



Sperm storage by females of the polyandrous noctuid moth *Heliothis virescens*

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Female tobacco budworm moths, *Heliothis virescens*, generally mate with more than one male, receiving from each mate both fertilizing sperm (eupyrene) and nonfertilizing anucleate sperm (apyrene), which is thought to play a role in sperm competition. One male typically gains sperm precedence, but it is not consistently the last or the first male to mate. I investigated the mechanism of this variable pattern of paternity by examining the patterns of storage of both types of sperm in the female's spermatheca as a function of multiple mating and male phenotype. The number of stored apyrene sperm varied with mating history, being greatest in twice-mated females and least in females mated to one nonvirgin male. In contrast, only one ejaculate's worth of eupyrene sperm was stored regardless of female mating history (once or twice mated). Thus, while they store two complements of apyrene sperm, twice-mated females apparently store only one ejaculate's worth of eupyrene sperm. This biased pattern of sperm storage may contribute to the variable pattern of paternity observed in this species. Eupyrene sperm storage also correlated positively with female size, male age and spermatophore size. Finally, a new sperm storage site was identified and described. It is a bulged region in the seminal duct.

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In most animal species, females mate with more than one male during any one fertile period (Smith 1984; Birkhead & Møller 1998). This frequently places the sperm from two (or more) different males in competition for fertilizations, and thereby extends sexual selection past the act of copulation, which has far-reaching implications. Depending upon the species, males may respond to the threat of sperm competition by attempting to limit other males' access to their mates by methods such as mate guarding (Birkhead & Parker 1997) and secreting materials, typically called mating plugs, that block the female's copulatory opening (Thornhill & Alcock 1983). Males may also respond by acting directly on competing sperm, either by removing it with penile brushes and scrapers (Waage 1979), by disabling it with seminal secretions (Harshman & Prout 1994; Clark et al. 1995; Rice 1996), by flooding it with large numbers of their own sperm (Gage & Baker 1991; Gage & Barnard 1996), or by producing sperm with greater competitiveness (LaMunyon & Ward 1998, 1999). These actions taken directly against the competing sperm occur within the confines of the female reproductive tract, where the female can manipulate the competition to give certain sets of sperm an advantage (Eberhard 1996). However,

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because these actions occur within the female it is extremely difficult to determine the mechanism by which the competition proceeds, and thus, the forms of sexual selection that continue past copulation.

Recent research has been directed at unravelling the mechanisms of sperm competition to understand how males and females influence sperm use. Many studies have focused on sperm transfer and use in insects (Harshman & Prout 1994; Cook & Wedell 1996; Arthur et al. 1998; Gage 1998; Price et al. 1999; Simmons et al. 1999), because insects generally mate more than once and have sperm storage organs, spermathecae, in which sperm are stored for use in fertilization and where sperm may reside for long periods, even for the life of the female. Thus, when female insects remate, sperm competition is a likely outcome (Parker 1970), and a male's fertilization success is likely to depend, in part, upon the number of his sperm that reach and remain in the spermatheca.

One order of insects that has been studied intensively is the Lepidoptera, whose members are very intriguing in terms of sperm competition. The magnitude of multiple mating by female Lepidoptera can be impressive (Drummond 1984): females of the tiger moth *Utetheisa ornatrix* mate up to 13 times, each mating requiring an entire night, and females live only 3–4 weeks (LaMunyon 1994). Thus, sperm from many males enter the female

reproductive tract. However, the fate of the incoming sperm is uncertain, due to the complexity of the lepidopteran female reproductive tract. In various Lepidoptera, sperm are known to fail to transfer out of the sperm package, or spermatophore (Mann 1984), to become mired in dead-end chambers (personal observations of *U. ornatrix*), or to be swept from the reproductive tract by passing eggs (Etman & Hooper 1979). While lepidopteran sperm may exert some influence over their movement from spermatophore to spermatheca, they are largely dependent upon the female's musculature to propel them (Tschudi Rein & Benz 1990; LaMunyon & Eisner 1993), giving the female the opportunity to exert influence over the successful transfer of sperm to the spermatheca. Even more bizarre is the fact that male Lepidoptera produce two sperm morphs: fertilizing, or eupyrene sperm, and nonfertilizing, anucleate apyrene sperm that may function in sperm competition (Silberglied et al. 1984; Cook & Wedell 1999). Not surprisingly, the common pattern of paternity observed within species of Lepidoptera is variable. One male generally takes sperm precedence, siring most or all 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). Thus, some males mate but receive no fertilizations, a costly loss for male Lepidoptera, because males of many species transfer nuptial gifts with the ejaculate (reviewed in Rooney & Lewis 1999).

