# Determinants of paternity in a butterfly

**Nina Wedell**[*] **and Penny A. Cook**

*Department of Zoology, University of Stockholm, S-106 91 Stockholm, Sweden*

Success in sperm competition is of fundamental importance to males, yet little is known about what factors determine paternity. Theory predicts that males producing high sperm numbers have an advantage in sperm competition. Large spermatophore size (the sperm containing package) also correlates with paternity in some species, but the relative importance of spermatophore size and sperm numbers has remained unexplored. Males of the small white butterfly, *Pieris rapae* (Lepidoptera: Pieridae), produce large nutritious spermatophores on their first mating. On their second mating, spermatophores are only about half the size of the first, but with almost twice the sperm number. We manipulated male mating history to examine the effect of spermatophore size and sperm numbers on male fertilization success. Overall, paternity shows either first male or, more frequently, second male sperm precedence. Previously mated males have significantly higher fertilization success in competition with males mating for the first time, strongly suggesting that high sperm number is advantageous in sperm competition. Male size also affects paternity with relatively larger males having higher fertilization success. This may indicate that spermatophore size influences paternity, because in virgin males spermatophore size correlates with male size. The paternity of an individual male is also inversely correlated with the mass of his spermatophore remains dissected out of the female. This suggests that females may influence paternity by affecting the rate of spermatophore drainage. Although the possibility of female postcopulatory choice remains to be explored, these results clearly show that males maximize their fertilization success by increasing the number of sperm in their second mating.

**Keywords:** Lepidoptera; *Pieris rapae*; sperm competition; body size; spermatophore size; sperm number

## 1. INTRODUCTION

Competition between ejaculates of several males for access to the female's ova is a widespread phenomenon occurring in most animal groups (Parker 1970; Smith 1984; Birkhead & Møller 1992, 1998). The pattern of paternity in species where females mate multiply within a single reproductive cycle is often highly variable both within, as well as between species (Lewis & Austad 1990). Insects either show mixed paternity to varying degrees, or the second male fertilizes most of the female's eggs (Simmons & Siva-Jothy 1998). A second male advantage can arise by males removing previous ejaculates. However, the mechanisms by which the second male achieves high paternity are unknown for most species where males do not remove sperm.

Theory predicts that transfer of high numbers of sperm to the female at mating is advantageous in sperm competition (e.g. Parker 1984). Larger ejaculate volumes may displace pervious male's sperm (Parker & Simmons 1991; Simmons & Parker 1992), or result in higher fertilization success when sperm mix in the female's spermatheca and are utilized numerically (Martin *et al.* 1974; Simmons 1987; Wedell 1991). Results from comparative studies support this, as species experiencing intense sperm competition have larger testes (e.g. Birkhead & Møller 1992; Møller 1988; Gage 1994) and larger ejaculate volumes (Svärd & Wiklund 1989) than more monandrous species. A similar pattern is also found within species, with males increasing

the number of sperm ejaculated depending on the risk of competition from rival males' ejaculates (Gage 1991; Gage & Baker 1991; Simmons *et al.* 1993; Cook & Gage 1995).

Large male size may be advantageous in sperm competition, because larger males often produce bigger ejaculates containing more sperm (Nylin & Wedell 1994; Wedell 1997). However, it is not known whether this is solely due to male size correlating with sperm numbers, or is an effect of large ejaculate volume per se. It is possible that large ejaculate volume itself is advantageous, since males producing larger ejaculates have higher fertilization success (Simmons 1987; Wedell 1991; Sakaluk & Eggert 1996). This may be because larger ejaculate volumes trigger longer period of unreceptivity in females (Oberhauser 1989; Wedell 1993), or because females prefer larger males and are able to bias paternity in their favour.

Male Lepidoptera transfer a spermatophore at mating containing sperm of two types. Most are anucleate, apyrene sperm that do not fertilize the eggs, whereas the fertilizing, eupyrene sperm only comprise about 10–15% (Cook & Gage 1995; Cook & Wedell 1996). The spermatophore is formed in the female's receptacle (bursa copulatrix) during mating. After mating, the spermatophore is ruptured by the lamina dentata, a set of sclerotized teeth inside the bursa (Rogers & Wells 1984), and sperm migrate or are transported to the spermatheca for storage until egglaying. Males of the polyandrous small white butterfly, *Pieris rapae* (Lepidoptera: Pieridae), produce large nutritious spermatophores on their first mating. On their second mating spermatophore size is reduced and sperm number dramatically increased. This

[*]Author for correspondence (nina.wedell@zoologi.su.se).

has been interpreted as a strategy by males to maximize their fertilization success, as the probability of encountering virgin females may be greater on a male's first mating (Cook & Wedell 1996). On second matings, when males may be more likely to mate with already mated females, they transfer significantly more sperm.

