore had remained inside the female for several days before it could be measured, the collum had collapsed on 10 of the spermatophores. Measurements were not made for these 10 cases.

The spermathecae were broken apart using fine forceps in a small volume of phosphate buffered saline (pH 7.0), which was

then drawn into a pipette, measured for volume, and transferred to a 1.5-ml tube. Both eupyrene and apyrene sperm in each sample were counted in a hemacytometer following methods described by LaMunyon (2000). Finally, the duct that transfers sperm away from the spermatophore receptacle, the seminal duct, has been shown to contain sperm in nearly half of all females observed (LaMunyon, 2000). The amount of sperm in this organ was estimated by the extent of its distention, rated from 0 (not distended) to 3 (maximally distended). Owing to the severe state of internal degradation found in seven of the moths, the sperm duct was indistinct, precluding its measurement.

Mated males were also dissected and the following genital measurements taken: the diameter of the aedeagus (mean: 1.18 mm, range: 1.12–1.28), the lengths of the two claspers, structures with which the male grasps the female abdomen during copulation (mean: 4.38 mm, range: 4.08–4.69), and the length of a hook-like structure at the distal end of the genitalia (mean: 1.57 mm, range: 1.50–1.68). Male pupal mass, aedeagal diameter, clasper length, and hook length were all correlated significantly (correlation coefficients ranged from 0.562 to 0.966; Bonferroni probabilities were all <0.004). The effects of the male traits on paternity were analysed using multiple regression analysis, and to avoid inclusion of correlated independent variables, the correlated male measures were combined into a composite male size score as follows: for each trait, the individual measure was standardised by dividing it by the mean for that trait, then all the standardised scores were averaged to give the composite. Finally, the difference in length between left and right claspers was calculated as a measure of fluctuating asymmetry (mean: 0.03 mm, range: 0–0.12 mm). These fluctuating asymmetry measures have been shown to be highly repeatable (LaMunyon, 2000).

Results

Offspring paternity varied considerably among the females; the per cent of the offspring sired by the second male ranged from 0 to 100 (Fig. 1). Indeed, sperm precedence predominated: $>80\%$ of the progeny were sired by one of the female's mates in 17 of the 24 females in the experiment. The second male was nearly twice as likely to gain sperm precedence (Fig. 1). Mixed paternity ($20\% <$ second male paternity $< 80\%$) was found in only seven females. The proportion of offspring sired by the second male did not change significantly with time after mating (Wilcoxon signed ranks test: $Z = -0.958$, $P = \text{NS}$; Fig. 2). In 16 females, the second male's paternity changed by $<10\%$ in the days after mating. For the remaining eight females, the change in the second male's paternity ranged from 13 to 68%.

While the overall pattern of paternity shown in Fig. 1 could be the result of differential fertilisation, it could also result from differential offspring survivorship, where one male's progeny do not survive to be assigned paternity. Offspring survivorship ranged from 86 to 36% (mean 66%). Because survivorship and paternity were assessed on progeny produced both immediately after the females' second matings and

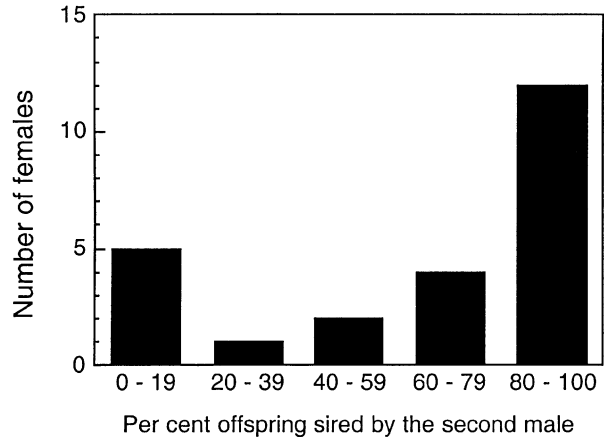


Fig. 1. The per cent of the offspring sired by a female's second mate for 24 twice-mated females.