What mechanisms could be at play to give rise to the variable pattern of paternity in the Lepidoptera? The number of sperm delivered by the male may be an important determinant of paternity (Cook & Wedell 1996; Wedell & Cook 1998), as can be the size of the spermatophore (LaMunyon & Eisner 1994; Wedell & Cook 1998). Sperm from different ejaculates apparently do not mix extensively in the spermatheca, because sperm precedence is so common (Cook et al. 1997). Sperm from different ejaculates might be layered in the spermatheca so that the last mate's sperm are the first to be used. However, this pattern of sperm storage would give rise to last-male sperm precedence (Walker 1980). Perhaps sperm storage is biased, with sperm from only certain males being stored in the spermatheca. These different scenarios would give rise to quite different patterns of sperm storage. For instance, layering of newer sperm over old would cause the total number of sperm stored in the spermatheca to rise with each mating, whereas biased sperm storage may not.

To understand the mechanisms that give rise to the variable patterns of paternity in the Lepidoptera, I investigated sperm storage in 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). Furthermore, females appear to experience essentially no refractory period following mating, because they will resume pheromone production and calling behaviour the very next night after mating (Raina & Stadelbacher 1990). Offspring paternity in this species is characteristic of the Lepidoptera, being biased towards one male, but it can be either the female's last mate or a previous mate (Flint & Kressin 1968; Pair et al.

1977). Therefore, when females seek more than one mating, sperm from one or more males lose out. By what mechanism do these sperm fail? Are all the sperm received by the female stored in the spermatheca? Or is there biased sperm storage? Here, I describe the patterns of sperm storage by female *H. virescens* in an attempt to answer these questions. I also present an investigation of the relationship of several male traits with the numbers of sperm stored to determine whether sperm from certain males are more likely to gain access to the spermatheca.

METHODS

Moths used in this study were graciously provided by the Western Cotton Research Laboratory (USDA), 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 on a 14:10 h light:dark cycle. Male pupae averaged 204 mg (range 148–307) and female pupae averaged 190 mg (range 138–262). Upon 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, males were 1–14 days posteclosion (mean: 3.7 days), and females were 1–8 days old (mean 2.5 days). Thirty-seven females mated once to virgin males. Twenty-two of the males that copulated with virgin females were mated to a second virgin female 1 day following their first mating. Twenty-four other females were mated to two virgin males, with a 2-day interval between matings. All twice-mated females laid eggs between matings, but five of the original 24 females laid infertile eggs during the interval and were not included in the study. (Eggs were scored as fertile if after 3 days they turned from green to brown, showing embryonic development.) After their final mating, the moths were placed in 1.5-ml microcentrifuge tubes and frozen either 5 h into the light phase of the morning after mating, or on subsequent days up to 3 days after mating (twice-mated females were frozen only on the morning after mating or 1 day later).

I dissected females and removed their sperm storage organs for sperm counts. I removed the main sperm storage organ, the spermatheca, together with the spermathecal duct, after separating them from the spermathecal gland (Fig. 1). The spermathecae of these moths have two chambers, the main chamber or utriculus, and the side chamber or lagena (Fig. 1). Often, the lagena was empty, but occasionally it contained sperm, and in these cases I removed it and stored it separately for sperm counts. In nine of 59 singly mated females (five mated to virgin males; four mated to nonvirgin males), the spermatheca was empty. The absence of spermathecal sperm was in most cases accompanied by the misplacement of the spermatophore, where the collum tip did not align with the seminal duct (Figs 1, 2). These cases were not included in the analyses, because the sperm in the spermatophore never had a chance to enter the seminal duct. I also found sperm in the seminal duct itself. Therefore, I

