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Prolonged mating in the milkweed leaf beetle *Labidomera clivicollis clivicollis* (Coleoptera: Chrysomelidae): a test of the "sperm-loading" hypothesis

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Summary. Female milkweed leaf beetles (*Labidomera clivicollis clivicollis*) frequently mate with more than one male, and pairs form mating associations which last for up to 42 h in the field. I tested the hypothesis that males remaining with females for long periods of time benefit by numerically overwhelming the sperm of their competitors. Male *L.c.clivicollis* copulated intermittently with females throughout an 11 hour period in the laboratory. When virgin females were allowed a single copulation, 94.3% of the sperm they received were located in the spermatheca immediately afterward. Males were not sperm-depleted, for they had large numbers of sperm available after one copulation (mean = 230,000 ± 43,200); the maximal number of sperm a male transferred to a female in 24 h was 30,500. There was a positive linear relationship between the number of sperm transferred and time up to 24 h after mounting ($r^2 = 0.178$, $P < 0.003$). These data suggest that males transfer increasing numbers of sperm throughout a 24-h-period. Mating duration was the most important determinant of paternity when females were placed with one male for 24 h and another male for 6 hours. Females whose first matings were longer showed first male sperm predominance (as determined by starch-gel electrophoresis), while females whose second matings were longer showed last male sperm predominance. In view of these data, it is puzzling that males do not inseminate with large numbers of sperm immediately after mounting the female. It is possible that female refractory behaviors make insemination difficult and favor prolonged mating by male milkweed leaf beetles.

Introduction

Prolonged mating occurs in all of the major orders of insects and, in most cases, evidence suggests that it involves precopulatory (Parker and Smith 1975; Sivinski 1983) or postcopulatory (Waage 1979) mate-guarding by males. However, in some species of insects (Alcock 1976; Johnson 1983; Smith 1979) and laboratory strains of muroid rodents (Lanier et al. 1979; Oglesby et al. 1981), males copulate repeatedly with the same female. For intermittently copulating species and those with highly variable copula durations, it is important to consider an alternative hypothesis that also involves minimizing loss of paternity to rival males: perhaps males that remain with females for long periods of time benefit by numerically overwhelming the sperm of their competitors. I refer to this hypothetical advantage of prolonged mating for males as "sperm-loading".

There is some evidence that multiple ejaculation confers paternity advantages to male Syrian golden hamsters (Oglesby et al. 1981) and laboratory rats (Lanier et al. 1979) in competitive mating situations in the laboratory. However, there has been no such demonstration for any insect species. In some species of insects, sperm transfer rates may be limited by the refractory behaviors of females. These conditions should favor prolonged mating by males if the number of offspring a male fathers depends upon the number of sperm he is able to place in the female's spermatheca (sperm storage organ). Prolonged mating and repeated insemination could increase the proportion of eggs fertilized by either late-mating or early-mating males: (1) if a female has mated previously, the proportion of eggs fertilized by the last male to mate could be greater if he inseminates with greater quantities of sperm (Parker 1970; Walker 1980) and (2) although remating by the female may cause an early male to lose fertilizations, prolonged mating by the earlier male could minimize that loss. The sperm-loading advantage will be most dramatic in species in which there is complete mixing of sperm

within the female. Sperm mixing occurs at least to some degree in several species of insects (Gromko et al. 1984; McCauley and O'Donnell 1984; Page and Metcalf 1982; Woodhead 1985). In such species, it is possible that females recruit sperm based upon their numerical representation in the spermatheca. However, if there is last male sperm predominance, sperm-loading can still be advantageous. For example, in two studies involving the dragonfly *Erythemis simplicicollis* (Odonata: Libellulidae) (McVey and Smittle 1984) and the beetle *Tribolium castaneum* (Coleoptera: Tenebrionidae) (Schlager 1960), researchers determined paternity of offspring from eggs laid at various times after a final mating. They found that "early male" sperm were utilized by females in increasing proportions after an initial period of strong last male sperm predominance. Schlager (1960) suggested for *Tribolium castaneum* that there was an initial position effect (in which the last male's sperm were in the best location to fertilize the eggs), followed by mixing of sperm within the female. After sperm mixing has occurred, the number of offspring an early male sires could depend upon the number of sperm he originally transferred.

