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Butterflies tailor their ejaculate in response to sperm competition risk and intensity

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Males of many insects eclose with their entire lifetime sperm supply and have to allocate their ejaculates at mating prudently. In polyandrous species, ejaculates of rival males overlap, creating sperm competition. Recent models suggest that males should increase their ejaculate expenditure when experiencing a high risk of sperm competition. Ejaculate expenditure is also predicted to vary in relation to sperm competition intensity. During high intensity, where several ejaculates compete for fertilization of the female's eggs, ejaculate expenditure is expected to be reduced. This is because there are diminishing returns for providing more sperm. Additionally, sperm numbers will depend on males' ability to assess female mating status. We investigate ejaculate allocation in the polyandrous small white butterfly *Pieris rapae* (Lepidoptera). Males have previously been found to ejaculate more sperm on their second mating when experiencing increased risk of sperm competition. Here we show that males also adjust the number of sperm ejaculated in relation to direct sperm competition. Mated males provide more sperm to females previously mated with mated males (i.e. when competing with many sperm) than to females previously mated to virgin males (competing with few sperm). Virgin males, on the other hand, do not adjust their ejaculate in relation to female mating history, but provide heavier females with more sperm. Although virgin males induce longer non-receptive periods in females than mated males, heavier females remate sooner. Virgin males may be responding to the higher risk of sperm competition by providing more sperm to heavier females. It is clear from this study that males are sensitive to factors affecting sperm competition risk, tailoring their ejaculates as predicted by recent theoretical models.

Keywords: sexual selection; *Pieris rapae*; body size; mate assessment; sperm, apyrene and eupyrene; polyandry

1. INTRODUCTION

Females commonly invest more resources in reproduction than males, eggs being considerably larger than sperm, explaining why females are often the choosy sex. However, in some species males also make substantial contributions to reproduction, such as nutrient donations by male insects (Vahed 1998). Sperm production itself can also pose a considerable cost to males and be limited in supply (Dewsbury 1982; Nakatsuru & Kramer 1982; Olsson *et al*. 1997). To optimize the number of offspring sired, males should allocate resources to ejaculate production prudently, both across successive matings, as well as within a single mating. Theory predicts that a high sperm number is advantageous in sperm competition (Parker 1982, 1990*a*). In polyandrous species, where sperm competition is prevalent, it is therefore advantageous for males to assess female mating history and vary the number of sperm provided accordingly.

Theoretical models predicting optimum ejaculate allocation within species show it is dependent on males' knowledge of the risk of sperm competition occurring (Parker 1990*b*; Parker *et al*. 1997). This also has empirical support. Males of several species are sensitive to the risk of sperm competition, increasing the number of sperm produced when there is a high probability of competition occurring. For example, males ejaculate more sperm in the presence of rival males (e.g. Gage 1991; Gage & Baker 1991; Gage & Barnard 1996), when the probability of encountering already mated females is higher (e.g. Baker & Bellis 1989; Bellis *et al*. 1990; Simmons *et al*. 1993; Cook & Wedell 1996), or in high-density populations (Gage 1995; He & Miyata 1997). Males also appear to be capable of discriminating between virgin and mated females in some species, providing mated females with more sperm (Cook & Gage 1995; Wedell 1998).

Across species, ejaculate expenditure is expected to increase with increasing degree of sperm competition (Parker 1982, 1998), a prediction which is corroborated by several animal groups (e.g. Harcourt *et al*. 1991; Møller 1991; Gage 1994; Stockley *et al*. 1997). However, within species the reverse may apply. Recent models suggest that males should not only respond to the risk of sperm competition (i.e. the probability of sperm competition occurring), but also vary their ejaculate expenditure depending on the intensity of sperm competition (Ball & Parker 1996, 1997; Parker *et al*. 1996). Sperm competition intensity increases as the number of ejaculates competing for a given set of eggs increases (Parker *et al*. 1996).

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During high sperm competition intensity, when ejaculates of several males compete for fertilization of the female's eggs, males are predicted to decrease their ejaculate expenditure. In general, males are predicted to increase ejaculate expenditure in response to sperm competition, but when the number of males increases above two, ejaculate expenditure per male gradually decreases (Ball & Parker 1996, 1997; Parker *et al*. 1996). This is because the advantage to an individual male of producing high sperm numbers decreases as the total number of competing sperm increases.

Females may also vary in quality in other respects than their mating history, influencing males' ejaculate expenditure. For example, in species where female fecundity covaries with body size (e.g. Simmons 1986), larger females may require more sperm to fertilize all their eggs (Shapiro *et al*. 1994). It will be advantageous for males to provide more sperm to larger females, since the potential gains are higher. It has been suggested that larger females have a larger reproductive tract, making transfer of high sperm numbers to fill it advantageous (Baker & Bellis 1995; Gage 1998). Alternatively, female body size may correlate with risk of sperm competition, if female mating frequency varies depending on their body size (Simmons & Kvarnemo 1997; Gage 1998).