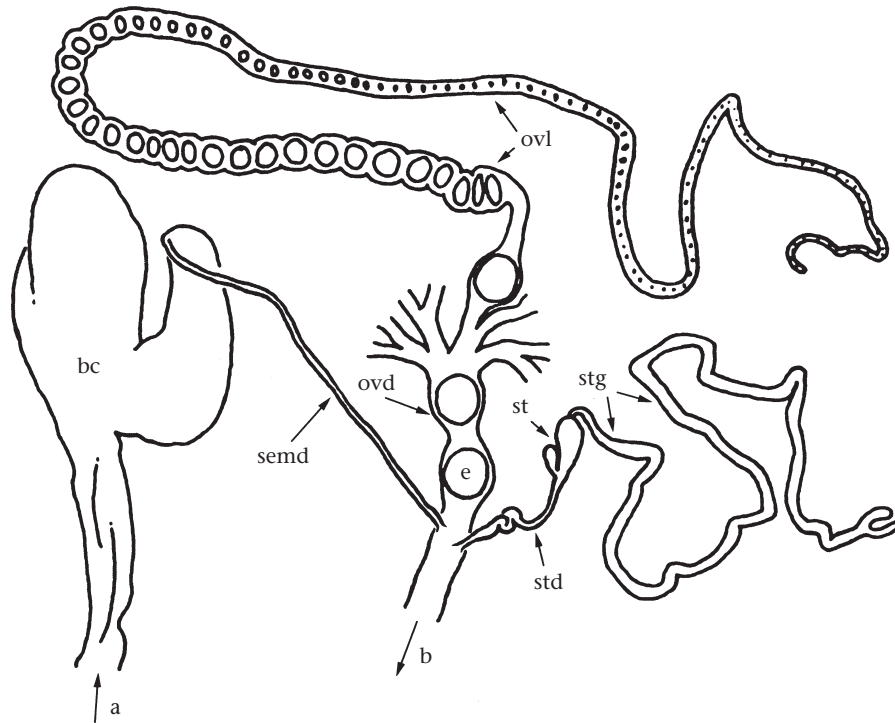


Figure 1. Female reproductive tract. Drawing made from a dissection of a virgin female. Spermatophores are deposited in the bursa copulatrix (bc) through opening a, and eggs (e) are laid through opening b. Sperm travel from the bursa copulatrix through the seminal duct (semd) to the oviduct (ovd), and then enter the spermathecal duct (std) on their way to the spermatheca (st), to which the spermathecal gland (stg) is attached. The ovarioles (ovl) supply unfertilized oocytes. Only one of the eight ovarioles is illustrated.

removed the seminal duct from every female for sperm counts. Finally, I dissected the spermatophore(s) out of the bursa copulatrix for measurements (see below).

I dislodged the sperm stored within the spermathecal chambers and the seminal ducts and diluted them in buffer for counting. I broke the organs apart with 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. I brought the total volume to 187.8 μ l and vortexed the sample for 5 min to free the sperm from the organ remains. I then brought the samples to 200 μ l with the addition of the DNA label 4,6-diamidino-2-phenylindole (DAPI; final concentration 10 μ g/ml) and dimethyl sulphoxide (final concentration 4%, v/v) to facilitate penetration of the DAPI. I vortexed each sample for 5 s and stored the samples in the dark for at least 4 min to allow sufficient DAPI labelling. The eupyrene sperm were easily distinguished from the apyrene sperm by their unmistakable nuclei and greater length and diameter (Fig. 3). Due to the relatively high concentration of DAPI, even the apyrene sperm fluoresced weakly, which allowed them to be counted with certainty (Fig. 3). I subsampled each tube by vortexing each for 5 s and immediately withdrawing 9 μ l from the middle of the sample. I placed the 9 μ l in a hemacytometer, observed it under epifluorescence, and counted the sperm. I subsampled each tube six times.