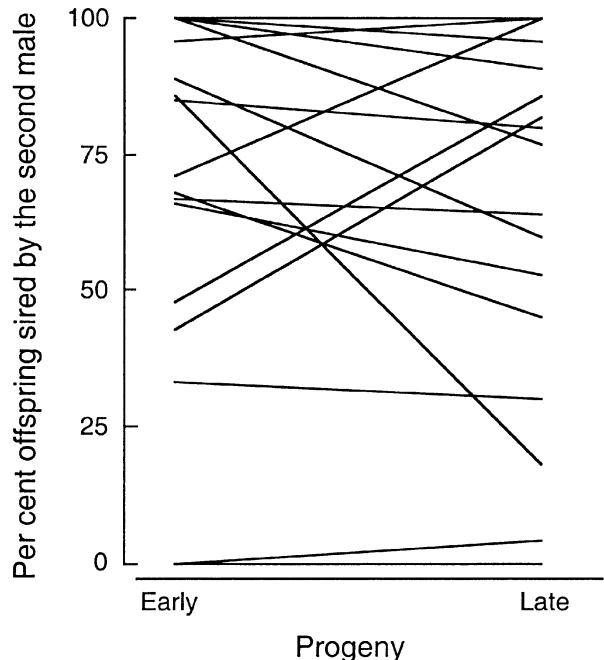


Fig. 2. The change in the per cent offspring sired by a female's second mate over time for the 24 twice-mated females. Early progeny were laid on the first day after each female's second mating and late progeny were laid ≥ 3 days later.

several days thereafter, the relationship between the two variables was investigated. Changes in progeny survivorship did not correlate with changes in the second male's paternity over time ($r = -0.1743$, $P = \text{NS}$), indicating that estimates of paternity were not influenced by the survivorship of the progeny. In the most extreme case, survivorship increased from 42% in the early progeny to 86% in the later progeny, but the second male's paternity remained constant at 0. Factors

other than offspring survivorship must therefore explain the variable pattern of paternity.

In an effort to find factors that determine paternity, several male and female characters were investigated as possible correlates of paternity, including female pupal mass and age at first mating, the interval between matings (generally 2 days, but 3 days for four females and 4 days for two females), fluctuating asymmetry in the length of the male claspers, the eupyrene and apyrene sperm remaining in the spermatheca, the ratio of eupyrene sperm to apyrene sperm, and the difference between the rival males' composite size scores and ages at mating. The size of the second spermatophore (as estimated by the diameter of the collum) and the degree of sperm in the seminal duct were also analysed, although these measures were not made on all females due to their state of internal degradation.

Paternity estimates from progeny produced the day after the females' second matings were analysed by stepwise multiple regression (Table 1). Two factors were identified as significant determinants of paternity: female pupal mass and the difference in age between the female's two mates (Table 1). The second male's paternity increased with the size of the

female (Fig. 3a) and with the age of the second male at mating relative to that of the first mate (Fig. 3b). Variation in female mass and male age explained nearly 50% of the variation in the observed pattern of paternity (overall $R^2=0.492$). In a similar analysis performed on the paternity estimates from progeny produced ≥ 3 days after mating, female mass continued to be a significant determinant of the second male's paternity but the age difference between the female's two mates was no longer a significant determinant (Table 1). Although the per cent offspring sired was arcsin transformed, this procedure did not normalise the data, so the relationships between the second male's paternity in the early progeny and both the male age difference and female mass were analysed using the non-parametric Spearman rank correlation. This analysis showed that the second male's paternity varied significantly with both the difference in age between a female's two mates ($r=0.413$, $P<0.05$) and the female's pupal mass ($r=0.628$, $P<0.01$), supporting the multiple regression results.

The absence of an effect of the number of sperm stored is interesting because the stored sperm are those that compete for fertilisations. The females' spermathecae, sampled ≥ 4 days after the second matings, contained a mean of 14 469 (range:

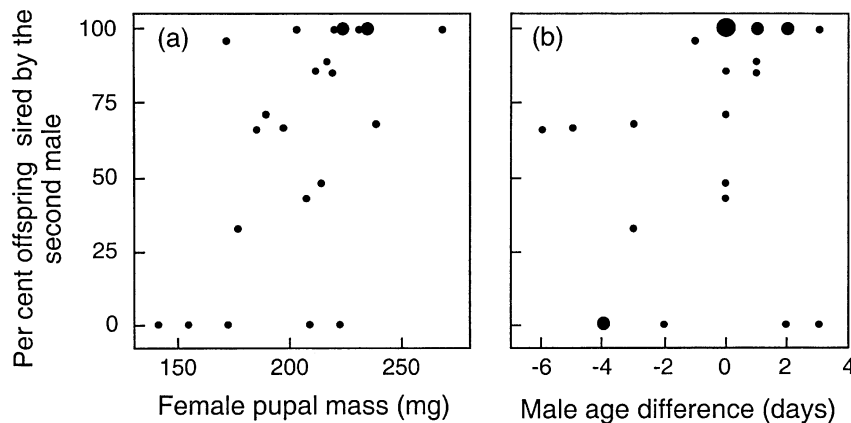


Fig. 3. The relationship between the per cent offspring sired by a female's second mate and (a) female pupal mass and (b) the difference in age between each female's two mates. For statistical treatment of these relationships, see Table 1. Increased dot size indicates overlapping data points.

