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Rate of ejaculate breakdown and intermating intervals in monarch butterflies

Karen S. Oberhauser

Department of Ecology, Evolution and Behavior, 109 Zoology Building, Minneapolis, MN 55455, USA

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Summary. Both mass and nitrogen content of ejaculates transferred by male monarch butterflies (*Danaus plexippus*) varied with male history. Older virgins and males that had waited longer after a previous mating transferred larger ejaculates with more nitrogen. After mating, ejaculates were broken down within the female bursa copulatrix; mass and nitrogen content decreased at constant rates until little material remained. Because the time required for breakdown of large spermatophores is longer than the intermating interval, a significant portion of a male's investment could be used to benefit offspring from other males.

Introduction

Lepidopteran sperm are transferred within a protein-rich (Johnson 1979; Marshall 1982) ejaculate that can represent up to 15% of male body mass (Boggs 1981; Sims 1979; Rutowski et al. 1983; Rutowski 1984; Svärd 1985; Rutowski and Gilchrist 1986; Svärd and Wiklund 1986, 1989; Oberhauser 1988); in fact an investment of 23% of male mass has been reported in a single mating (Forsberg and Wiklund 1989). After mating, sperm are transferred to the sperm storage organ, the spermatheca, and the rest of the ejaculate, or spermatophore, remains in the female bursa copulatrix. Accessory gland products in spermatophores have been shown to function in sperm activation and stimulation of oviposition (reviews in Leopold 1976; Drummond 1984); but males transfer more material than is necessary for these functions. Even very small spermatophores result in egg fertilization and oviposition (Rutowski et al. 1987; Oberhauser 1989).

Boggs (1981) suggested two ways in which investment in large ejaculates could benefit male Lepidoptera. First, this investment could increase the proportion of a female's eggs fertilized by the spermatophore donor by increasing the time before female remating. Second, it could represent paternal investment, providing material benefits that allow a female to lay more or higher-quality eggs. There is experimental confirmation for both of these functions. Polyandry is common in the Lepidoptera (Pliske 1973; Ehrlich and Ehrlich 1978; Drummond 1984). Combined with the fact that there is often sperm precedence of the last male to mate (reviewed in Parker 1970; Drummond 1984; Gwynne 1984), this suggests that males should benefit by delaying female remating as long as possible. While many factors have been implicated in the mediation of female receptivity in the Lepidoptera (e.g. the presence of sperm in the spermatheca, Taylor 1967; mating plugs, references in Ehrlich and Ehrlich 1978; male-contributed hormones or "anti-aphrodisiacs," Gilbert 1976; Obara 1982; the presence of sperm or testicular fluids in the bursa copulatrix. Riddiford and Ashenhurst 1973; Sasaki and Riddiford 1984), these factors do not explain the quantity of material transferred by male Lepidoptera. Several studies have indicated that large ejaculates delay female remating (Labine 1964; Sugawara 1979; Rutowski 1980; Rutowski et al. 1981; Rutowski 1984; Oberhauser 1989) and two of these (Labine 1964; Sugawara 1979) showed that, in at least some species, this effect is mechanical; the amount of material in a female's bursa copulatrix affects her receptivity to courting males. Females incorporate amino acids from male ejaculates into their eggs and somatic tissue within 24 h after mating (Boggs 1981; Boggs and Gilbert 1979; Boggs and Watt 1981; Greenfield 1982; Boggs 1990), and receiving more of these nutrients has been shown to increase female fecundity in three species (Rutowski et al. 1987; Watanabe 1988; Oberhauser 1989) and to allow decreased foraging expenditure in another (Boggs 1990).

The two potential functions of spermatophore investment are not mutually exclusive; spermatophores could represent both mating and paternal effort. This was suggested by Rutowski et al. (1987), who showed that large ejaculates produced by male *Colias eurytheme* increased female reproductive output in the first 5 days after mating, when females are unlikely to remate. However, if the delayed remating effect of spermatophores is mechanical, there is an inherent conflict between these two

functions of male investment; a spermatophore that has been broken down to provide nutrients will not be as effective at delaying remating.