So far the relative importance of sperm numbers and spermatophore size for male fertilization success has not been examined. Spermatophore size is known to influence paternity (LaMunyon & Eisner 1994; Bissoondath & Wiklund 1997), but little attention has been paid to the influence of sperm number (Cook *et al.* 1997). In this study we investigate the relative contributions of variation in sperm numbers and spermatophore size to male fertilization success in *P. rapae*. Because males produce larger spermatophores with fewer sperm on their first mating, compared with smaller, sperm-rich spermatophores on second mating, it is possible to separate the effects of spermatophore size and sperm number by manipulating male mating history. Here we examine the effects of spermatophore size and sperm number independently, as well as the effect of mating order and male body size on subsequent paternity.

## 2. METHODS

Larvae of *P. rapae* were reared in 0.5 l plastic cups and fed ad lib. on garlic mustard, *Alliaria petiolata*. Development took place at 25 °C with an 18L:6D light cycle to promote direct development. On the day of eclosion, adults were placed in a cold-room at 4 °C, weighed the following day and given a unique mark on their wing with a permanent marker pen. Adults were then returned to the cold-room for up to 6 d until used in the experiments.

Males were sterilized using 30 krad of gamma radiation on the evening before the experiment and returned to the cold-room until the following morning. Mating experiments took place in 0.5 m × 0.5 m × 0.5 m flight cages in a greenhouse. Each cage contained flowers with 25% sugar solution added twice a day, and cages were regularly sprayed with a fine mist of water. On the morning of the mating experiment, sterilized and untreated males were placed in cages (ten males in each) and allowed to feed for 30 min on the 25% sugar solution added to flowers. Untreated and sterilized males were placed in separate cages. Ten virgin females were then added to each of the cages. Cages were inspected every 15 min for copulating pairs which, on discovery, were isolated in plastic cups until they separated. After their first mating, males were placed in a holding cage, provided with sugar solution added to flowers and allowed to feed for the rest of the day.

At the end of the day, all males were returned to the cold-room. Females mated to either a virgin normal male or a virgin sterilized male were put in cages, provided with sugar solution added to flowers and horse-radish leaves (*Armoracia rusticana*) for egglaying. Females also spent the night in the cold-room. On the day following their first mating, males were mated for a second time in the same way as before. Females mated to previously mated males were treated in the same way as females mating to virgin males. The next day a new batch of unmated males were sterilized in the evening and treated in the same way as before. The following morning these males, together with untreated males, were placed in the mating cages and half of the previously mated females (half mated to sterilized or normal virgin males, and half mated to sterilized or normal mated males) were added

in the same density as for the first mating and allowed to mate for a second time. These females had experienced one day of egglaying after their first mating before remating. The other half of the females were placed in the cold-room prior to being used the following morning, in order to control the time available for oviposition between first and second matings. After males had mated with the singly mated females they were treated in exactly the same way as previously, before being allowed to mate again the following day with the rest of the singly mated females that had been kept in the cold-room. So, all females mated for the first time on their first day out of the cold-room; half of the females remated to virgin males after one day of egglaying, the other half remated to mated males after one day of egglaying and one day spent in the cold-room. All individuals spent the night in the cold-room simulating the temperature drop experienced at night in the wild. In total, 151 of 166 singly mated females remated.

Doubly mated females were placed in individual egg laying cages (30 cm × 30 cm × 50 cm), provided with 25% sugar solution added to flowers twice a day and a horse-radish leaf for egglaying. The leaves were replaced daily and all eggs laid counted. Each female was removed after laying more than 100 eggs. The individual leaves were incubated at 25 °C for 7 d until all the eggs originating from 'normal matings' had hatched. Twenty females failed to lay more than 100 eggs in 6 d and were not included in the analyses. After egglaying, all females were dissected to ensure that both males had transferred a spermatophore at mating. In Lepidoptera, remains of old spermatophores are left in the bursa copulatrix, making it possible to determine the number of matings performed. Spermatophores from first and second matings can be distinguished, as the second spermatophore pushes the first to the back of the bursa. Spermatophore remains from the two matings were removed and weighed to the nearest 0.01 mg. Sixteen of the 151 doubly mated females received only one or no spermatophore and were excluded from the analyses.

