

Determinants of Sperm Transfer by Males of the Noctuid Moth *Heliothis virescens*

Craig W. LaMunyon¹⁻³ and Tammy S. Huffman¹

Accepted July 18, 2000, Revised October 10, 2000

*Ejaculate composition can be an important determinant of male reproductive success in the face of sperm competition, which varies with the mating history of the female. Here we examine the effect of various male and female mating histories and morphological traits on ejaculate sperm numbers in the polyandrous moth *Heliothis virescens*. We show that when mating with nonvirgin females, males passed larger sperm packages (spermatophores) but did not alter either the sperm count or the ratio of nucleated-to-nonnucleated sperm. Males also passed fewer sperm in their second ejaculates. Finally, older males passed more sperm than did younger males. Earlier research found that females store more sperm from older males and that older males are more likely to gain sperm precedence over younger rivals. These earlier results, taken together with the present results, indicate that the advantage enjoyed by older males is due to an increased sperm count.*

KEY WORDS: *Heliothis virescens*; sperm precedence; apyrene; eupyrene; sperm count.

INTRODUCTION

Females of most species will mate with more than one male before their eggs are fertilized (Birkhead and Møller, 1998). This polyandrous behavior has profound implications for mating system evolution because it places ejaculates in competition for fertilizations (Parker, 1970). Males subject to such

¹Department of Molecular and Cellular Biology, University of Arizona, Tucson, Arizona.

²Department of Entomology, University of Arizona, Tucson, Arizona.

³To whom correspondence should be addressed at Division of Biological Science, Florida Atlantic University, Davie, Florida 33314. e-mail: clamunyo@fau.edu. Fax: (954) 236-1099.

sperm competition are expected to evolve adaptations that improve their chances at fertilization. Such adaptations include mate guarding (Frankino and Sakaluk, 1994), removal of rival sperm from the female reproductive tract (Waage, 1979), the production of large sperm (Gage, 1994; LaMunyon and Ward, 1998, 1999), and the transfer of larger numbers of sperm in ejaculates (Parker, 1982, 1984). In butterfly species where females are highly polyandrous and the risk of sperm competition is great, males produce larger ejaculates and ejaculate recovery is faster after mating than in less polyandrous species (Svård and Wiklund, 1989). In fact, males of some species, including humans, even adjust ejaculate sperm counts depending upon the risk of sperm competition at a given mating (Gage, 1991; Baker and Bellis, 1993; Cook and Gage, 1995).

Here we investigate the numbers of sperm delivered by males of the tobacco budworm moth *Heliothis virescens* F. under different mating conditions. Females of this economically important species have a life span of 2 to 3 weeks in the laboratory and, in nature, 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 and Kressin, 1968; Pair *et al.*, 1977; LaMunyon, 2000b). This pattern of sperm precedence is a consequence of female sperm storage: twice-mated females store only one ejaculate's worth of fertilizing, or eupyrene, sperm but nearly two ejaculates worth of apyrene sperm (LaMunyon, 2000a). Apyrene sperm are nonnucleated and thought to function in sperm competition (Silberglied *et al.*, 1984). The numbers of sperm stored by the female are predicted by both her mass and the relative ages of her mates (LaMunyon, 2000). Furthermore, female mass and male age have been identified as independent determinants of paternity: a female's second mate is more likely to gain sperm precedence over the first mate if the female is larger and if he is older than his rival at the time of mating (LaMunyon, 2000b). What is the effect of female mass and male age? Do they simply correlate with the amount of sperm transferred by the male, or is their effect independent of sperm number? Here we test the hypotheses that males transfer more sperm if they are older and if they mate with larger females.

METHODS

Moths used in this study were kindly provided by the Western Cotton Research Laboratory (USDA), Phoenix, 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 light phase of 14 h and a dark phase of 10 h. Male pupae averaged 209 mg (range, 140–296 mg), and female pupae averaged 193 mg (range, 137–291 mg). Upon eclosion, the moths were given cotton wicks moistened with sugar water (10% sucrose).