Table 1. Results of stepwise multiple regression analyses of the per cent of either early (first day after second mating) or late (third or later day after second mating) offspring sired by the second mate of 24 twice-mated females. The entire list of independent variables included female pupal mass and age at first mating, the interval between mating, fluctuating asymmetry in the length of male claspers, the numbers and ratios of sperm remaining in the spermatheca, the difference between the rival males' size and age, the size of the second spermatophore, and the amount of sperm in the seminal duct (see text for details). The per cent of offspring sired by the second male was arcsin transformed for statistical treatment.

Variable	Early progeny			Late progeny		
	Coefficient (SE)	T	P	Coefficient (SE)	T	P
Constant	1.571 (0.689)	-2.279	0.033	-1.063 (0.761)	-1.398	0.177
Female pupal mass	0.012 (0.003)	3.672	0.001	0.009 (0.004)	2.558	0.018
Male age difference	0.084 (0.038)	2.187	0.040	0.075 (0.042)	1.768	0.092

1250–27 500) eupyrene sperm and 290 781 (range: 48 750–600 000) apyrene sperm. In another stepwise multiple regression analysis, these numbers were not related to any of the male and female traits listed above, including female mass, indicating that larger females stored no more sperm on average than did smaller females.

The change in the second male's paternity observed with time since mating (Fig. 2) was also examined by multiple regression analysis. None of the independent variables described above was a significant predictor of variation in paternity over time.

Discussion

Twice-mated female *H. virescens* displayed a pattern of variable sperm precedence, where one of the two mates generally sired most or all of the offspring but the second mate was more likely to gain sperm precedence. This pattern is similar to results from earlier studies of this moth (Flint & Kressin, 1968; Pair *et al.*, 1977) and to the pattern of paternity found in other Lepidoptera (LaMunyon & Eisner, 1993; Bissoondath & Wiklund, 1997; Cook *et al.*, 1997; Wedell & Cook, 1998). Furthermore, this pattern is the result of differential fertilisation, and is not likely to be due to failed matings or differential offspring survival. The twice-mated females all received two successful matings, and while offspring survival was only $\approx 65\%$, changes in progeny survival over time since mating did not correlate with changes in the paternity of the second male, suggesting that progeny survival did not influence measures of paternity. Thus, the pattern of paternity reflects the outcome of ejaculate competition, however the mechanism of ejaculate competition is not clear. Results from this study showed that twice-mated females store on average nearly 14 000 eupyrene sperm, which is similar to that stored by singly mated females (LaMunyon, 2000). Therefore, females store only a fraction of the sperm they receive in two matings, which may contribute to the pattern of paternity.

What factors or mechanisms explain this pattern of paternity? With the results presented here and elsewhere (LaMunyon, 2000), several factors can be ruled out. The present paternity results were not affected by fluctuating asymmetry in the length of the male's claspers, male size, or the interval between matings. Because the experimental protocol limited the inter-mating interval to 2 to 4 days, the effect of a longer inter-mating interval on paternity remains unknown. The size of the male spermatophore, estimated here by the diameter of the collum, also did not affect paternity, although this conclusion should be taken with some caution because only 14 spermatophores were measured. In an arctiid moth, male size and spermatophore size are correlated positively and have been shown to influence paternity (LaMunyon & Eisner, 1993, 1994) but neither trait was important to paternity here.

Fluctuating asymmetry is also apparently unimportant to paternity. Fluctuating asymmetry is the presence of small departures from perfect bilateral symmetry believed to result

from developmental stress and thought to reflect male quality (Møller & Swaddle, 1997). The absence of an effect of fluctuating asymmetry is not unexpected because in other moth species it has no influence on either paternity (LaMunyon, 1994) or sperm delivery (Gage, 1998), and in *H. virescens* it does not influence the number of sperm stored by females (LaMunyon, 2000). Also, while the interval between matings varied by only 3 days, this variation did not influence paternity, suggesting that depletion of the first male's sperm between matings does not affect the pattern of sperm precedence. A study of sperm storage in female *H. virescens* showed no significant reduction in the numbers of sperm stored as a function of time since mating (LaMunyon, 2000), indicating that sperm usage is minimal in comparison with the number stored ($\approx 15 000$ eupyrene sperm), and supporting the conclusion that sperm depletion does not affect sperm precedence.