I dissected mated males and made measurements of their genitalia (Fig. 2). I measured the diameter of the aedeagus (mean 1.19 mm; range 1.06–1.28), and the lengths of both claspers (mean 4.28 mm; range 3.83–4.67)

and a hook-like structure at the distal end of the genitalia (mean 1.58 mm; range 1.48–1.70). In addition, I estimated the size of each spermatophore by taking the average of two diameters measured in perpendicular directions across the body (mean 1.25 mm; range 1.02–1.55). I also measured the diameter of the exit tube, or collum, of each spermatophore (mean 0.22 mm; range 0.18–0.26). Male pupal mass, aedeagal diameter, clasper length and hook length were all significantly correlated (correlation coefficients ranged from 0.375 to 0.947; Bonferroni probabilities were all less than 0.008). For statistical analysis, I combined these correlated male measures into a composite male size score as follows: for each trait, I standardized the individual measure by dividing it by the mean for that trait, and then averaged all the standardized scores to obtain the composite. Finally, I calculated the difference in length between left and right claspers as a measure of fluctuating asymmetry (FA; mean 0.02 mm; range 0–0.05). To estimate repeatability of the FA measures, I measured the length of the right and left claspers of an additional 25 males twice. The first and second calculated measures of asymmetry were strongly correlated ($R=0.903$, $P<0.001$). Thus, the FA measurements were highly repeatable.

RESULTS

To find variables that determine sperm numbers stored by the female, I first examined mating history. Sperm storage varied significantly with female mating history, but only for the non-nucleated apyrene sperm (Fig. 4). When

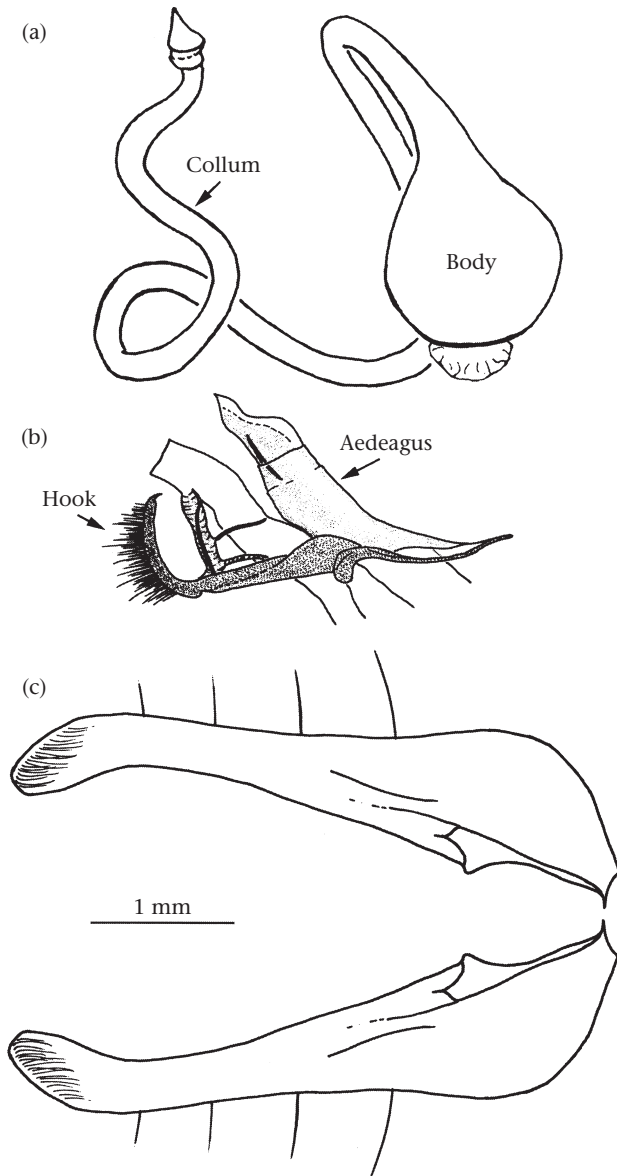


Figure 2. The spermatophore (a) and genitalia (b, c) of male *H. virescens*. All are drawn at the same scale. (b) Lateral view of the aedeagus and a hook-like structure terminating the genitalia. The claspers (c), shown here from the dorsal view, normally surround the structures shown in (b).

mated to one virgin male, females contained significantly more apyrene sperm than when mated to a nonvirgin male, but less than when mated to two virgin males (Fig. 4). In contrast, the number of eupyrene sperm stored in the spermatheca did not vary significantly with mating history. Females in all categories averaged approximately 15 000 eupyrene sperm. Because the eupyrene sperm were sometimes broken, only the nuclei were counted. However, the broken eupyrene tails were not mistaken for apyrene sperm because the eupyrene sperm appear to be of much greater diameter than do the apyrene sperm. Thus, even a piece of eupyrene sperm tail of comparable length to an apyrene sperm was easily distinguished.