Moths were paired for matings in 0.25-liter cylindrical containers to obtain ejaculates for sperm counts. At their first matings, females were 1 to 9 days posteclosion (mean, 3.6 days). Thirty-two females mated once to virgin males (5 males were 1 day old, 17 were 2 days old, and 8 were 3 days old). Twenty-eight of these males copulated with a second virgin female 1 day following their first mating. Therefore, we examined the first and second ejaculates from 28 males. Thirty-two other females were mated to two virgin males, with an interval between matings ranging from 1 to 5 days (the males ranged from 1 to 5 days old). To determine whether paired moths actually mated, they were observed under red light at 15-min intervals during the final 4 h of their dark phase, a time when all mating pairs are still *in copula* (personal observation, C.W.L.). Mating pairs were observed, and when they finished copulating, the females were frozen (unless they were to copulate with a second male). Thus, all females were frozen within 15 min of the end of copula, before the sperm had a chance to transfer out of the spermatophore into the female's reproductive tract (see next paragraph).

Females were thawed and dissected and their reproductive organs removed for sperm counts. Initially, the main sperm storage organ, or spermatheca, from every female was observed for the presence of sperm. This step was omitted after the first 20 spermathecae were found to be empty, indicating that no sperm had transferred out of the spermatophores. We opened the spermatophore receptacle, or bursa copulatrix, and removed the spermatophore, which consists of a tube attached to a spherical body that contains the sperm and seminal fluid. The size of each spermatophore was estimated by taking the average of two diameters measured in perpendicular directions across the spermatophore body (mean, 1.11 mm; range, 0.60–1.48 mm). Each spermatophore was then placed in a small volume of phosphate-buffered saline (PBS) and snipped open with fine scissors. We then transferred each spermatophore and its contents to a microfuge tube and added PBS to give a final volume of 200 μ l. The eupyrene sperm released from the spermatophores remained packaged in bundles (Katsuno, 1978; Osanai *et al.*, 1989). We vortexed the spermatophore samples for 4 min each, a procedure that freed the sperm from the spermatophore remains and unbundled the sperm. The sperm were then counted in a hemacytometer after nuclear labeling with DAPI (4,6-diamidino-2-phenylindole; 10 μ g/ml in PBS) as described elsewhere (LaMunyon, 2000a). Briefly, 9- μ l subsamples were loaded into a hemacytometer, and the eupyrene and apyrene sperm counted under epifluorescence. Each original sample was subsampled eight

Table I. Results of Stepwise Multiple Regression Analysis on Ejaculate Sperm Counts in *H. virescens*^a

Sperm type	Dependent variable	Mating participants		
		Virgin male, virgin female	Nonvirgin male, virgin female	Virgin male, nonvirgin female
Eupyrene	Male age	11,560 (3,642)**	ns	ns
	Male mass	ns	ns	154 (64)*
	Female age	ns	ns	ns
	Female mass	ns	ns	ns
	Spermatophore diameter	ns	ns	ns
	Number of eupyrene in first ejaculate	—	0.25 (0.09)**	—
Apyrene	Male age	155,742 (37,928)***	33,146 (13,617)*	ns
	Male mass	1,940 (669)**	ns	2,068 (636)**
	Female age	ns	ns	ns
	Female mass	ns	ns	ns
	Spermatophore diameter	ns	ns	ns
	Number of apyrene in first ejaculate	—	ns	—

^aFor cases where the female was nonvirgin, the mass and age of the females' first mates were also included as independent variables. Finally, when the male was the nonvirgin, the size of the spermatophore and number of sperm from his first previous mating were included as variables. Standard errors of the coefficients are listed parenthetically.

* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

times and the counts were averaged. Due to the high concentration of DAPI in this protocol, even the nonnucleated apyrene sperm fluoresce, enabling unambiguous counting of both eupyrene and apyrene sperm (LaMunyon, 2000a). Total sperm in the ejaculates were calculated and analyzed for differences among mating regimes and for relationships with spermatophore size and male and female age and mass by multiple regression analysis (see Table I for the traits examined).