In assessing potential benefits to males of providing females with nutrients, we need to know not only whether the nutrients increase female fecundity, but also whether they are used in offspring of the donating male. This would be true if the female did not remate until she had used the nutrients from a previous mate. The nutrient function, or the nutrient and delay effects acting in concert to benefit males, predicts that the average female intermating interval in a species should be greater than the time required for spermatophore degradation. If this interval is shorter than degradation time, the delay effect is probably more important.

In this study, I estimate the proportion of a male's nutrient contribution that is available exclusively to his own offspring by measuring the proportion of spermatophore nutrients have been removed from the bursa copulatrix by the time female remating occurs. Boggs and Gilbert (1979) showed that male-derived nutrients were present in monarch butterfly eggs the day after mating and increased in level for approximately 5 days after mating, at which point their levels declined sharply. Since this period corresponds roughly to the time over which spermatophores are degraded, I assume that male-derived nutrients are used soon after being removed from the bursa copulatrix.

Methods

General methods. Monarch butterflies were reared and maintained as described previously (Oberhauser 1988). Experiments were carried out during the summers of 1986 (in Minneapolis, Minnesota) and 1988 (in central Wisconsin). Butterflies were kept in outdoor screen cages (2 m × 2 m × 2 m or 2 m × 3 m × 2 m), and fed a 30% honey solution to satiation daily. All butterflies were offspring of females captured from the wild.

Rate of spermatophore degradation. In order to measure the rate at which ejaculates were degraded, 28 males in each of five groups were allowed to mate with virgin females aged 5–8 days. The five male groups included three groups of virgins (aged 5, 8 and 12–15 days), and two groups of previously-mated males (mated 1 and 4 days prior to the experimental mating). Initial sample sizes varied because it was not always possible to get 28 males with the same history to mate. After mating, females were kept in outdoor screen cages and provided with fresh milkweed (*Asclepias syriaca*) on which to oviposit.

A 3-day intervals 1–19 days after mating, four females from each group were randomly selected for dissection. In some cases fewer than four could be selected, due to initial sample sizes of fewer than 28 in some groups and subsequent female mortality. All dissections were done in the morning, and day values refer to the number of days after mating initiated (monarchs begin copulating in the afternoon and separate at approximately sunrise the following morning). Thus data obtained on day 1 represent initial male investment. The remaining ejaculate material was removed from the bursa copulatrix into insect saline solution, blotted to uniform dryness on tissue paper, weighed to the nearest 0.01 mg on a Mettler semi-micro analytical balance, and frozen for later analysis of nitrogen content. Sample sizes were further reduced because some spermatophores had become so degraded that it was impossible to remove them intact from the bursa. This was true for many of those from males mated 1 day previously; for this reason I was unable to carry out some of the statistical analyses on this group. Micro-Kjeldahl anayses of nitrogen content were done by the Crop Research Laboratory at the University of Minnesota.

Intermating intervals. I measured female intermating intervals in two ways: (1) time between the first and second matings of females that received either large or small spermatophores in the first mating, and (2) time between all of the matings of females allowed to mate *ad libitum* throughout their lives.

To measure the interval between first and second matings, 70 six-to-seven day old females were allowed to mate with either 6–10 day old virgin males or males that had mated one to two days previously (spermatophore masses of approximately 30–37 mg and 7–15 mg respectively; Oberhauser 1988). Females were then put into mating cages with males each day after the first mating until they remated. To measure intermating intervals throughout females' lives, 38 females mated for the first time at age 7–10 days and were then put into mating cages with males every day thereafter. The experiment was ended when all females were either dead or over 5 weeks old, when few matings occur. In all tests of intermating intervals butterfly densities were 40 or fewer per cage and males used for second and subsequent matings were either virgins or had not mated within 4 days. Fresh milkweed plants were provided daily for oviposition. All matings were recorded. (See Oberhauser 1989 for more details.)