Male small white butterflies, *Pieris rapae* (Lepidoptera: Pieridae), produce a spermatophore at mating containing two types of sperm and nutrients. The majority of the sperm are anucleate, apyrene sperm that do not fertilize the female's eggs (Cook & Wedell 1996). Males vary the size of the spermatophore produced and the number of eupyrene and apyrene sperm transferred depending on their mating status. Previously mated males produce spermatophores of about half the size of the spermatophore they produced as virgins, but with twice the number of sperm. This has been interpreted as a strategy by males to maximize their fertilization success when experiencing an increased risk of sperm competition (Cook & Wedell 1996). On their second mating, the probability of encountering non-virgin females is higher (N. Wedell, P. A. Cook and C. Wiklund, unpublished data). Hence, it may be advantageous to provide these females with large numbers of sperm and reduced nutrient donations. Mated males have higher fertilization success in competition with virgin males' ejaculates, suggesting that high sperm number is advantageous in sperm competition in this species (Wedell & Cook 1998). In this study we examine whether males respond to the direct risk of sperm competition as determined by the mating status of females—either virgin or previously mated. We also investigate the influence of intensity of sperm competition, using two levels of sperm competition intensity: (i) low intensity, as experienced by males mating to females previously mated to a virgin male that provided few sperm, and (ii) high intensity, as experienced by males mating to females previously mated to a mated male receiving many sperm (equivalent to two virgin males ejaculates). The sperm competition intensity models were developed for a situation where males compete with ejaculates of two or more males, whereas this study examines a two-male competitive situation. However, since mated males provide sperm numbers equivalent to two matings by virgin males, this situation might be analogous to competition between several ejaculates and therefore of high intensity (G. A. Parker, personal communication). Sperm have equal probability of fertilization in the models, so it is the proportion of sperm in relation to total sperm number that is important. Therefore, we examine the effect of number of ejaculates (i.e. the number of sperm) a male competes with, rather than the number of competing males. Finally, we investigate whether female quality, in terms of body size, affects male ejaculate expenditure. The results are discussed in relation to predictions arising from models of both sperm competition risk (increased ejaculate expenditure under high risk) and intensity (reduced ejaculate expenditure under high intensity).

2. METHODS

Larvae of *P. rapae* were reared on garlic mustard, *Alliaria petiolata*, as previously described (Cook & Wedell 1996; Wedell & Cook 1998). Emerging individuals were weighed on day of eclosion and given a unique mark on the wing with a permanent marker pen. Matings 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. 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 allowed to mate again for up to three days after which they were removed from the experiment. Females, mated to either virgin or mated males, were placed in cages, provided with flowers, sugar solution, garlic mustard leaves for egg laying, and virgin or mated males, and allowed to mate for a second time. The duration of the female refractory period between first and second mating was recorded. We generated two treatment groups. When investigating the effect of female mating status on the ejaculate allocation of virgin males, females were either mated once to a virgin male V ($n = 21$), once each to two virgin males V–V ($n = 25$), or to a mated and a virgin male M–V ($n = 18$). Similarly, when investigating the effect of female mating status for mated males, females were either mated once to a mated male M ($n = 14$), a virgin and a mated male V–M ($n = 12$), or once each to two mated males M–M ($n = 22$).

Singly and doubly mated females were decapitated and dissected within 20 min of termination of copulation, before sperm migration from the spermatophore takes place. To obtain spermatophore mass, the bursa copulatrix was dissected out and the intact spermatophore carefully removed and weighed to the nearest 0.01 mg on a Cahn electrobalance. The intact spermatophore was placed on a drop of modified Barth saline (Gurdon 1991) on a cavity slide. The sperm-containing ampulla was ruptured with a fine needle and gently stirred to ensure full dispersal of the sperm. The total number of eupyrene sperm bundles were counted at ×40 magnification and the number multiplied by 256 (the number of individual sperm per bundle) to give the total number of eupyrene sperm (Cook & Wedell 1996). To count apyrene sperm, the sample was washed from the slide with Barth saline into a 30 ml specimen tube and diluted with distilled water. The sample was gently agitated to disperse sperm. Six 10 µl subsamples were removed from each sample with an autopipette and allowed to dry on slides under dust covers. The dry slides were dipped for about 3 s in distilled water to dissolve salt crystals and dried again. The dried samples were examined using dark-field phase contrast microscopy at ×100 magnification and all the apyrene sperm in each subsample counted. The total number of apyrene sperm per