RESULTS

Nearly every spermatophore we examined contained sperm. Only two males passed no eupyrene sperm and two others passed <1000 eupyrene sperm. The remaining 88 spermatophores contained thousands of eupyrene sperm and 10-fold more apyrene sperm. Virgin males passed nearly 25,000 eupyrene and 300,000 apyrene sperm, whether they mated with a virgin female or a nonvirgin female (Fig. 1). There were significant differences in the numbers of sperm transferred among mating histories for both the eupyrene sperm ($F_{2,89} = 6.08$, $P = 0.003$) and apyrene sperm ($F_{2,89} = 10.22$, $P =$

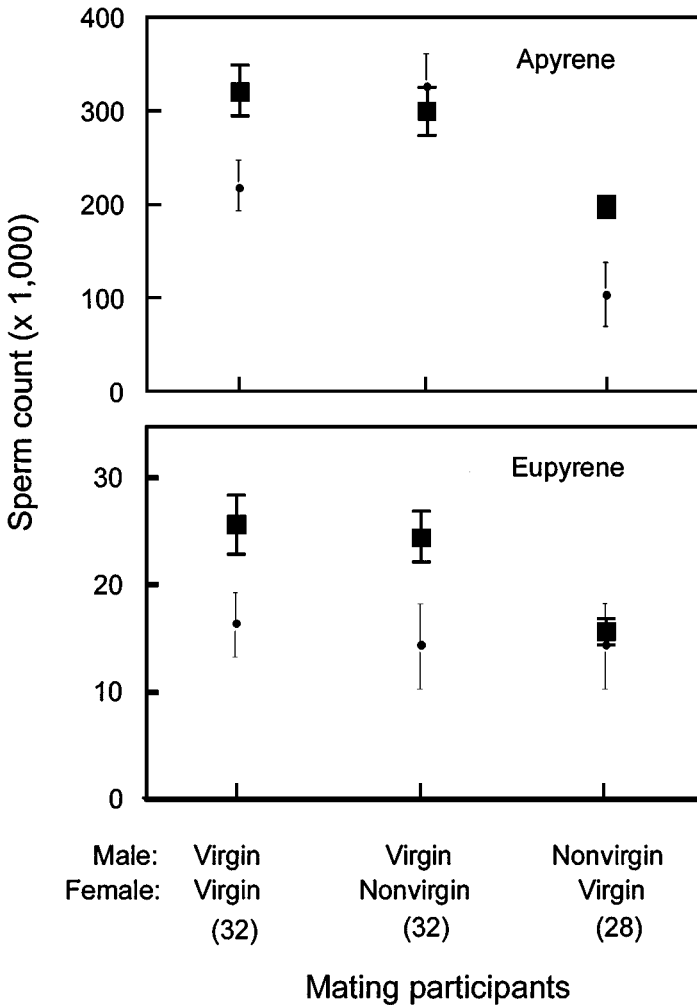


Fig. 1. Mean numbers of eupyrene and apyrene sperm transferred in the spermatophore (boxes; data collected in this study) and stored in the female spermatheca [circles; data included from LaMunyon (2000a)] as a function of mating history. Error bars represent 1 SE. Sample sizes listed parenthetically.

0.0001) (Fig. 1). For both sperm types, the only significant differences were between virgin and nonvirgin males (Fisher's LSD, $P < 0.01$), where nonvirgin males passed significantly fewer sperm than did virgins (Fig. 1). The numbers passed by virgins did not differ with the mating history of their mates (Fig. 1). The numbers of sperm passed by a male were generally more