Spermatophore masses transferred by wild males. To estimate the mass of spermatophores that wild females receive, I captured wild males and allowed them to mate with females in captivity. Males were put with virgin females as soon as possible after capture. When males mated within 2 days of capture, their mates were dissected to determine the spermatophore mass transferred.

Results

Size of male investment and rate of spermatophore degradation

Means of initial ejaculate mass, total amount of nitrogen and nitrogen as a percentage of wet mass are shown in Table 1. Recently-mated males transferred significantly smaller spermatophores than virgins, and 4 days after mating, males transferred spermatophores with masses

Table 1. Comparison of initial male investment

Group	*n*	Mass (mg)	Nitrogen (mg)	% Nitrogen
1NV	4	9.33 ± 2.21 a	0.23 ± 0.107 a	2.53 ± 0.95 a
4NV	4	22.99 ± 1.32 b	0.98 ± 0.037 b	4.26 ± 0.35 b
5V	3	19.72 ± 0.82 b	0.89 ± 0.043 c	4.54 ± 1.75 b
8V	5	24.88 ± 4.13 b	1.06 ± 0.083 d	4.34 ± 0.04 b
12–15V	4	39.09 ± 9.13 c	1.72 ± 0.318 e	4.43 ± 0.32 b

Numbers followed by NV and V indicate the number of days between the previous and current mating of nonvirgin males (NV) and the age of virgin males (V) at the time of mating. All values are given as group means followed by 1 SD. They were compared by one-way analyses of variance (Mass $F_{4,15} = 20.17$, $P < 0.001$; Nitrogen $F_{4,15} = 44.26$, $P < 0.001$; % Nitrogen $F_{4,15} = 8.78$, $P < 0.001$). Means followed by the same letter are not significantly different at the 0.05 level of confidence (Spjotvoll-Stoline *T'*-test for minimum significant difference for groups with unequal sample sizes)

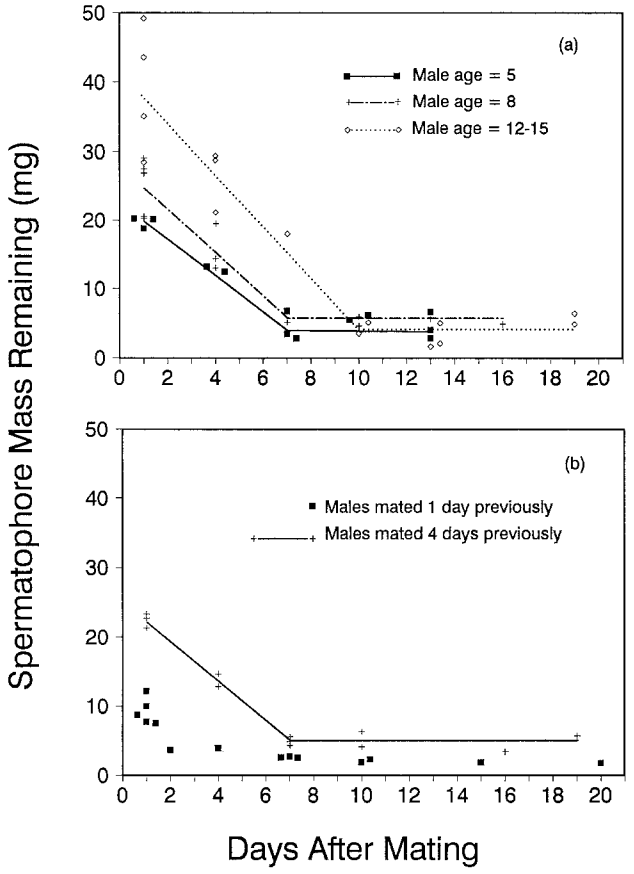

Fig. 1 a, b. Spermatophore mass remaining at different times after mating from **a** virgin males of different ages and **b** males that had mated previously. Regressions were calculated using only spermatophores removed during the time that mass was decreasing (see Table 2 and text for more explanation). Marks on the figure were moved slightly right or left when there was overlap

equal to those transferred by virgin males approximately 1 week old. Spermatophore mass increased with age in virgin males, although the difference between spermatophores from 5- and 8-day-old virgins was not statistically significant.