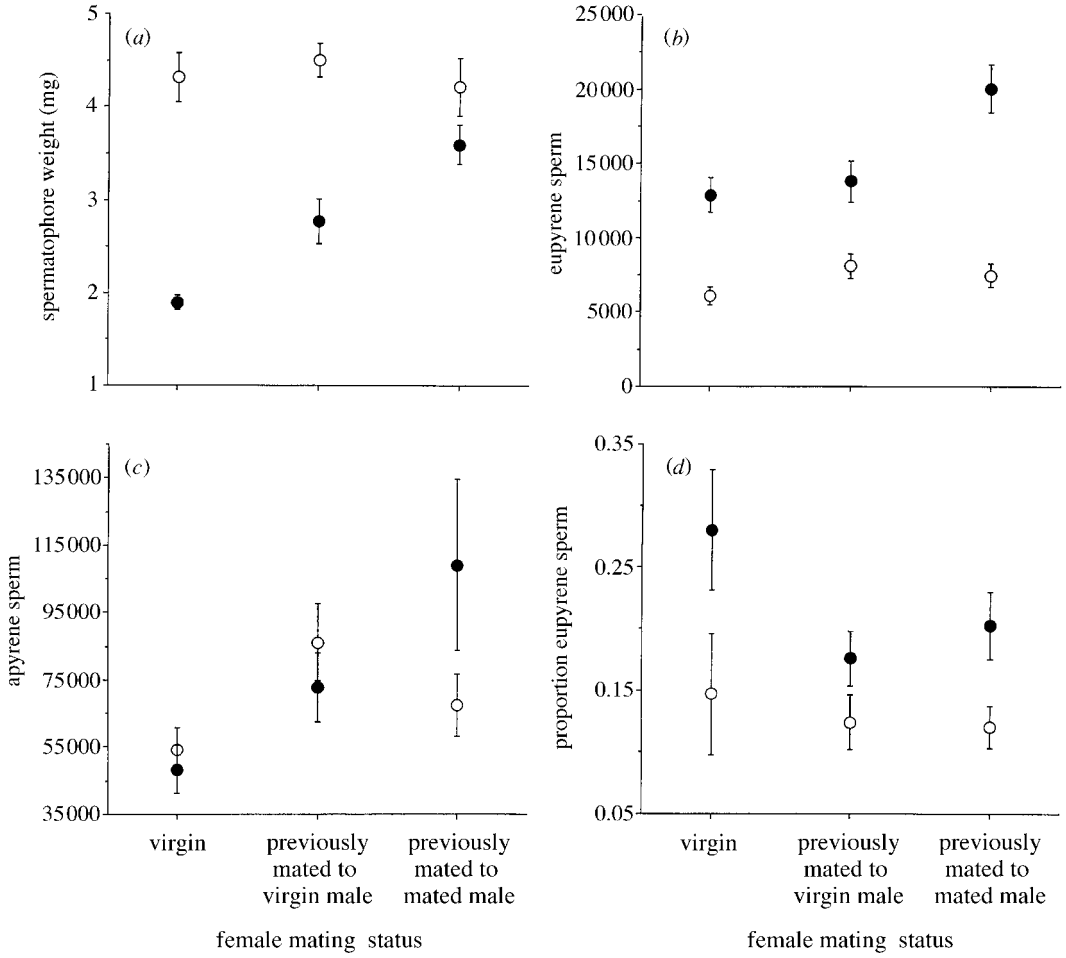

Figure 1. Ejaculate characteristics of males mated to females of varying mating histories. Open circles represent virgin males and black dots mated males. Means ± s.e. Virgin males do not vary their spermatophore characteristics depending on female mating status. Mated males, on the other hand, vary (*a*) spermatophore size, (*b*) eupyrene and (*c*) apyrene sperm number, but not (*d*) proportion of eupyrene sperm transferred depending on female mating history.

spermatophore were calculated by multiplying the average number of the six 10 µl sperm counts by its dilution factor. The number of eupyrene and apyrene sperm in spermatophores from virgin and mated males mated to either virgin or mated females were counted in the same way.

All males mated within three days of emergence and rematings took place within three days of the first mating. There were no differences in age of males with treatment for either virgin males (ANOVA: $F_{2,61}=0.855$, $p>0.4$) or mated males ($F_{2,45}=1.417$, $p>0.2$). There were no significant differences in female or male body weight with treatment for either virgin males (ANOVA: female weight $F_{2,61}=0.137$, $p>0.8$; male weight $F_{2,61}=2.297$, $p>0.1$) or mated males (ANOVA: female weight $F_{2,45}=1.896$, $p>0.1$; male weight $F_{2,45}=1.949$, $p>0.1$). However, virgin male body weight was significantly correlated with female body weight ($r=0.42$, $p=0.0005$, $n=64$), whereas no such correlation existed for mated males ($r=0.10$, $p>0.5$, $n=48$). We therefore used multiple correlations to investigate the effect of male and female body weight on spermatophore characteristics for virgin males. Sperm numbers were log transformed to improve normality and proportions square-root arcsine transformed before being used in analyses. Results are presented as means ± s.e.