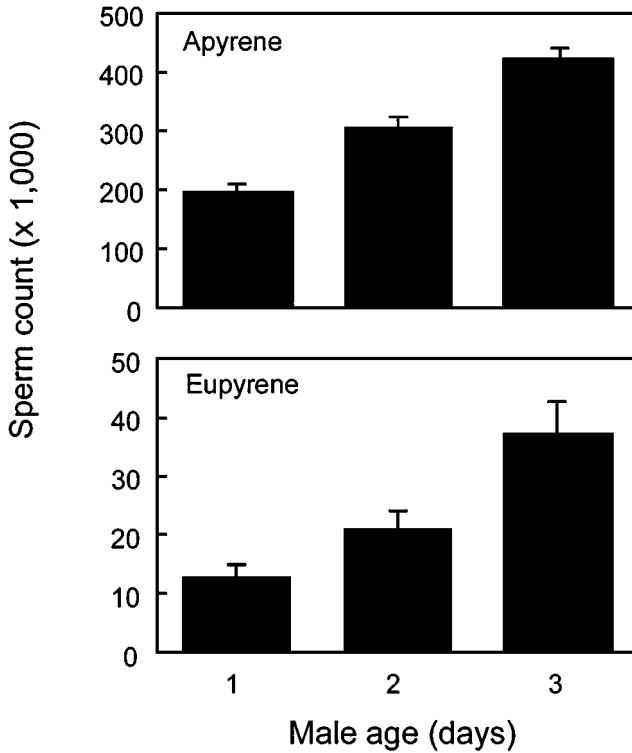


Fig. 2. The numbers of sperm passed by virgin males to virgin females as a function of male age. Sperm counts varied significantly with male age for both eupyrene sperm ($F_{2,29} = 7.55$, $P = 0.002$) and apyrene sperm ($F_{2,29} = 4.38$, $p = 0.022$). Error bars represent 1 SE.

than the numbers stored in the female's spermatheca [Fig. 1; sperm storage data included from LaMunyon (2000a)]. However, this was not the case for nonvirgin males. Nearly all the eupyrene sperm passed by nonvirgin males became stored in the spermatheca, indicating that the process of eupyrene sperm storage itself is not a wasteful process.

In this study, we set out to determine whether sperm counts increased with female mass and male age. While there was no effect of female mass on the number of sperm transferred, male age was an important predictor of sperm count in the 32 cases where virgin males mated with virgin females (Table I). The older virgin males passed significantly more sperm of both types to virgin females than did the younger virgin males (Fig. 2). Of these 32 males, 28 mated with a second virgin female 1 day later. Thus, we analyzed both the first and the second ejaculates from 28 males. For the second

ejaculate, the number of apyrene, but not eupyrene, sperm was predicted by male age (Table I). It must be noted that the males were 1 day older at their second mating than at their first. Interestingly, when virgin males mated with nonvirgin females, there was no effect of male age on the numbers of either apyrene or eupyrene sperm ejaculated (Table I).

Several other traits were predictors of the numbers of sperm passed. Larger males (measured as pupal mass) passed significantly more apyrene sperm in virgin–virgin matings (Table I). The numbers of both sperm types also increased significantly with male mass when virgin males mated with nonvirgin females (Table I). The number of eupyrene sperm in a male's second ejaculates was predicted by the number of eupyrene sperm in his first ejaculate (Table I). Further investigation of the correlation between sperm numbers revealed that eupyrene number, but not apyrene number, was significantly correlated between a male's ejaculates (Fig. 3). Also, apyrene number correlated positively with eupyrene number at an approximate 10:1 ratio in all ejaculates (Fig. 4). This ratio did not differ among the three mating regimes (virgin–virgin, virgin–mated, mated–virgin; $F_{2,86} = 0.38$, $P = 0.68$). Thus, males do not adjust the ratio of sperm types depending upon their mating history or that of their mate.

In addition to sperm counts, spermatophore size was also variable. Analysis of covariance with mating regime as treatment and the masses and ages of the male and female as covariates showed that spermatophore size varied significantly among the three mating regimes ($F_{2,79} = 60.08$, $P < 0.0001$)