Total nitrogen content was significantly different in spermatophores from all five groups, with males that had mated 1 day previously transferring the least nitrogen, and old virgins transferring the most. All males except those mated 1 day previously transferred spermatophores with approximately equal proportions of nitrogen. Spermatophores from males that had just mated contained a significantly lower proportion of nitrogen.

Figure 1a and b illustrates rates of mass decrease in spermatophores transferred by virgin and previously-mated males. In all groups, spermatophores were broken down at a constant rate until they reached approximately 5 mg (2 mg when males had mated 1 day previously), when mass stopped decreasing. This occurred from approximately 7 to 10 days after mating for all but the smallest spermatophores. I did not obtain enough intact spermatophores from males mated 1 day previously to determine when mass stopped decreasing in this group, but it may have happened as soon as 2 days after mating (Fig. 1b).

Regression coefficients obtained from linear regressions of spermatophore mass on the number of days after mating (DAM) are shown in Table 2a. In calculating these coefficients, I only used data from the days during which the plots of spermatophore mass versus time (Fig. 1) showed that mass was decreasing. After this, slopes were not significantly different from zero in any of the groups. Lines obtained from the regressions are plotted on Fig. 1. None of the slopes are significantly

Table 2

Group	Days	*n*	Predictor	Coefficient	SE	*P*	ADJ R^2
[a] Post-mating spermatophore mass decrease							
5V	1–7	8	constant	22.49	0.90	<0.001	
			DAM	−2.57	0.19	<0.001	0.963
4NV	1–7	8	constant	35–43	0.57	<0.001	
			DAM	−2.93	0.12	<0.001	0.988
8V	1–7	9	constant	28.22	2.00	<0.001	
			DAM	−3.32	0.59	0.001	0.781
12–15V	1–10	10	constant	42.60	3.12	<0.001	
			DAM	−3.81	0.57	<0.001	0.829
[b] Post-mating nitrogen decrease							
5V	1–7	8	constant	1.036	0.072	<0.001	
			DAM	−0.103	0.015	<0.001	0.866
4NV	1–7	8	constant	1.079	0.026	<0.001	
			CAM	−0.116	0.006	<0.001	0.984
8V	1–7	9	constant	1.194	0.077	<0.001	
			DAM	−0.128	0.023	<0.001	0.792
12–15V	1–10	10	constant	1.899	0.116	<0.001	
			DAM	−0.158	0.021	<0.001	0.860

V and NV are defined in Table 1. DAYS refers to the time during which spermatophores were decreasing in mass (see Fig. 1). DAM refers to the number of Days After Mating that the spermatophore was removed from the female. Coefficients for DAM have units of mg/day (Table 2a) and mg N/day (Table 2b)

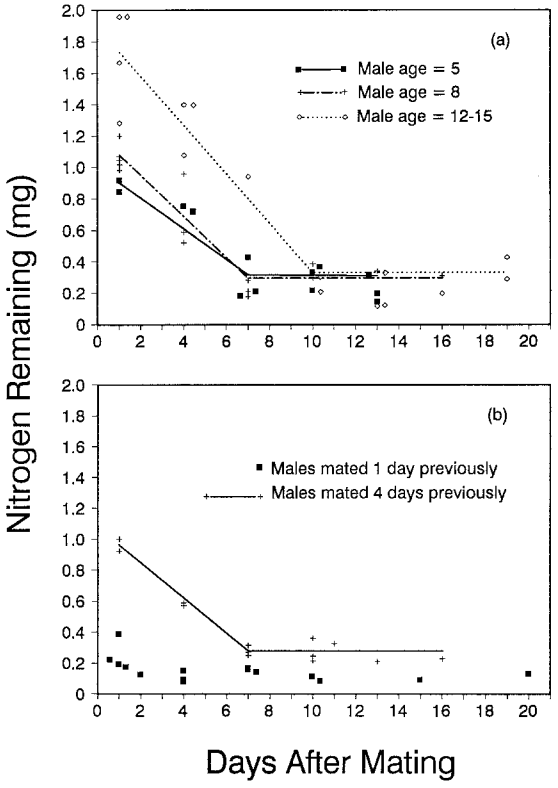

Fig. 2a, b. Amount of nitrogen remaining in spermatophores at different times from **a** virgin males and **b** males that had mated previously. Regressions were calculated in the same manner as those shown in Fig. 1. Marks on the figure were moved slightly right or left when there was overlap