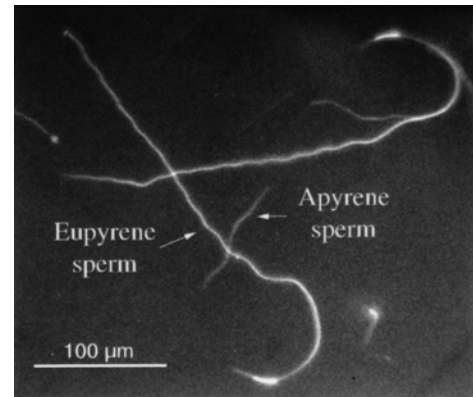


Figure 3. Epifluorescent images of eupyrene and apyrene sperm stained with the DNA label DAPI.

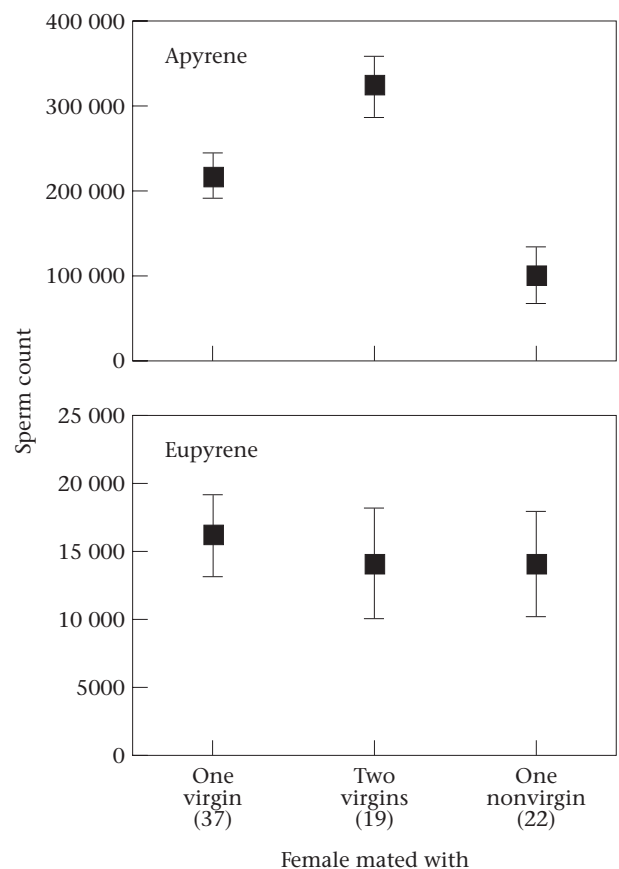


Figure 4. Mean \pm SE numbers of eupyrene and apyrene sperm in the spermatheca as a function of mating history. Eupyrene sperm number did not vary with mating history ($F_{2,59}=0.12$, $P=0.89$), while apyrene sperm number varied significantly across all three categories ($F_{2,59}=10.22$, $P=0.002$). Sample sizes listed parenthetically.

While the number of eupyrene sperm stored did not vary with mating history, it did vary with other factors. The factors analysed included female pupal mass, male and female age, the interval between mating and sampling (henceforth, sampling lag), male ejaculate number (first or second), spermatophore body diameter, spermatophore collum diameter, male size score, and fluctuating asymmetry in clasper length. Table 1 shows

Table 1. Results of stepwise multiple regression analysis on spermathecal sperm counts in once-mated females

Sperm type	Dependent variable	Coefficient	<i>T</i>	<i>P</i>
Eupyrene	Female pupal mass	175 (51)	3.41	0.001
	Male age	1274 (597)	2.13	0.039
	Collum diameter	4397 (1805)	2.44	0.019
Apyrene	Male ejaculate number	-101 746 (37 179)	-2.74	0.009
	Male age	10 722 (5873)	1.82	0.074