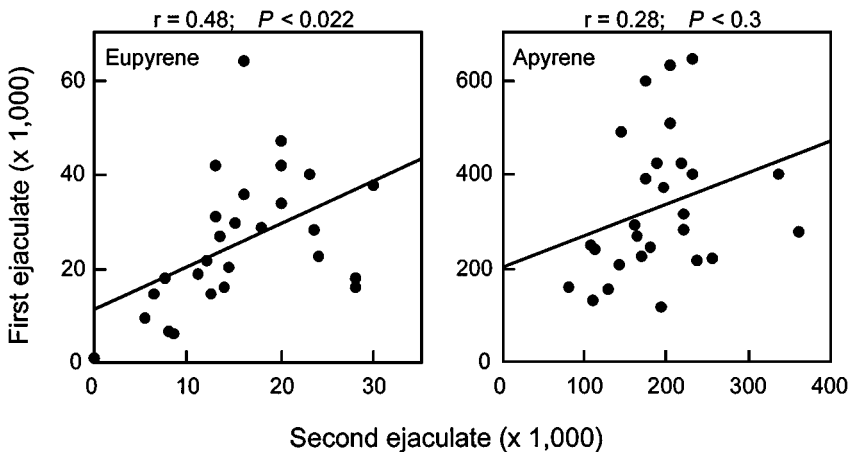


Fig. 3. The relationship of the numbers of sperm in the first versus the second ejaculate transferred by a male to virgin females.

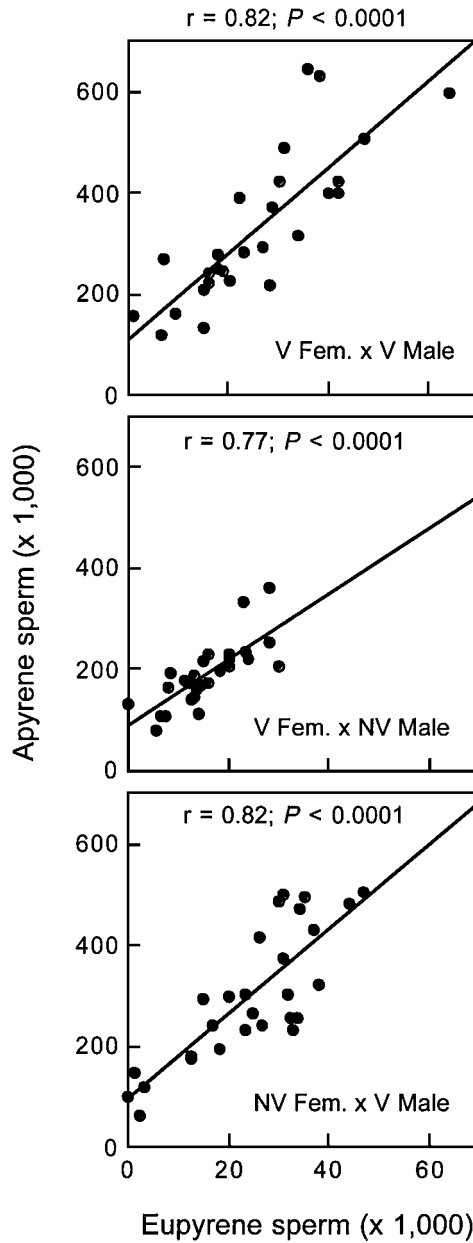


Fig. 4. The relationship between apyrene sperm and eupyrene sperm across the three mating regimes.

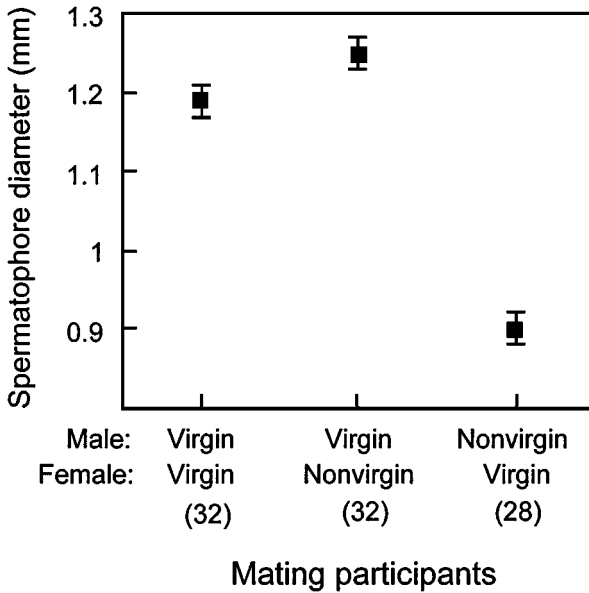


Fig. 5. Mean spermatophore body diameter across the three mating regimes. Error bars represent 1 SE.

and with male mass ($F_{1,79} = 10.18$, $P = 0.002$). Post hoc analysis revealed that the diameter of the body of the spermatophore decreased significantly with a male's second mating (Fisher's LSD, $P < 0.001$; Fig. 5). In addition, when mating with a nonvirgin female, males transferred a slightly but significantly larger spermatophore than when they mated with a virgin female ($P < 0.05$; Fig. 5).