Female milkweed leaf beetles (*L.c.clivicollis* Coleoptera: Chrysomelidae) frequently mate with more than one male, and pairs engage in prolonged mating associations which last from 1 to 42 h in the field. Males alternate copulation with bouts of mounted courtship or riding on the females' backs, as occurs in several other beetle species (Alcock 1976; Johnson 1983). In this paper, I present evidence that the number of sperm a male transfers increases with time (up to 24 h) and that mating duration is an important determinant of paternity in *L.c.clivicollis*. These findings suggest that both mating order and mating duration should be considered when determining patterns of sperm utilization for intermittently copulating species of insects.

Methods

Field observations

Mating frequency and mating duration. Because copulation is intermittent, "mating" includes mounting and all of the interactions that occur between a male and female while the male is riding on her back. In order to estimate mating frequencies and mating durations under natural conditions, two assistants and I conducted field observations between June 7 and July 13, 1984. We monitored mating of marked beetles in a patch of 343 *Asclepias incarnata* (swamp milkweed plants) in a roadside ditch on Petrie Road, Bridgeport, New York. Milkweed leaf beetles feed on other plants within the family Asclepiadaceae, but females preferentially oviposit on or near *A. incarnata* in New York state, and population densities tend to be higher on *A. incarnata* than on *A. syriaca*, the common milkweed (Eickwort 1977). Beginning June 7, when *A. incarnata* first sprouted, we numbered all plants and checked them for beetles once per hour 10 times per day. The beetles are active day and night, but when the weather is warm they are generally present in greatest numbers during the evening hours. For this reason, 7 of the 10 censuses were taken during evening hours (18:00–00:00), and the remaining 3 censuses were scattered throughout other portions of the day or night. Beetles were captured, sexed, their elytral lengths were measured, and green or yellow Opalithplättchen ("Von Frisch tags", numbered 1–99) were glued to their pronota with cyanoacrylate glue. Before glueing on a tag, we abraded the pronotum to remove grease or wax. Positions (plant number) and behaviors (mating) of all beetles in the plot were recorded during each scan using instantaneous sampling methods described by Altman (1984). Data were not taken during thunderstorms.

Laboratory experiments

Copulation and sperm transfer. Animals used in these experiments were from the second and third laboratory generations of a culture started in June 1984 with adults collected from *A. incarnata* and *A. syriaca* in Bridgeport, New York. Cultures were kept on long day [ambient conditions (June–August) or 16L:8D (September–December)] at 23–25° C and were reared on *A. syriaca* (June–August) and *A. incarnata* (September–December).

Information on the number of copulations per hour was obtained by watching 11 male-female pairs in separate 9 cm Petri dishes lined with moist Whatman No. 1 filter paper and containing swamp milkweed leaves. Seven of the 11 matings were between virgin males and females. The 11 pairs were sampled for 1.08–11 h (mean = 6.14, SE = 1.15). The time period over which an individual pair was observed varied because males mounted at different times after the 12-h sampling-period had begun and because some males dismounted before the sampling period ended. I scanned each dish every 5 min for 12 h, recording complete intromissions and partial intromissions. Unless specified, the term "copulation" will be used to refer to both partial and complete intromissions. Males were removed and sampling was terminated if they dismounted or if the females succeeded in shaking them off their backs. I divided the observations into 1-h categories by taking the time the male first mounted a female as time zero. Using a Kruskal-Wallis test, I determined whether the mean number of instantaneous samples during which copulation was observed differed among 1-h intervals from time zero to the eleventh hour of mating.

In order to determine the time and mode of insemination, I placed a virgin female with a virgin male and observed the pair ($n = 9$) through the first copulation. Immediately following the first copulation, I dissected the female, and counted the number of spermatozoa in the spermatheca (sperm storage organ) separately from the number in the common oviduct (vagina). I also counted sperms in the seminal vesicles (paired sperm storage organs of males) to determine whether males retained sperm after a first copulation.