The entire list of independent variables included female pupal mass, male and female age, the interval between mating and sampling, male ejaculate number (first or second ejaculate), spermatophore body diameter, spermatophore collum diameter, male composite size score, and fluctuating asymmetry in clasper length. Standard errors of the coefficients are given in parentheses.

the subset of independent factors that gave a significant multiple regression model in stepwise analyses of sperm numbers for once-mated females (Wilkinson 1989). Eupyrene sperm number increased significantly with the age of the male and the diameter of the collum. Eupyrene sperm count also correlated positively with female pupal mass. In contrast, apyrene sperm number varied significantly only with ejaculate number, decreasing with a male's second ejaculate as shown in Fig. 4. Apyrene sperm number also increased with male age, but the significance of this effect was marginal (Table 1).

In addition to sperm stored in the main chamber of the spermatheca, I also found sperm stored in unexpected locations within the female reproductive tract. One such location was the small chamber of the spermatheca, the lagena (Fig. 1), which was empty in a majority of females. However, it did bear sperm in 8% of females. In these cases, the lagena held on average 2200 eupyrene sperm and 1000 apyrene sperm. The presence of sperm in the lagena might simply be due to the fact that they were in transit to the main chamber, or utriculus, and were not allowed sufficient time for transfer. However, sperm remained in the lagena of one female dissected 3 days after mating and a second female 2 days after mating. Thus, sperm may persist in the lagena for at least 3 days.

Sperm were also found lodged within the seminal duct (Fig. 1). Duct sperm were found in 44% of once-mated females and in 42% of twice-mated females. Generally, when sperm were present in the seminal duct, it was greatly swollen and contained 2300 eupyrene sperm and 6000 apyrene sperm in once-mated females, and 2200 eupyrene sperm and 3400 apyrene sperm in twice-mated females. In stepwise multiple regression analyses, none of the male and female traits measured was a significant predictor of the number of either type of sperm stored in the seminal duct. As with sperm in the lagena, sperm in the seminal duct might have been present because they were caught in transit to the main chamber. Again, because the interval between mating and dissection, or sampling lag, was not a significant predictor of the presence of sperm in the seminal duct, sperm persist in the seminal duct.

The spermatophore itself varied significantly with mating history (Fig. 5). In analyses of covariance with ejaculate number as the treatment and male and female traits as covariates, both the diameters of the body of the

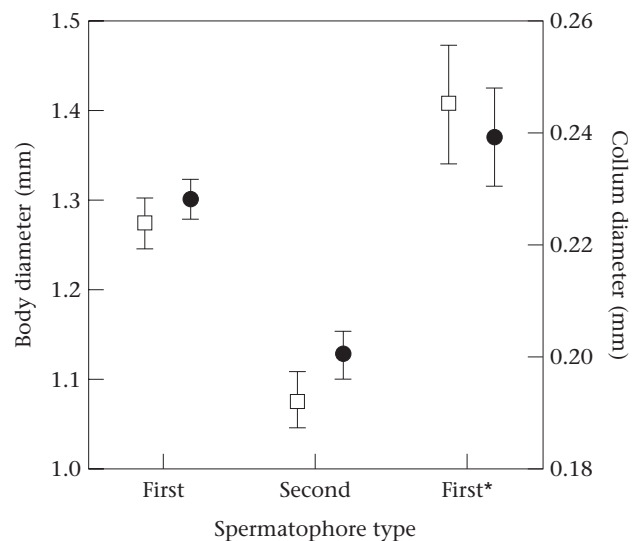


Figure 5. Spermatophore size as a function of mating history. Data represent mean \pm SE diameters of the spermatophore body (□) and of the exit tube, or collum (●). *Passed to a nonvirgin female.

spermatophore, and of the collum, or exit tube, decreased with a male's second mating (body: $F_{2,44}=29.28$, $P<0.001$; collum: $F_{2,43}=30.38$, $P<0.001$). However, spermatophores produced by virgin males mating with once-mated females were slightly larger than those passed to virgin females, although this tendency was not significant for either diameter measured (Fig. 5). The only male trait that had a significant relationship with spermatophore size was aedeagal diameter, which was a significant covariate for both spermatophore measures (body: $F_{1,44}=5.90$, $P=0.019$; collum: $F_{1,43}=10.06$, $P=0.003$).