Six control groups were performed to assess the effect of male mating status and gamma radiation on egg viability. Control females were mated twice to two virgin normal males (VN–VN, $n=5$), twice to two mated normal males (MN–MN, $n=6$) or once each to a virgin normal and a mated normal male (VN–MN, $n=5$). Similarly, for the sterilized control groups, females were mated to two virgin sterilized males (VS–VS, $n=5$), two mated sterilized males (MS–MS, $n=5$) or to a virgin sterilized and a mated sterilized male (VS–MS, $n=5$). Four treatments were used in the experiment. A virgin female was mated twice to one of the following combinations: (i) virgin–virgin males (V–V); (ii) mated–mated males (M–M); (iii) mated–virgin males (M–V); or (iv) virgin–mated males (V–M). Females were either first mated with a normal male (N) followed by a sterilized male (S), or vice versa (S–N). All combinations were successfully achieved with the following sample sizes: VN–VS ($n=10$), VS–VN ($n=7$), MN–MS ($n=8$), MS–MN ($n=10$), VN–MS ($n=9$), VS–MN ($n=9$), MN–VS ($n=6$), MS–VN ($n=11$).

To calculate paternity, the number of eggs fertilized ($x$) by the N male in the mating sequence (N–S or S–N) was estimated from the proportion of viable eggs ($a$) using average viabilities from N–N matings ($b$) and S–S matings ($c$). Following Sillén-Tullberg (1981), $x=(a-c)/(b-c)$. As there was no difference in egg viability between the three N–N control groups ($F_{(2,14)}=1.198$, $p=0.336$), the estimates were pooled and the mean viability, 0.879 (s.e. 0.034, $n=16$), was used in the calculation of paternity. Similarly, there was no difference in viability of the three S–S control groups ($F_{(2,13)}=0.767$, $p=0.486$), and hence the mean,
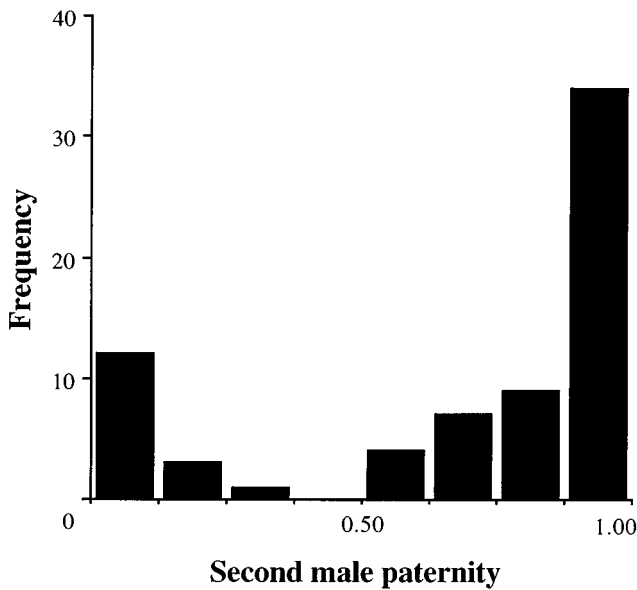
Figure 1. Paternity shows a bimodal distribution with either a last male or a first male sperm priority. There is also a clear effect of mating order with the second male siring most of the offspring.

0.058 (s.e. 0.017, $n=15$), was used in paternity calculations. There was no effect of mating order of sterilized males in the four treatment categories (Mann–Whitney U, $z=0.993$, $p=0.321$, $n=70$), and the reciprocal matings (i.e. N–S and S–N) of the four treatments (V–V, M–M, V–M and M–V) were therefore pooled. Data were checked for normality and proportions arcsine transformed before used in ANOVAs.

## 3. RESULTS

### (a) *Mating order and male mating status*

The proportion of eggs sired by the second male ($P_2$) ranged between 0% and 100% and showed a bimodal distribution, with either a first male or a last male sperm priority (figures 1 and 2). There was a clear effect of mating order on $P_2$ in all four treatments, but the distribution of $P_2$ differed between treatments (contingency table with $P_2$ divided into four categories (0–25%, 26–50%, 51–75%, 76–100%): $\chi^2=19.44$, $p=0.022$, d.f.$=9$, figure 2). There was no difference in the distribution of $P_2$ when both males had the same mating status (comparing V–V and M–M: $\chi^2=1.13$, $p=0.570$, d.f.$=3$, figure 2a,b). However, male mating status did have an effect when females were mated to males with different mating histories. Previously mated males gained significantly more fertilizations than males mating for the first time. There was a significant difference in the distribution of $P_2$ values between V–M and M–V treatments ($\chi^2=11.98$, $p=0.007$, d.f.$=3$, figure 2c,d). When the second male had mated previously, significantly more fertilizations were gained when competing with the ejaculate of a virgin male, compared with when the second male was virgin competing with a previously mated male's ejaculate.