DISCUSSION

Mating in *H. virescens* does not guarantee reproductive success for males. Mating males sometimes sire no offspring (LaMunyon, 2000a). This inability to sire progeny does not stem from a lack of sperm. Nearly every ejaculate we examined here contained substantial amounts of sperm. However, twice-mated female *H. virescens* store only one ejaculate's worth of sperm (LaMunyon, 2000a), and it can be from the first, the second, or both of her mates (LaMunyon, 2000b). The problem for mating males, then, is the transfer of their sperm to the spermatheca and the sperms' tenure in this storage organ, processes that are governed by the female musculature in

the Lepidoptera (Etman and Hooper, 1979; Tschudi Rein and Benz, 1990; LaMunyon and Eisner, 1993).

In earlier studies, sperm storage and paternity in *H. virescens* varied with male age (LaMunyon, 2000a, b). Sperm from older males were stored by the female in greater numbers and were more likely to take precedence. Data collected here show that the advantage enjoyed by older males is one of sperm number. Each day a male ages before mating significantly increases his sperm count. Thus, performance in sperm competition is based, in part, on the number of sperm transferred. However, sperm competition is not a lottery where every sperm received by the female enters storage and competes for fertilizations, because twice-mated females store only one ejaculate's worth of sperm (LaMunyon, 2000a). When a male ejaculates more sperm, perhaps they are more likely to evict preexisting sperm from the spermatheca and/or to resist eviction by sperm from subsequent mates. Males obviously ejaculate more sperm than are required simply to fill the spermatheca. This extra is likely due to the demands of sperm competition (He and Miyata, 1997).

While the numbers of sperm increased with male age, the size of the spermatophores that bore these sperm did not increase similarly. Thus, spermatophores of similar size may contain vastly different numbers of sperm. While spermatophore size has not been found to affect paternity in *H. virescens* (LaMunyon, 2000b), it is an important determinant of paternity in other lepidopterans (LaMunyon and Eisner, 1994; Wedell and Cook, 1998). In these other species, it is unclear whether larger spermatophores simply deliver greater quantities of sperm [as they do in some species (Svård and Wiklund, 1986; He and Miyata, 1997)] or whether they have some effect independent of sperm. Data presented here for *H. virescens* and by Cook and Wedell (1996) for *Pieris rapae* show that, for at least these two lepidopterans, spermatophore size and sperm count are unrelated. Indeed, in *P. rapae* there is evidence that both sperm count and spermatophore size are independent determinants of paternity (Wedell and Cook, 1998).

Here we found that spermatophore size is adjusted depending upon the mating status of the female. Males transfer larger spermatophores to previously mated females, a trend found in an earlier study (LaMunyon, 2000a). This suggests that males detect the presence of a rival ejaculate and adjust their own output in response [a process known to occur in another moth (Cook and Gage, 1995)]. These spermatophores contained no more sperm than those passed to virgin females, and a study of paternity found no relationship between spermatophore size and paternity (LaMunyon, 2000b). However, paternity could be affected by spermatophore size in situations that were not examined in that study (e.g., instances where females mate with nonvirgins, etc.).

Neither the total number of sperm transferred nor the ratio of eupyrene to apyrene sperm changed with the mating history or the mass of the female. Thus, males do not adjust sperm output depending upon the condition of their mates. This finding makes results from an earlier paternity study difficult to interpret. In that study, the second of a female's mates was more likely to gain sperm precedence when she was larger (LaMunyon, 2000b). Thus, the effect of female size on paternity must be explained by some aspect other than sperm count or spermatophore diameter. Male *H. virescens* may simply lack the ability to adjust sperm numbers. They may transfer the most competitive ejaculate possible and do not "hold back" in matings with lower risk of sperm competition, even though males of other Lepidoptera do tailor sperm output to mating conditions. For example, in the pyralid moth *Plodia interpunctella*, males pass more sperm to females that are larger or have a greater store of preexisting sperm (Cook and Gage, 1995; Gage, 1998). Male *H. virescens* might alter some other component of their ejaculate (i.e., nutrient load) depending on female size or mating history, providing an advantage in sperm competition.