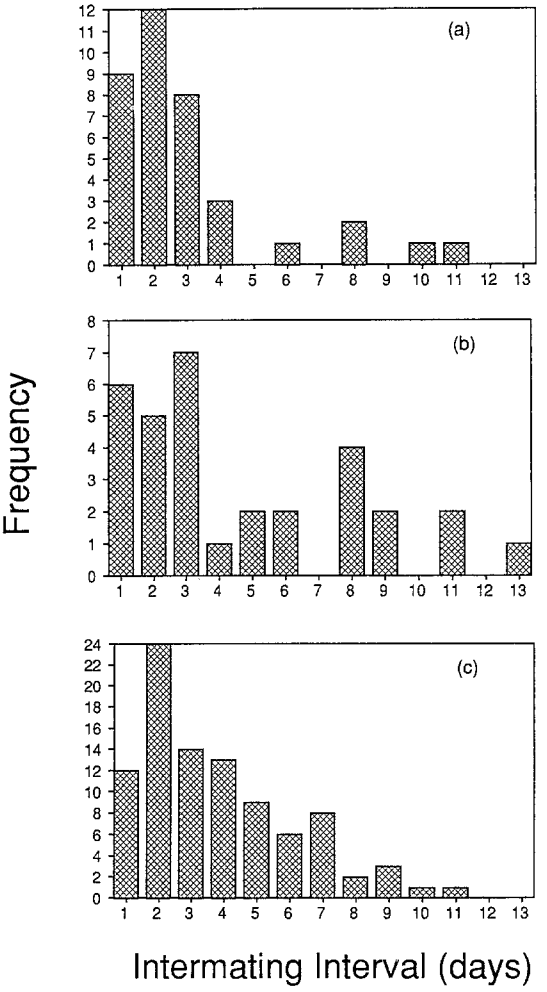

Fig. 3a–c. Female intermating intervals between **a** the first and second mating when the first spermatophore was small, **b** the first and second mating when first spermatophore was large, and **c** all matings for females allowed to mate *ad libitum* throughout their lives

different; a model in which all regressions were analyzed separately was not significantly better than one in which it was assumed that all slopes were equal ($F_{3,30}=1.39$, $P=0.265$). The regression coefficient for DAM in the model that combines data from all four groups was -3.29 (1 SE = 0.25), which means that mass was decreasing at a rate of approximately 3.3 mg per day in all groups.

Spermatophore nitrogen levels are shown in Fig. 2a and b. They were analyzed in the same way as mass data, with regressions using only data obtained before the decrease stopped (Table 2b). No slopes were significantly different from zero after this. Slopes obtained from the different male groups were not significantly different; comparison of the same two models described for mass decrease gave a similar result ($F_{3,30}=1.94$, $P=0.144$). The regression coefficient for DAM in the model that combined data from all four groups was -0.134 (1 SE = 0.010), which means that spermatophores were losing nitrogen at a rate of approximately 0.13 mg per day in all groups.