DISCUSSION

Sperm storage in *H. virescens* is complex. Females that have mated twice store approximately the same number of fertilizing eupyrene sperm as do females that have mated only once. While an earlier study found increases in the number of eupyrene sperm stored with each mating (Raulston et al. 1975), another reported data similar to mine, namely that eupyrene sperm number does not necessarily increase with multiple mating, and may even decrease (Pair et al. 1977). These observations prompt the question: whose eupyrene sperm are stored in

the spermatheca? Are they from only one of the female's two mates, or are they a mixture? Answers to these questions require selective sperm labelling to distinguish between sperm from different ejaculates. It is possible that the eupyrene sperm results were affected by failed matings. In my experiments, nearly 20% of the matings between virgins failed to supply sperm to the spermatheca. While the twice-mated females all had a successful first mating (they laid fertile eggs between matings), their second matings may have had a similar 20% failure rate. However, this level of failure cannot explain the fact that the vast majority of twice-mated females stored only one ejaculate's worth of eupyrene sperm. Also, the oviposition that occurred between matings probably had little effect on either the likelihood that females remated or the total sperm count because there was no noticeable depletion of sperm in once-mated females even 3 days after mating. If and when females do store sperm from their second mates, which occurs in those instances where the second male gains sperm precedence (Flint & Kressin 1968; Pair et al. 1977), then an entire ejaculate's worth of sperm are lost from storage, and it is not clear how they might be eliminated.

In contrast to eupyrene sperm, the number of non-nucleated, or apyrene, sperm stored increases with a female's second mating. The function of apyrene sperm is not clear. It has been suggested that they serve a purpose in sperm competition, perhaps in a 'kamikaze' role, sacrificing themselves in a 'war' with rival sperm for the good of their own ejaculate (Silberglied et al. 1984). Indeed, in the moths *Plodia interpunctella* (Gage 1995) and *Pseudalea separata* (He & Miyata 1997), sperm counts rise when males are reared in crowded conditions with increased risk of sperm competition, and in *P. interpunctella*, the number of apyrene sperm in a male's second ejaculate increase when, due to population phenology, the risk of sperm competition is greatest (Cook & Wedell 1996). Moreover, in the butterfly *Pieris napi*, increased numbers of apyrene sperm induce longer delays before the female resumes mating activity (Cook & Wedell 1999). Here, I show that in *H. virescens*, the number of apyrene sperm stored from a male's second ejaculate decrease, a trend whose significance is not clear. In any case, it is not obvious why the eupyrene sperm number should stay the same with the female's second mating while the number of apyrene sperm increases, unless the second ejaculate provides a much larger portion of apyrene sperm.

In addition to the main chamber of the spermatheca, sperm were also found in the smaller chamber, the lagena and in the seminal duct. An earlier description of sperm use in the closely related *H. zea* (Callahan & Cascio 1963) concluded that sperm remain only transiently in the lagena, and there was no mention of sperm storage in the seminal duct. Because both types of sperm persisted in both organs for at least 3 days, they were not just transient inhabitants of either accessory location but remained there indefinitely. While sperm were only rarely found in the lagena, they were lodged in the seminal duct in nearly half of all females dissected. It remains unclear whether this sperm can be utilized in fertilizations.

Considering male effects on sperm storage, more sperm are stored from certain males than from others. Sperm storage varied principally with two male traits: male age and spermatophore size. The older the male, the more eupyrene and apyrene sperm that were stored, although the increase in apyrene sperm was only marginally significant. Also, the larger the diameter of the spermatophore exit tube, or collum, the greater the storage of eupyrene sperm but not apyrene sperm. It is not clear whether these two male traits somehow induce the female to store a greater portion of the sperm that males pass, or whether they correlate with increased numbers of sperm in the ejaculate, because the number of sperm passed in the ejaculate has not been studied in this species. However, it is not unreasonable that older males simply have amassed a greater supply of sperm than have younger males, and that larger spermatophores deliver more sperm. The effect of sperm count on paternity is also unclear in *H. virescens*, but in the butterfly *Pieris rapae*, mated males, which provide more eupyrene sperm than do virgin males, are more apt to gain sperm precedence (Wedell & Cook 1998). If sperm count is important in *H. virescens*, males that are older and those that make larger spermatophores should be more apt to gain sperm precedence. These results could be important in the design of control efforts for this species. Allowing males to age before release in a sterile insect control programme might provide increased ejaculate competitiveness for the sterilized males, which have been plagued by low ejaculate competitiveness in early pilot studies (Flint & Kressin 1967, 1969).