### (b) *Male body weight*

Fertilization success was affected by male body weight. Because fertilization success shows a bimodal distribution, paternity was categorized into first male sperm priority
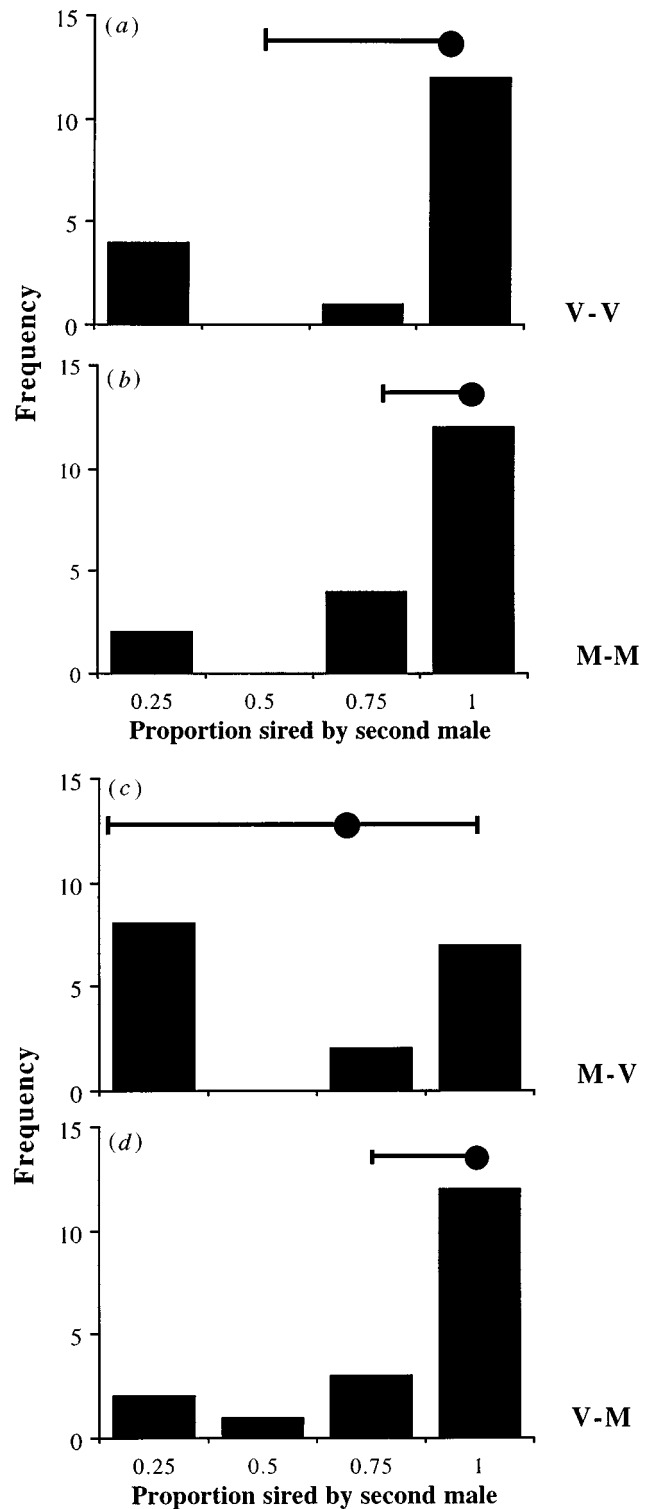


Figure 2. The frequency distribution of proportion of eggs sired by the second male, $P_2$ (divided as in contingency analyses), in doubly mated females. Median and upper and lower quartile also shown. (*a*) Virgin–virgin treatment, (*b*) mated–mated treatment, (*c*) mated–virgin treatment and (*d*) virgin–mated treatment.