Counts of spermatozoa within the females were obtained by dissecting out their reproductive tracts, and detaching them at the ovipore and at the branching point of the lateral oviducts. The bursa copulatrix (copulatory pouch) is not well-defined in *L.c.clivicollis*: there is a slight swelling of the common oviduct where the spermathecal duct joins it. A few drops of Tween 80 (Polyoxyethylene [20] sorbitan monoleate) were added to

200 ml of *Aedes* saline (Hagedorn et al. 1977) in order to disperse clumps of sperm. The entire common oviduct (with the spermatheca attached) was placed in 200 µl of saline/Tween 80. The spermatheca was immediately detached at the junction of the spermathecal duct with the common oviduct, and placed in a second depression dish with 200 µl of saline/Tween 80. The contents of the common oviduct were expressed into the saline. When a white amorphous mass of accessory gland secretion had been deposited by the male, the mass was torn apart into small pieces to release any sperm that were imbedded in it. Examination of these pieces at 100X (Nomarsky) revealed very few sperms. The spermatheca of *L.c.clivicollis* is a small, sclerotized structure. I cracked the spermatheca open, broke it into small pieces to release the sperm, and removed the pieces of cuticle from the depression dish. After stirring the solution for at least a minute, I placed a 3 µl aliquot of the dilute sperm solution on a glass slide, and covered the droplet with 1/4 of a 22 × 22 mm square coverslip. It was important to stir the solution for at least a minute each time a sample was taken; failure to do so resulted in a high variance in sperm number for individual females due to settling and clumping of sperm. I counted four 3 µl aliquots from each depression dish, and multiplied the mean by the appropriate dilution factor. If sperm concentrations were higher than 100 spermatozoa/3 µl aliquot, 400–600 µl of saline/Tween 80 were used. The mean percent error using this method was 12 ± 1 (SE) % ($n = 43$) for counts of sperms in the spermathecae.

The numbers of spermatozoa remaining in the males after a first copulation were estimated by expressing the contents of the seminal vesicles into 10 ml saline/Tween 80 and counting sperm in four 3 µl aliquots as described above. The sperms in the testes were not counted, because they are immature and not available for insemination.

The relationship between mating duration and the number of sperms transferred to females was determined using virgin females and virgin males. Pairs were placed with swamp milkweed leaves in 9 cm. Petri dishes lined with moist Whatman No. 1 filter paper. They were observed continuously until the male mounted the female. The time of mounting was recorded and the pair was transferred to an incubator at a temperature of 23° C. Pairs were separated 1, 3, 6, 12, 15, 18, 21, and 24 h after mounting. The females were refrigerated immediately to retard digestion or expulsion of sperm and to prevent oviposition. Sperms in the spermatheca and common oviduct were counted separately, as described above. No effort was made to record the time actually spent in copula. The relationship between the total number of sperm transferred to the female (common oviduct + spermatheca) and mating duration was analyzed using the *F*-test for a linear regression of number of sperm vs time.

Mating duration and paternity. The virgin males and females used in this experiment were the adult offspring of overwintered adults collected in the field in June, 1984. I used a reciprocal design to determine the effect of mating duration on paternity of offspring of twice-mated females. In the first treatment, a female was placed with a male in a 9 cm. Petri dish as described above. I recorded the time he first mounted her, and replaced him with a second male 6 h later. I watched to make sure that the second male mounted the female, and allowed pairs to remain together for 24 h. After 24 h, I removed the male, and continued to supply the female with food until she laid 3 consecutive egg clutches, which she did in 3–7 days (mean = 4.2, SE = 0.2). The second treatment was exactly like the first, except that the first male was removed 24 h after mounting, and a second male was added. The second male was then removed 6 h after mounting. 13 females were used for each treatment. In all cases the second male mounted within 10 min of introduction. Five pairs of males were used twice, once for each treatment. These males were isolated for at least a week before reuse, and were used in the same order in 4 out of 5 of these cases. I reared the offspring to adulthood on common milkweed leaves and froze the offspring and parents in liquid N_2 for electrophoretic analysis of paternity. The mean number of offspring reared from the different clutches ranged from 6.34 ± 0.91 (SE) [Clutch 1:6 h:24 h treatment] to 10.1 ± 1.91 (SE) [Clutch 2:24 h:6 h treatment]. One clutch from each of two females yielded no adult offspring. Beetles were transferred to an ultracold freezer (−75° C) in 1–4 weeks and held for 3 months before analysis.