Finally, it is still unclear what function, if any, the apyrene sperm play in sperm competition. Their presence in the female's spermatheca does not inhibit female remating as it does in *P. rapae* (Cook and Wedell, 1999), because *H. virescens* will resume mating activity the day after mating (Raina and Stadelbacher, 1990), regardless of apyrene storage. Thus, apyrene sperm likely have different functions depending upon the species. In *H. virescens*, the apyrene sperm may interact directly with rival sperm in a "kamikaze" fashion (Silberglied *et al.*, 1984). The sheer numbers of apyrene sperm suggest that they are important: they compose 90% of the sperm in the ejaculates of *H. virescens* (data reported here) and other Lepidoptera (Cook and Gage, 1995; Cook and Wedell, 1996; He and Miyata, 1997). The function of apyrene sperm in *H. virescens* and in other Lepidoptera will be discerned only when their numbers can be altered and the effect on sperm competition determined.

ACKNOWLEDGMENTS

We thank S. Ward for his continued and generous support, which included microscopy supplies, M. Ramaswami for the use of a microscope, and C. Boswell for reviewing an early version of the manuscript. The Western Cotton Research Laboratory supplied the *Heliothis* through the Department of Entomology at the University of Arizona. This research was supported by USDA NRI Grant 97-35302-4869.

REFERENCES

- Baker, R. R., and Bellis, M. A. (1993). Human sperm competition: Ejaculate adjustment by males and the function of masturbation. *Anim. Behav.* **46**: 861–885.
- Birkhead, T. R., and Møller, A. P. (1998). *Sperm Competition and Sexual Selection*, Academic Press, San Diego.
- Cook, P. A., and Gage, M. J. G. (1995). Effects of risks of sperm competition on the numbers of eupyrene and apyrene sperm ejaculated by the moth *Plodia interpunctella* (Lepidoptera: Pyralidae). *Behav. Ecol. Sociobiol.* **36**: 261–268.
- Cook, P. A., and Wedell, N. (1996). Ejaculate dynamics in butterflies: A strategy for maximizing fertilization success. *Proc. R. Soc. Lond. B* **263**: 1047–1051.
- Cook, P. A., and Wedell, N. (1999). Non-fertile sperm delay female remating. *Nature* **397**: 486–486.
- Etman, A. A. M., and Hooper, G. H. S. (1979). Sperm precedence of the last mating in *Spodoptera litura*. *Ann. Entomol. Soc. Am.* **72**: 119–120.
- Flint, H. M., and Kressin, E. L. (1968). Gamma irradiation of the tobacco budworm: sterilization, competitiveness and observations on reproductive biology. *J. Econ. Entomol.* **61**: 477–483.
- Frankino, W. A., and Sakaluk, S. K. (1994). Post-copulatory mate guarding delays promiscuous mating by female decorated crickets. *Anim. Behav.* **48**: 1479–1481.
- Gage, M. J. G. (1991). Risk of sperm competition directly affects ejaculate size in the mediterranean fruit fly. *Anim. Behav.* **42**: 1036–1037.
- Gage, M. J. G. (1994). Associations between body size, mating pattern, testis size and sperm lengths across butterflies. *Proc. R. Soc. Lond. B* **258**: 247–254.
- Gage, M. J. G. (1998). Influences of sex, size, and symmetry on ejaculate expenditure in a moth. *Behav. Ecol.* **9**: 592–597.
- He, Y., and Miyata, T. (1997). Variations in sperm number in relation to larval crowding and spermatophore size in the armyworm, *Pseudaletia separata*. *Ecol. Entomol.* **22**: 41–46.
- Katsuno, S. (1978). Studies on eupyrene and apyrene spermatozoa in the silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae). VII. The motility of sperm bundles and spermatozoa in the reproductive organs of males and females. *Appl. Entomol. Zool.* **13**: 91–96.
- LaMunyon, C. W. (2000a). Sperm storage by females of the polyandrous noctuid moth *Heliothis virescens*. *Anim. Behav.* **59**: 395–402.
- LaMunyon, C. W. (2000b). Determinants of sperm precedence in a noctuid moth (*Heliothis virescens*): A role for sperm count. *Ecol. Entomol.* (in press).
- LaMunyon, C. W., and Eisner, T. (1993). Post-copulatory sexual selection in an arctiid moth (*Utetheisa ornatrix*). *Proc. Nat. Acad. Sci. USA* **90**: 4689–4692.
- LaMunyon, C. W., and Eisner, T. (1994). Spermatophore size as a determinant of paternity in an arctiid moth (*Utetheisa ornatrix*). *Proc. Natl. Acad. Sci. USA* **91**: 7081–7084.
- LaMunyon, C. W., and Ward, S. (1998). Larger sperm outcompete smaller sperm in the nematode *Caenorhabditis elegans*. *Proc. R. Soc. Lond. B* **265**: 1997–2002.
- LaMunyon, C. W., and Ward, S. (1999). Evolution of sperm size in nematodes: Sperm competition favours larger sperm. *Proc. R. Soc. Lond. B* **266**: 263–267.
- Osanai, M., Kasuga, H., and Aigaki, T. (1989). Induction of motility of apyrene spermatozoa and dissociation of eupyrene sperm bundles of the silkworm, *Bombyx mori*, by initiatorin and trypsin. *Invert. Reprod. Dev.* **15**: 97–103.
- Pair, S. D., Laster, M. L., and Martin, D. F. (1977). Hybrid sterility of the tobacco budworm: effects of alternate sterile and normal matings on fecundity and fertility. *Ann. Entomol. Soc. Am.* **70**: 952–954.
- Parker, G. A. (1970). Sperm competition and its evolutionary consequences in the insects. *Biol. Rev.* **45**: 525–567.
- Parker, G. A. (1982). Why are there so many tiny sperm? Sperm competition and the maintenance of two sexes. *J. Theor. Biol.* **96**: 281–284.
- Parker, G. A. (1984). Sperm competition and the evolution of animal mating strategies. In Smith, R. L. (ed.), *Sperm Competition and the Evolution of Animal Mating Systems*, Academic Press, San Diego, pp. 1–60.