($P_2=0$–50%), and second male priority ($P_2=51$–100%). Overall, larger second males had higher fertilization success (two-way ANOVA, second male weight: paternity $F_{(1,62)}=7.78$, $p=0.007$; treatment $F_{(3,62)}=0.47$, $p=0.707$; interaction $F_{(3,62)}=0.29$, $p=0.831$). This was also true for relative weight: relatively larger males had higher
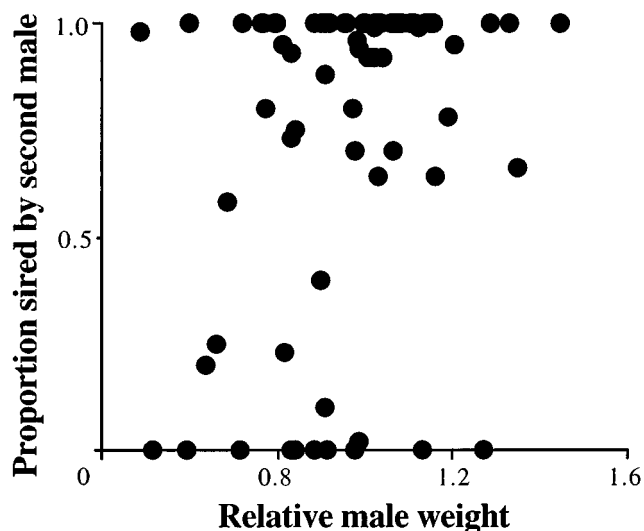
Figure 3. Relatively larger males (body weight of the second male/the body weight of the first male) have higher paternity (proportion sired by the second male).
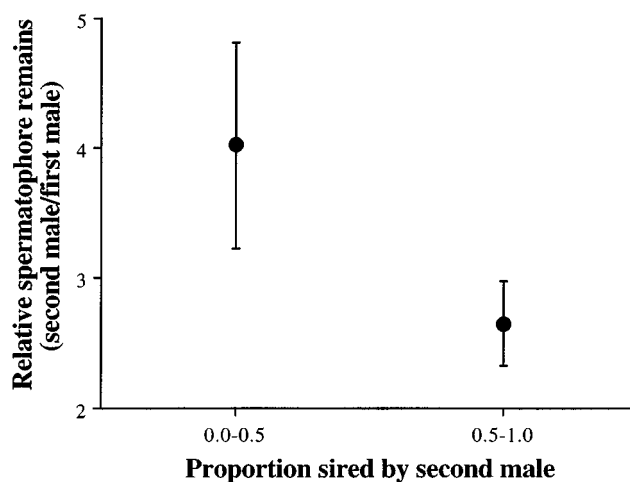


Figure 4. Males with relatively lighter spermatophore remains (weight of the second males' spermatophore/weight of the first males' spermatophore) have higher paternity (proportion sired by the second male).

fertilization success (paternity $F_{(1,62)} = 4.65$, $p = 0.035$; treatment $F_{(3,62)} = 0.21$, $p = 0.888$; interaction $F_{(3,62)} = 0.75$, $p = 0.529$; figure 3 showing the uncategorized data). The pattern with larger males having higher paternity was not due to differences in body weight between the treatment groups.

### (c) *Spermatophore remains*

The weight of the spermatophore remains dissected out of females was related to male fertilization success. Relatively lighter spermatophore remains (weight of the second males' spermatophore/weight of the first males' spermatophore) were associated with higher second male paternity (ANOVA: paternity $F_{(1,61)} = 4.09$, $p = 0.048$; treatment $F_{(3,61)} = 11.24$, $p = 0.0001$; interaction $F_{(3,61)} = 5.74$, $p = 0.002$; figure 4). The interaction is almost certainly due to male mating status influencing the size of spermatophore produced; males mating for a second time produce smaller spermatophores (Cook & Wedell 1996). A closer examination made between males of the same mating status (i.e. V–V and M–M), revealed that spermatophore weight remains were related to fertilization success also within these treatments. For both groups paternity was negatively related to remaining spermatophore weight (V–V matings: $F_{(1,15)} = 7.84$, $p = 0.014$, $n = 17$; M–M matings: $F_{(1,16)} = 11.03$, $p = 0.004$, $n = 18$). Overall, the weight of the spermatophore remains were unrelated to either body weight of males or time since mating (females' first spermatophore from virgin males: body weight $r = 0.23$, $p = 0.185$, time $r = 0.12$, $p = 0.513$, $n = 34$; first spermatophore from mated males: body weight $r = 0.15$, $p = 0.384$, time $r = 0.08$, $p = 0.649$, $n = 35$; second spermatophore from virgin males: body weight $r = 0.28$, $p = 0.115$, time $r = 0.23$, $p = 0.191$, $n = 34$; second spermatophore from mated males: body weight $r = 0.06$, $p = 0.727$, time $r = 0.24$, $p = 0.157$, $n = 35$).