Electrophoresis was performed at the Cornell Laboratory for Ecological and Evolutionary Genetics (CLEEG). The frozen parents were homogenized in extraction buffer on ice and the electrophoretic mobility of 12 of their enzymes was scored using starch gel electrophoresis and methods described by May et al. (1979). These twelve enzyme systems were chosen using data from a previous screen of 40 individuals at 20 allozyme loci. Eleven of the thirteen sets of parents from each treatment group possessed sufficient variation in their electromorphs that paternity of their offspring could be calculated. Paternity was clearly distinguishable at one locus for 8 of the 22 families. In these cases, the males were completely different from each other and the females were homozygous or possessed variants that were completely different from those of one of the two males. Paternity was ambiguous for a portion of the offspring of the 14 remaining families (i.e. some of the offspring could have been fathered by either male). For each of these families, the offspring were homogenized (as described above) and scored at the single enzyme locus at which the most offspring should be distinguishable. The enzyme systems that were actually used in the paternity studies were: glucose phosphate isomerase (GPI), octanol dehydrogenase (ODH), peptidase with glycyl-leucine (PEP-GL), and glycerol-3-phosphate dehydrogenase (G3P).

A maximum likelihood function, based upon the expected mendelian ratios, was used to calculate the proportion of offspring fathered by the second male (Lindgren 1968). A generalized form of the likelihood equation is as follows:

$$L = [F_u(1-p)]^x \cdot [F_a(1-p) + S_a p]^y \cdot [S_u p]^z \quad (1)$$

where 'F_u' is the proportion of the first male's offspring that are expected to possess electrophoretic variants that *could not* have been derived from the second male and 'F_a' is the proportion of the first male's offspring expected to possess electrophoretic variants that *could* have been derived from either male. 'S_u' and 'S_a' are analogously defined for the second male's offspring. The proportion of offspring fathered by the second male is represented by 'p'. The exponents, 'x' and 'z' are equal to the numbers of offspring that can be unambiguously assigned to the first and second male, respectively, whereas 'y' is equal to the number of offspring that are of ambiguous paternity. If 'p' was a known parameter, then Eq. (1) would be the probability of observing x, y, and z individuals in each of these three categories.

Consider the case of a 3 allele system where the first male is scored 'ab', the second male is scored 'bc', and the female is scored 'ab'. Male 1's offspring should possess "genotypes" 'aa', 'ab', and 'bb' in a 1:2:1 ratio. Male 2's offspring should be 'ab', 'bb', 'ac', and 'bc' in a 1:1:1:1 ratio. Any 'ab' or 'bb' offspring could belong to either male. One-quarter of male 1's offspring should be distinguishable and $^3/_4$ should be ambiguous, whereas $^1/_2$ of male 2's offspring should be distinguishable and $^1/_2$ ambiguous. If electrophoretic analysis gives 3 distinguishable male 1 offspring ('aa'), 7 offspring of ambiguous

paternity ('ab', 'bb'), and 2 distinguishable male 2 offspring ('ac', 'bc'), the maximum likelihood function is:

$$L=[^1/_4(1-p)]^3\cdot[^3/_4(1-p)+{}^1/_2p]^7\cdot[^1/_2p]^2 \quad (2)$$

where 'p' is the proportion of offspring fathered by the second male. The value of 'p' that will maximize the function can be obtained by taking the Log of 'L', setting derivative of the resulting function equal to zero, and solving for 'p'. My data showed a single maximum in each case. Values of 'p' at these maxima were combined with 'p' values calculated for the 8 families with clearly distinguishable paternity. The proportions of offspring sired by the second males were compared among the two treatment groups using a Mann-Whitney U-test. I compared data on the 3 different egg clutches using a one-way analysis of variance. Both Mann-Whitney U-tests and two-sample t-tests were used to compare the proportions of offspring fathered by the "24-h" males between treatment groups.

If paternity is determined by mating duration alone, the expected proportion of offspring fathered by the "24-h" males would be 0.80 ($^{24}/_{30}$). I used a one-sample t-test to determine whether the proportion of offspring fathered by the "24-h" males was statistically different from 0.80.

Results

Field observations

Mating frequency and mating duration. Multiple mating was verified in the field for both males and females. Although data on mating frequencies are not complete, we saw marked females mating with up to 7 different males over the 5-week monitoring period (Fig. 1). Pairs remained together for 1–42 h. It was not possible to get an accurate estimate of the distribution of mating durations because females walked around with males on their backs and frequently oviposited on plants other than their host plants. Females usually disappeared from sight before a mating terminated and frequently appeared after a mating had begun. Consequently, the probability of seeing a mating from start to finish decreased as mating duration increased, biasing samples in favor of low durations.

Females that mated with more than one male remated 0 to 591 hours after a previous mating [mean = 101 ± 18 (SE) h, $n=39$]. Some matings went undetected, so that the gap between matings is overestimated by the data.