Several other male traits did not affect sperm storage. These included male size, sampling lag, and FA. The lack of an effect of male size is consistent with the absence of a relationship between male body size and the number of sperm males deliver in the moth *P. interpunctella* (Gage 1998). Although the numbers of sperm delivered by male *H. virescens* are not known, my data suggest either that ejaculate sperm numbers do not correlate with male size, or if they do, that the numbers of sperm stored do not reflect the numbers received from a single mating. Because sampling lag did not affect sperm count, the number of sperm remained relatively constant in the spermatheca, even though some females oviposited for 3 days before being sampled. Therefore, sperm use must be relatively conservative, so that very little sperm is lost due to fertilization and oviposition. Male FA also did not affect sperm storage. FA is thought to reflect male quality, where small departures from perfect bilateral symmetry are believed to result from developmental stress (Møller & Swaddle 1997). Here, FA was measured in the claspers, appendages used by the male to grasp the tip of the female abdomen. Thus, asymmetry in the claspers may be perceived by the female. The absence of an effect of FA is not unexpected, because in other studies it has influenced neither paternity (LaMunyon 1994) nor sperm delivery (Gage 1998).

Along with sperm numbers, spermatophore size fluctuated with mating history. A male's second spermatophore was significantly smaller than one he produced the day

before. In the Lepidoptera, it is typical for spermatophore size to decrease with the second ejaculate (Rutowski & Gilchrist 1986; Svård & Wiklund 1986; Oberhauser 1988; Marshall & McNeil 1989; LaMunyon & Eisner 1994; Delisle & Bouchard 1995). However, there was a tendency in the data presented here: a spermatophore passed to a previously mated female was slightly larger than that passed to a virgin female. While the tendency was not statistically significant, it is intriguing and suggests that males might detect the presence of a rival ejaculate and adjust their output to compete better, which is known to occur in a *P. interpunctella* (Cook & Gage 1995).

The paternity of offspring from multiply mated *H. virescens* females is highly variable. For twice-mated females, the offspring may be sired entirely by the first, or the last male to mate (Flint & Kressin 1968; Pair et al. 1977). The present results provide some clue to what the mechanism of sperm precedence might be. Only one ejaculate's worth of eupyrene sperm are normally found in the spermatheca of a twice-mated female, suggesting that an entire ejaculate's worth of sperm are somehow lost. If the spermatheca has a limited capacity, the explanation may be simple: if the first male's ejaculate fills the spermatheca, no more sperm will enter, and first-male sperm precedence will occur. Second-male sperm precedence would occur if the first male's sperm do not completely fill the spermatheca, and it is 'topped off' by the second male's sperm, which, if they do not mix, are then the first to be used in fertilization (Simmons et al. 1996). Alternatively, the second male's sperm might somehow displace the first male's sperm from storage. If more than one ejaculate's worth of sperm can fit in the spermatheca, something more complex is occurring. In this case, sperm storage may be biased, perhaps under control of the female (Eberhard 1991, 1996), with some sperm expelled, as it is in at least one other lepidopteran (Etman & Hooper 1979). There is evidence that the spermatheca can hold more than one ejaculate's worth of sperm. An earlier study found two ejaculate's worth of sperm occasionally stored in multiply mated females (Raulston et al. 1975), and my results indicate that the spermatheca can accommodate nearly two ejaculate's worth of eupyrene sperm, which are passed in much greater number than are the eupyrene sperm. While the present results cannot distinguish between these possibilities, sperm use in *H. virescens* is not a simple lottery where all sperm enter the storage organ and then are used at random. Only further study will elucidate the exact mechanism of sperm use in this species.

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