The significant relationships between paternity and both female mass and male age suggest other possible mechanisms of sperm use. Results presented here show that the older of a female's two mates was more likely to gain sperm precedence in the progeny laid immediately after the female's second mating (all males were virgins at the time of mating). This relationship was not found in the progeny laid ≥ 3 days after the female's second mating, perhaps as a function of either sperm mixing in the spermatheca or sperm depletion, although, as noted above, sperm depletion is unlikely to have a significant effect. The earliest progeny represent the immediate competition between ejaculates, and the older males had the advantage with these progeny. In an earlier study of sperm storage by female *H. virescens*, females stored more sperm from older virgin males (LaMunyon, 2000). In addition, the number of sperm transferred in the spermatophore increases with male age (C. W. LaMunyon and T. Huffman, unpublished). It is likely that older males have more sperm available for ejaculation due to daily sperm release from the testes, as is the case in other Lepidoptera (Giebultowicz *et al.*, 1989; Hiroyoshi, 1995; Wedell & Cook, 1999). Taken together, these results suggest that performance in sperm competition depends, in part, on the number of sperm transferred. In its simplest form, the competition could approximate a lottery, where all a male's sperm enter the spermatheca and fertilise eggs as a function of their numeric representation.

A simple lottery cannot function in *H. virescens* sperm competition because females store only one ejaculate's worth of eupyrene sperm (LaMunyon, 2000). Thus, some sperm either do not enter the spermatheca or are displaced from it when the female remates. Such a pattern of biased sperm storage could occur in several ways. The female's sperm storage organ may have a capacity that approximates one ejaculate and correlates with female size. If the first male's sperm fill the spermatheca, no more will enter and first male sperm precedence will ensue. If, on the other hand, the first male does not pass enough sperm to fill the spermatheca, a portion of the second male's sperm will enter, and, if the last sperm to enter are the first to be used, second male sperm precedence will occur. Larger females would have larger spermathecae, biasing paternity toward the second male. Interestingly, paternity did not vary with the number of either

type of sperm stored in the spermatheca, even when the effect of female mass was removed by multiple regression. Indeed, the number of eupyrene sperm stored in the spermatheca varied by over an order of magnitude (1250–27 500), with no effect on paternity.

Alternatively, the second male's sperm could displace the first male's sperm from storage. There is evidence that sperm enter and leave the spermatheca via different passageways (Callahan & Cascio, 1963). If the spermatheca has a limited capacity, it is possible that sperm entering the spermatheca force pre-existing sperm out of the spermatheca. In this model, sperm count would be an important factor because the more pre-existing sperm present, the more sperm the second male would have to transfer in order to gain sperm precedence.

Another potential mechanism for biased sperm storage is active manipulation on the part of the female. Females may either limit the number of sperm that reach the spermatheca or eject stored sperm when they remate, as females are known to do in another noctuid moth (Etman & Hooper, 1979). In a number of insects, including several Lepidoptera, the sperm do not propel themselves to the spermatheca but are transported there by the muscular contractions of the female's reproductive tract (Davey, 1958; Tschudi Rein & Benz, 1990; LaMunyon & Eisner, 1993). In an earlier study, sperm were found lodged indefinitely in the seminal duct (LaMunyon, 2000), through which the sperm migrate towards to the spermatheca. These may be at least some of the sperm the female withholds from storage.

The apyrene sperm may also participate in the determination of paternity. Apyrene sperm have been shown in other Lepidoptera to reduce the likelihood of female remating and thereby protect the eupyrene sperm from competing ejaculates (He *et al.*, 1995; Cook & Wedell, 1999). Apyrene sperm do not serve this function in *H. virescens* because females will mate on consecutive nights with no apparent refractory period (Raina & Stadelbacher, 1990). Alternatively, the apyrene sperm may interact directly with the rival sperm in a *kamikaze* role (Silberglied *et al.*, 1984). In this case, the number of apyrene sperm may determine a male's success in gaining sperm precedence. It is interesting that the number of apyrene sperm stored by twice-mated females is nearly twice the amount stored by once-mated females (LaMunyon, 2000). The ratio of eupyrene sperm to apyrene sperm in the spermatheca did not explain variation in paternity, although this conclusion requires caution. Sperm counts were performed either 4 days after the second mating ($n = 12$) or at the time of natural death ($n = 12$). Thus, the interval between the second mating and sperm counts varied, however this interval has been shown to have no significant effect on the numbers of sperm found in the spermatheca (LaMunyon, 2000). Therefore the importance of the apyrene sperm remains unclear.

Obviously, only additional data will distinguish among these hypothetical mechanisms of sperm use, however it seems likely that the mechanism will be a mix of male and female processes. Males influence the outcome through sperm number: the greater the number, the better the chance of gaining sperm precedence. Female size influences paternity, and females may influence the process actively through biased

sperm storage, by either withholding access to the spermatheca or ejecting sperm from it. The criterion on which the females might base the decision to withhold is not clear, neither is the potential benefit they realise from such post-copulatory choice of sire.

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