Laboratory experiments

Copulation and sperm transfer. In the laboratory, males copulated with females a mean of 2.51 ± 0.15 (SE) times per hour for an average of 15 ± 2.60 min per copulation. Pairs spent 57% ± 5% of the time in copula; 54% of the copulations were complete intromissions, while 46% were partial intromissions. Males spent the remaining 43% of the time riding high on females' backs, or riding further down with or without their genitalia hooked beneath the females' wing covers (elytra). Males

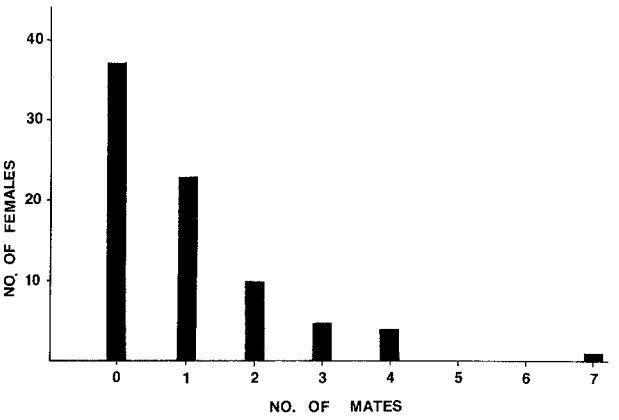

Fig. 1. The number of marked females that acquired 0–7 different mates during the 5-week sampling period. Note that these data do not represent complete mating histories of females, but are minimum numbers of mates per female

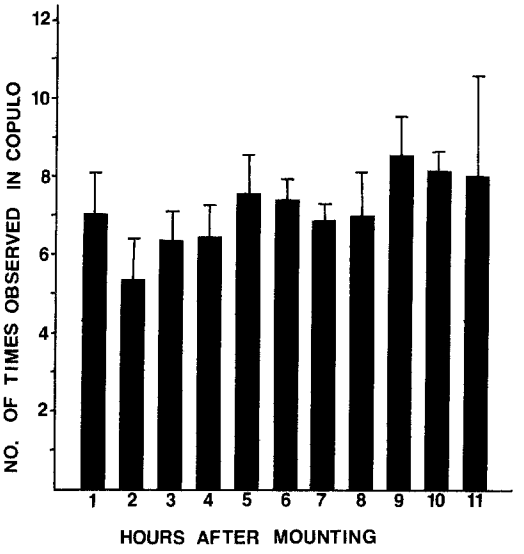

Fig. 2. The mean number of times pairs were found in copula (out of 12 instantaneous samples/pair/hour). Times are the first through the eleventh 1-hour intervals following mounting

also stroked females rhythmically with their hind legs, tapped them rapidly with their antennae, turned around on their backs (seeming to attempt to copulate with their head regions), and bit their pronota while rocking violently on their backs. One male bit a female's legs repeatedly before mounting her.

Copulation in petri dishes occurred throughout the 11-h test period (Fig. 2). There was no statistically significant difference in the frequency of copulation between the 11 different 1-h intervals (Kruskal-Wallis test, $0.50 < P < 0.90$).

Males inseminated directly into the spermatheca and did not make spermatophores. One of the 9 virgin males failed to inseminate the female during the first copulation. For the remaining eight pairs, the mean proportion of sperm found in the

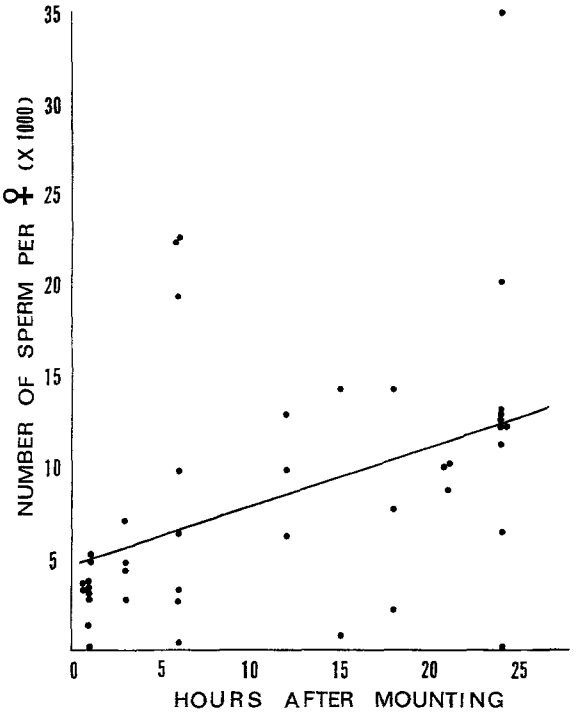

Fig. 3. Linear regression of the number of sperm transferred to females with time after mounting first occurred. The regression is significant by the F-test ($P<0.003$, $r^2=0.178$)