## 4. DISCUSSION

Males of the small white butterfly produce significantly more sperm on their second mating than males mating for the first time, and this strategy results in higher fertilization success (figure 2c,d). Although there is a clear effect of mating order on paternity, males producing more sperm (mated males) do better in competition with males producing larger spermatophores but with fewer sperm (virgin males). This result demonstrates that increasing sperm numbers is advantageous in sperm competition.

Male body weight correlates with paternity, with larger males gaining more fertilizations. This pattern has been found also in other species (Simmons & Parker 1992), including lepidopterans (LaMunyon & Eisner 1994; Bissoondath & Wiklund 1997). In *P. rapae*, larger males produce bigger spermatophores on their first mating. However, there is no relationship between male body weight and sperm number (Cook & Wedell 1996). The effect of male body weight on paternity therefore suggests that large spermatophore size per se provides advantages in sperm competition.

Because sperm numbers influence paternity, why do virgin males produce few sperm? There are two possible reasons for this. First, the average number of matings by females in this species is 2.13 times (Svärd & Wiklund 1989), and animals emerge synchronously (Heath *et al.* 1984). Virgin males are therefore unlikely to encounter females mated to previously mated males. Second, other factors apart from sperm number influence male fertilization success: virgin males can increase their paternity by inducing longer non-receptive periods in females than mated males. In the related species *P. napi*, virgin males produce bigger spermatophores than already mated males, resulting in longer periods of female unreceptivity (Kaitala & Wiklund 1995), and preliminary results suggests that this is true also for *P. rapae* (P. A. Cook & N. Wedell, unpublished data). Sperm production is likely to pose a cost to males (Dewsbury 1982; Olsson *et al.* 1997), hence it may be advantageous for virgin males to reserve sperm for future matings when the risk of sperm competition is higher.

Butterflies often show a pronounced last male or first male sperm priority, with low degree of sperm mixing (Drummond 1984; Simmons & Siva-Jothy 1998). The mechanism by which this is achieved is not known. It has

been suggested that the cause of complete first male sperm priority is due to the second male failing to successfully transfer a spermatophore (Drummond 1984). As we dissected all females to ensure that both males had successfully transferred a spermatophore, this cannot explain the mating order effect in this study. A first male priority could also arise if first males' sperm fill the female's sperm storage organ, the number increasing with the remating interval due to the spermatheca becoming completely filled (Retnakaran 1974). Alternatively, if sperm are lost and/or used in fertilization, a longer time between matings can result in higher second male priority. As the remating interval was controlled in this study, these explanations do not apply. Male butterflies also do not have the possibility of removing previous males' ejaculates, because sperm storage is completely separate from the bursa copulatrix where the spermatophore is formed during mating. Bimodal distributions of paternity have been found in other lepidopteran species (e.g. LaMunyon & Eisner 1994; Svärd & McNeil 1994; Cook *et al.* 1997), and it has been argued that this suggests that females are exercising postcopulatory choice (LaMunyon & Eisner 1993; Eberhard 1996).

Relatively lighter spermatophore remains also correlate with paternity. There is no relationship with male body weight, hence the weight of the spermatophore remains do not appear to be related to the size of the spermatophore transferred at mating. There is also no effect of time since mating, suggesting that spermatophores are not being utilized at a constant rate. After mating the female ruptures the spermatophore by contracting the bursal muscle (Sugawara 1979). It is conceivable that by varying the pressure on the bursa, females influence the rate of spermatophore drainage. Smaller spermatophore remains may consequently represent spermatophores of favoured males. The rate of sperm transfer from the spermatophore to the spermatheca could increase the probability of these sperm being used for fertilization. Female butterflies may be able to make a more accurate assessment of male quality after copulation (Eberhard 1996). In the related species *P. napi*, females appear unable to detect male mating status and are just as likely to mate with already mated males (Kaitala & Wiklund 1995), even though they receive smaller nutrient donations resulting in lower fecundity (Wiklund *et al.* 1998). In the comma butterfly, females exercise postcopulatory choice by varying their reproductive investment in relation to amount of nutrients received from the male (Wedell 1996).

This study shows that males maximize their fertilization success by increasing the number of sperm delivered on their second mating, although the mechanism whereby high sperm number results in increased paternity is unknown. Females may also affect male fertilization success by varying spermatophore drainage. However, a possible causal relationship between sperm number and female postcopulatory choice remains to be explored.

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