- Raina, A. K., and Stadelbacher, E. A. (1990). Pheromone titer and calling in *Heliothis virescens* (Lepidoptera: Noctuidae): Effect of mating with normal and sterile backcross males. *Ann. Entomol. Soc. Am.* **83**: 987–990.
- Raulston, J. R., Snow, J. W., Graham, H. M., and Lingren, P. D. (1975). Tobacco budworm: Effect of prior mating and sperm content on the mating behavior of females. *Ann. Entomol. Soc. Am.* **68**: 701–704.
- Silberglied, R. E., Shepherd, J. G., and Dickinson, J. L. (1984). Eunuchs: The role of apyrene sperm in Lepidoptera. *Am. Nat.* **123**: 255–265.
- Svärd, L., and Wiklund, C. (1986). Different ejaculate delivery strategies in first versus subsequent matings in the swallowtail butterfly *Papilio machaon* L. *Behav. Ecol. Sociobiol.* **18**: 325–330.
- Svärd, L., and Wiklund, C. (1989). Mass and production rate of ejaculates in relation to monandry/polandry in butterflies. *Behav. Ecol. Sociobiol.* **24**: 395–402.
- Tschudi Rein, K., and Benz, G. (1990). Mechanisms of sperm transfer in female *Pieris brassicae* (Lepidoptera: Pieridae). *Ann. Entomol. Soc. Am.* **83**: 1158–1164.
- Waage, J. K. (1979). Dual function of the damselfly penis: Sperm removal and transfer. *Science* **203**: 916–918.
- Wedell, N., and Cook, P. A. (1998). Determinants of paternity in a butterfly. *Proc. R. Soc. Lond. B* **265**: 625–630.