spermatheca as opposed to the common oviduct was 0.943 ± 0.017 immediately after the first copulation. Forty-three counts of sperm in females at various times after mounting also showed a preponderance of sperm in the spermatheca as opposed to the common oviduct.

Males were not depleted of sperm after a first copulation. In fact, the number of sperm in the seminal vesicles of males that were allowed a single copulation ranged from 190,000 to 302,700 (mean $=229{,}600 \pm 43{,}200$, $n=7$). The largest number of sperm found within a female was 30,500, 24 h after mounting. This amounts to at most 16% of the total number of sperm a virgin male has available for insemination. Sperm in the seminal vesicles were not in bundles and were vigorously active *in vitro*. The testes of males were robust and full, indicating that spermatogenesis is an ongoing process.

There was a statistically significant linear regression of the number of sperm within the female with time after mounting ($r^2=0.178$, $P<0.003$, Fig. 3). The r^2 increased to 0.518 when a regression analysis was performed without 5 "outlier" points (points having high standardized residuals). A ln transformation of sperm number remedied the problem of unequal variances, and resulted in an r^2 of 0.226. The linear regression of ln (sperm number) on time was also statistically significant ($P<0.001$).

Mating duration and paternity. Although there were only 8 families with unambiguous paternity (such that maximum likelihood estimates were unnecessary), the mean proportions of offspring fathered by the second males for these 8 families were in close agreement with the proportions for all families combined (Table 1). I used data on all families for statistical comparisons between treatments and egg clutches. The mean proportion of offspring fathered by the second males differed markedly between the 2 reciprocal treatments (Fig. 4). The last male's sperm predominated in fertilization for each of the 3 egg clutches when the first male was with the female for 6 h and the second male was with the female for 24 h. The reciprocal treatment, in which the first male was with the female for 24 h and the second male for 6 h, resulted in a reversal of the trend. When the first mating association was longer in duration, the first male's sperm predominated in fertilization. The proportions of offspring sired by second males were significantly different between the two treatments for each of the 3 egg clutches (Mann-Whitney U-test, $0.001<P<0.003$). On the other hand, there was no statistically significant difference among the 3 egg clutches in the proportions of offspring fathered by the second males (ANOVA, $P>0.25$).

The mean proportion of offspring fathered by the "24-h" males was greater when the first mating was long than when the second mating was long, but these differences were not statistically significant for any of the 3 egg clutches (Mann-Whitney U-test, $0.13<P<0.77$ or two-sample t-test,

Table 1. Mean proportion of offspring fathered by the second males for 8 families with unambiguous paternity compared with proportions for all families combined. n: number of families

Treatment	Clutch 1 $\bar{x} \pm$ SE (n)	Clutch 2 $\bar{x} \pm$ SE (n)	Clutch 3 $\bar{x} \pm$ SE (n)	3 Clutches combined $\bar{x} \pm$ SE (n)
Unambiguous 6 h:24 h	0.78 ± 0.08 (5)	0.75 ± 0.07 (5)	0.62 ± 0.13 (5)	0.69 ± 0.08 (5)
All families 6 h:24 h	0.74 ± 0.10 (11)	0.74 ± 0.09 (11)	0.62 ± 0.09 (11)	0.69 ± 0.09 (11)
Unambiguous 24 h:6 h	0.21 ± 0.15 (3)	0.16 ± 0.11 (3)	0.07 ± 0.06 (2)	0.16 ± 0.09 (3)
All families 24 h:6 h	0.21 ± 0.07 (11)	0.15 ± 0.05 (10)	0.19 ± 0.06 (10)	0.18 ± 0.04 (11)