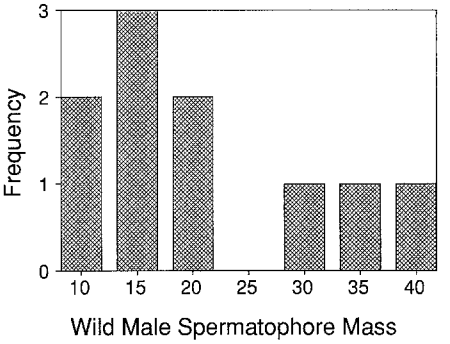

Fig. 4. Spermatophore masses (±2.5 mg) transferred by 10 males captured from the wild and mated with captive females within two days of their capture. (Mean = 17.86 mg)

Intermating intervals

Figure 3a and b shows frequency distributions of intermating intervals for females that received small and large spermatophores. Mean (±1 SD) intervals were 3.0±2.4 days (median = 2) for females receiving small spermatophores initially, and 4.6±3.4 days (median = 3) for females receiving large spermatophores. Figure 3c shows the distribution of intervals for females that mated *ad libitum* throughout their lives. The mean interval for this group was 3.8±2.4 days (median = 3).

Spermatophore sizes transferred by wild males

Ten males captured from the wild mated within 2 days of capture. Figure 4 shows the distribution of spermatophore masses from these males. Mean spermatophore mass was 17.9 mg

Discussion

Two things must be known to estimate how much of a male's material investment is available exclusively to his offspring: (1) the time between his mating with a female and her next mating and (2) the amount of spermatophore material that has been removed from the female bursa copulatrix at this time. Any material remaining at this point could potentially be used in the offspring of another male, depending on the degree of sperm precedence. Both (1) and (2) are a function of initial spermatophore size (Oberhauser 1989 and present results). Data presented above show that the proportion of a male's investment that will be removed from the bursa copulatrix before the female remates depends on the size of that investment. Spermatophores of approximately 15 mg or less are likely to be degraded before a female remates; most of the material is removed from the bursa copulatrix within 2 or 3 days, which is close to mean and median intermating intervals for females receiving small spermatophores (see Fig. 1 and 2). Thus the predictions suggested by the nutrient function, or the nutrient and delay functions acting in concert, are met when males transfer small spermatophores: intermating intervals are greater than or equal to spermatophore degradation time. However, it is likely that a significant proportion of larger spermatophores will remain in the bursa copulatrix after the female remates; females remate after 3–4 days when only half of the mass of larger spermatophores has been removed. In this case, the intermating interval is shorter than degradation time, which suggests that the delay effect is most important to males.

If some spermatophores within size ranges likely to be transferred by wild males meet predictions of the nutrient effect, and some meet predictions of the delay effect, can we draw any conclusions as to the function of the large material investment by male monarch butterflies? Several factors suggest that the delay effect is likely to be more important in this species. First, because males transfer all of their available accessory gland material during a mating (Oberhauser 1988), it is probably advantageous for males to transfer large spermatophores. Second, because it is likely that females will remate before they have used all of the material in a large spermatophore, the nutrient function suggests that male monarchs are transferring suboptimally large spermatophores. Finally, when females receive either one large or one small spermatophore, there is no detectable difference in fecundity (Oberhauser 1989). This suggests that the benefits of the fecundity effect are not very important to males.

My conclusion on the fate of male-donated nutrients in monarchs is based on two assumptions. (1) There is at least some degree of last-male sperm precedence in monarchs. This is true of most Lepidoptera that have been studied (Drummond 1984; Gwynne 1984 and references therein). Several authors have suggested that this is due to the elongate lepidopteran spermatheca; sperm from previous males are pushed back to where they are unlikely to fertilize eggs when a female remates (Walker 1980 and references therein). Drummond (1984) suggested that this shape might have evolved as a mechanism to ensure last-male precedence; this would benefit females receiving material benefits from their mates if it made males likely to court and mate with nonvirgin females. (2) Intermating intervals of captive female monarchs are representative of those in the wild. Two aspects of my experiments could affect these intervals. First, male monarchs can sometimes force unwilling females to copulate (Pliske 1975, personal observations), and this could happen more frequently in captivity if it is more difficult for females to escape. However, even captive females are usually able to avoid the advances of unwelcome suitors (personal observations). Second, males used in the experiment in which females mated *ad libitum* probably transferred larger spermatophores than wild males because they were either virgins or had waited at least 4 days since a previous mating. These two effects should be in opposite directions; forced copulations would decrease intermating intervals, and larger spermatophores should increase them (Oberhauser 1989). Supporting assumption (2) is the fact that the total number of matings by captive females allowed to mate *ad libitum* (lifetime mean of 3.5, Oberhauser 1989) are similar to those observed in old females captured from the wild by Pliske (1973) (mean = 3.1, $n = 23$; females in Pliske's "worn" and "very worn" categories were used to calculate these means).