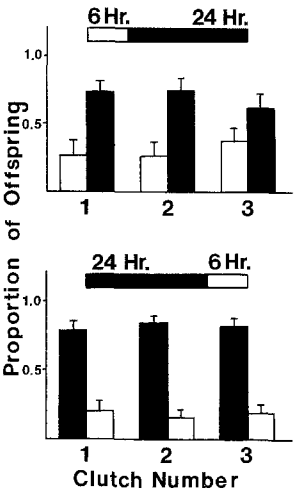

Fig. 4. The upper graph shows the proportions (+SE) of offspring fathered by the first and second males for each of three consecutive egg clutches laid by the females when the first mating was short (6 h) and the second mating was long (24 h). The lower graph shows the proportion of offspring fathered by the first and second males when the first mating was long (24 h) and the second mating was short (6 h)

$0.10 < P < 0.62$). The mean proportion of offspring fathered by the "24-h" males in the two groups combined was similar to the expected proportion of offspring fathered by the long-mating males if paternity is determined by time alone (i.e. $^{24}/_{30}$ or 0.80). The mean proportions for the three egg clutches were 0.765 ± 0.057 (clutch 1), 0.789 ± 0.052 (clutch 2) and 0.713 ± 0.059 (clutch 3), and did not differ statistically from 0.80 ($0.155 < P < 0.84$, one-sample *t*-test). One "24-h" male in the 6 h:24 h treatment did not father any offspring.

Discussion

Surprisingly little is known about the mechanisms which determine sperm utilization patterns in insects, with the exception of those for a few species of damselflies and dragonflies (McVey and Smittle 1984; Waage 1979, 1984). Five mechanisms that have commonly been invoked are: (1) the position effect, in which the last male's sperm lie nearest the site of fertilization (in/near the spermathecal duct or in the bursa) and thus predominate in fertilization (sperm precedence) (Schlager 1960; Walker 1980), (2) removal and replacement, in which a male removes (Waage 1979) or flushes away (Parker 1970) the sperm of earlier males, at least to some degree, before inseminating with his own sperm, (3) expulsion of later males' sperm by the female (Parker 1970), (4) inactivation, in which an earlier male's sperm are inactivated by substances in the ejaculate of later males (Silberglied et al. 1984) and (5) dilution, in which a male dilutes the sperm of earlier (or later) males and derives a paternity advantage based upon his numerical representation in the spermatheca (Parker 1970; Walker 1980). The evidence provided by this study suggests that dilution is likely to be the mechanism of sperm predominance in *L.c.clivicollis*. Although the evidence for this mechanism is circumstantial, the reciprocal experiment provides evidence against the hypotheses of sperm precedence, sperm replacement, sperm expulsion, and sperm inactivation. If any of these four mechanisms were involved in determining sperm utilization patterns in the milkweed leaf beetle, there should be substantial differences in the proportions of offspring fathered by the "24-h" males between the two treatment groups. This was not the case. The early male's sperm predominated in fertilization when the early mating was longer and there was no statistically significant difference in the number of offspring fathered by "24-h" first males as compared with "24-h" second males. This suggests that mating order is not important in this species. The data are consistent with a simple "dilution" model. The fact that the mean proportion of offspring fathered by "24-h" males did not differ significantly from 0.80 is consistent with complete sperm mixing and utilization of sperm from different males in direct relation to the proportion in which they occur. The mean was slightly lower than 0.80 for each of the three egg clutches, but this may be explained by the fact that one "24-h" male in the 6 h:24 h treatment fathered no offspring at all and thus was probably infertile. The spermatheca of *L.c.clivicollis* is a narrow tube bent into a "U" at the halfway point. Sperm mixing in this species is not consistent with Walker's (1980) hypothesis that sperm mixing occurs to a lesser degree among species with tubular spermathecae.

A small proportion ($r^2 = 0.178$) of the variance in the number of sperm transferred with time was explained by the linear regression. It is important to note that the time actually spent in copula was not measured. Variation in the amount of time spent copulating could account for much of the variation in the number of sperm transferred over a given period of time. In addition, insect sperm counts tend to be variable. The sperm are long and thin, and clumping of the tangled filamentous spermatozoa may be a source of error. Males may also vary considerably in their abilities to inseminate females.

Copulation and sperm transfer seem to occur throughout the prolonged mating association in *L.c.clivicollis*. A last male advantage has been

demonstrated for the intermittently copulating belostomatid, *Abedus herberti* (Hemiptera) (Smith 1979), but last male paternity is not the rule for the milkweed leaf beetle. Male belostomatid water bugs invest considerable time in paternal care, and Smith (1979) proposed that they copulate intermittently in order to repeatedly place their own sperm nearest the site of fertilization. The sperm utilization patterns of female milkweed leaf beetles suggest that intermittent copulation serves some other function.

It is puzzling that males inseminate females repeatedly, over a long period of time. According to the paternity data, males would do best to inseminate with large numbers of sperm as early as possible, even if they reap additional benefits by then remaining with females to protect them from rival males. They are not limited by sperm number, but they might be limited by the availability of accessory gland secretions. I believe this to be unlikely, because males do not make spermatophores and insemination is a matter of pumping the sperm through the ejaculatory duct into the female. The rate of insemination could be limited by the physical problem of pushing a viscous ejaculate through a very narrow recurved tube (the virga, which is only everted once the penis is inside the female). The advantages of inseminating directly into the spermatheca may have some associated costs; perhaps selection for insemination into the spermatheca and concomitant changes in the male's genitalic morphology have placed limitations on sperm transfer rate. However, this still would not explain why copulation is intermittent and interspersed with mounted courtship.

It is more likely that female refractory behaviors make insemination difficult. Males inseminate directly into the spermatheca; placement of the virga in the spermathecal duct of the female is likely to be an exacting process. This may be why copulation does not always lead to insemination. Females, including virgins, kick males' legs and penises with their hind legs throughout mounted courtship and copulation. They occasionally drop onto their sides in efforts to jar males off their backs, and pull their abdomens up against their wing covers to prevent intromission. In addition, females may use muscles to keep their genital openings closed, thereby preventing or delaying intromission. If female refractory behaviors make insemination difficult for males, the prolonged mating association might involve a form of "female choice" favoring males that are superior in their abilities to subdue females and achieve or maintain contact with the female's spermathecal duct. In field situations, "takeovers" occur, but are not common at the densities present in my study site. Still, it is possible that less "adept" males run the risk of being displaced by rivals or thrown off by females before they can place enough sperm in the spermatheca to ensure that they will get some fertilizations.

There is no courtship prior to mounting in *L. c. clivicollis* and males are only occasionally dispelled. They crouch on females' backs, out of reach, and grip the females' elytra with large tarsal pads that are characteristic of the Chrysomelidae. It is only when males crouch lower on the females' backs to assume the copulatory position that they come within reach of the females' kicking legs. If "female choice" occurs in *L. c. clivicollis* it most likely occurs during the copulation, insemination, and sperm utilization phases of reproduction.

This study has shown that male milkweed leaf beetles sire more offspring by remaining with females for longer periods to inseminate them with greater numbers of sperm, but males may also gain by remaining with females until oviposition to guard them from mating attempts by rival males (Hughes 1981; Johnson 1982; Kirkendall 1984; Parker 1970; Sillen-Tullberg 1981; Waage 1979). In addition, males might benefit due to pooling of chemical defenses with those of females or increased vigilance against predators or parasites (Eisner 1965; Sivinski 1983). These hypothetical, benefits for males are not mutually exclusive; all three could simultaneously favor prolonged mating. At this point, it is unclear whether the refractory behaviors of females constitute "coyness" that serves to keep the males on their backs. Both virgin and mated females exhibit refractory behaviors. It is unlikely that females are better able to feed and oviposit as a result of protection from mating attempts by competing males (Wilcox 1984) because riding males do not seem to reduce the frequency with which they copulate with females as the mating progresses. Females may gain from dual defense, or they may derive physical protection from predators or parasites by engaging in prolonged mating associations (Walker 1980). It is also possible that the risk of interspecific mating favors females that require a long assessment period and delay insemination. Studies are in progress to test these hypotheses and to measure to associated costs of prolonged mating for male and female milkweed leaf beetles.

In insects, prolonged mating associations fall into four basic categories: (1) In some species a brief copulation is followed by a long postcopulatory passive or guarding phase, (2) in others there

is a long guarding or passive phase followed by a single brief copulation, (3) in some, pairs form long mating associations and genital contact is maintained for the entire period, and (4) in other species, like the milkweed leaf beetle, males copulate repeatedly with the same female and engage in courtship or aggressive behaviors toward the female or ride passively between intromissions. The sperm-loading hypothesis should be considered for species falling into the latter two categories. The numerical advantage will be most pronounced where there is complete sperm mixing. However, even if there are mechanisms which ensure a last male advantage, as has been suggested for most insect species (Parker 1970), the number of sperm a male transfers can still affect the number of offspring he sires.

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