If males benefit by delaying female remating, and females benefit by using spermatophore nutrients, there is potential for conflict between the sexes in polyandrous Lepidoptera. This conflict could potentially lead to an evolutionary "arms race" (Dawkins and Krebs 1979; Parker 1979) with interesting evolutionary consequences. Males control spermatophore size and content, but females exert control over spermatophore use and fate. If spermatophores contain nutrients useful to females, selection should increase their ability to obtain these nutrients as quickly as possible and then remate, thereby increasing the total amount of male-derived nutrients obtained in their lives. Since it can be harmful for females to receive too much ejaculate material (captive females that mated several times in close succession were killed when their bursae ruptured: Oberhauser 1989), it would still be beneficial for females to use the amount of material in their bursa as a cue to tell them when to remate. Males should then be selected to produce undigestible, cheap spermatophores.

The outcome of this type of sexual contest depends on the costs and benefits incurred by both parties, which are not expected to be symmetric for males and females (Parker 1984). It is difficult to measure the costs of pro-

ducing or breaking down spermatophores of different compositions, but benefits can be estimated. The benefit to males of delaying female remating is gaining a larger portion of the total offspring produced by the female. The magnitude of this benefit will depend on the length of the delay and daily fecundity. Females benefit from using male nutrients quickly by increasing their lifetime reproductive output. The magnitude of this benefit is probably low relative to that of males, given the contradictory results of studies that have looked for an effect of male-derived nutrients on female fecundity in the Lepidoptera [four of these studies (Greenfield 1982; Jones et al. 1986; Svärd and Wiklund 1988, 1991) have found no effect, while three of them have found one (Rutowski et al. 1987; Watanabe 1988; Oberhauser 1989)]. This suggests that males should "win" this arms race. However, there may be evolutionary constraints determined by other, probably ancestral, functions of male accessory gland products. Lepidopteran spermatophores have important effects that are chemical in nature (Leopold 1976; Drummond 1984), including sperm activation (Sheperd 1975; Herman and Peng 1976) and stimulation of oviposition (MacFarlane and Tsao 1974; Yamaoka and Hirao 1977). Of the studies cited above, only Sheperd (1975) determined the chemical nature of the substance, and it was a polypeptide. Even though these chemical functions do not explain the large amount of accessory gland material transferred in lepidopteran spermatophores (small amounts have the same effects, above references), they could explain their high protein content.

Svärd and Wiklund (1989) presented comparative data showing a positive correlation between the degree of polyandry in butterflies and male ejaculate mass, suggesting that males do not invest as heavily when there is less advantage to delayed female remating. However, because spermatophores do not remain intact within the female bursa copulatrix, it is also important to compare rates of degradation when determining the function of this investment. If it is to delay female remating, there should be a negative correlation between degradation rates and the degree of polyandry across species; the selection pressure to make long-lasting spermatophores should be stronger in more polyandrous species. There are few data on the rates at which spermatophores are degraded, but there is some evidence of this negative correlation. Boggs (1981) measured spermatophore degradation in two heliconiine butterflies, one monandrous and one polyandrous, and found slower degradation in the polyandrous species.

While detailed studies of spermatophore content and degradation rates are scarce [see Rutowski (1984) for an exception] there is evidence of a great deal of variability within the Lepidoptera that could provide an interesting basis for comparative study. Spermatophores of *Heliothis zea* contain chitin (Callahan 1958), which is presumably difficult for females to break down; spruce budworms (Outram 1971), and possibly some skippers (Dana 1989), produce spermatophores that may not be degraded at all. On the other extreme, gypsy moth spermatophores are completely degraded within hours after mating (Taylor 1967; Loerch and Cameron 1984).

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