3. RESULTS

(a) *Female mating history*

Virgin males did not vary the size of spermatophore (ANOVA: $F_{2,61}=0.372$, $p>0.6$), the number of eupyrene (ANOVA: $F_{2,61}=1.084$, $p>0.3$) or apyrene sperm (ANOVA: $F_{2,61}=1.797$, $p>0.1$), or the proportion of eupyrene sperm produced (ANOVA: $F_{2,61}=0.298$, $p>0.7$) depending on the mating status of the female or the female's first mate (figure 1). Mated males, on the other hand, varied both the size of spermatophore and the number of sperm produced in relation to the females' mating history (figure 1). Mated males provide larger spermatophores to females previously mated to mated males (ANOVA: $F_{2,45}=15.000$, $p=0.0001$; figure 1*a*) compared to males mating with virgin females (post-hoc Bonferroni-adjusted $p=0.0001$). Similarly, mated males provide larger spermatophores when mating to females mated to virgin males compared to virgin females (post-hoc Bonferroni $p=0.033$). They increase the number of both eupyrene (ANOVA: $F_{2,45}=5.950$, $p=0.005$; figure 1*b*) and apyrene sperm produced (ANOVA: $F_{2,45}=5.093$, $p=0.01$; figure 1*c*). Males mating with females previously mated to

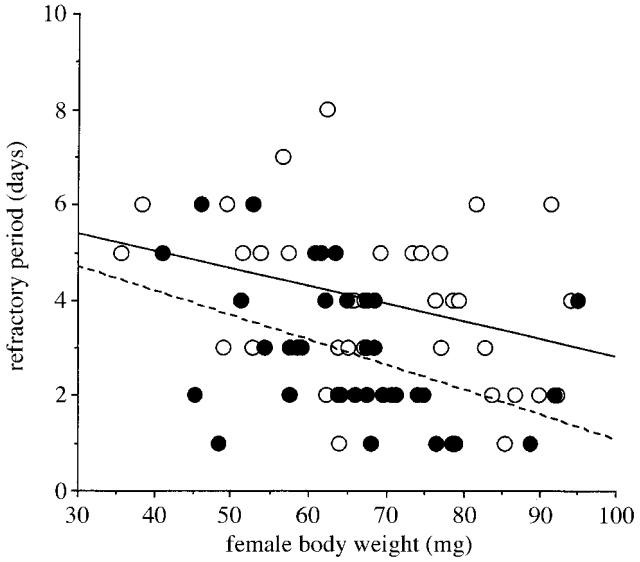

Figure 2. Duration of female refractory period in relation to female body size. Heavier females display shorter periods of sexual unreceptivity. This is true for females mated to both virgin males (open circles and solid line; regression $y = -0.037x + 6.512$) and mated males (black dots and broken line; $y = -0.052x + 6.276$), even though females mated to virgin males have longer refractory period than females mated to non-virgin males.

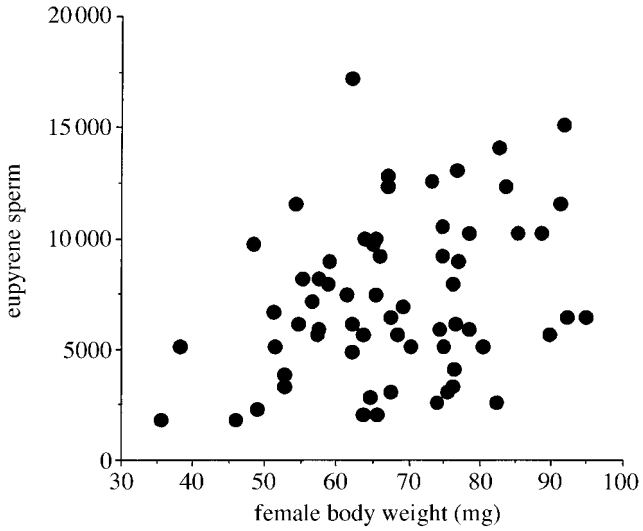

Figure 3. Number of eupyrene sperm provided by virgin males to females of increasing body weight. Virgin males provide heavier females with more eupyrene sperm.

mated males provide more eupyrene sperm than males mating either with females previously mated to a virgin male (post-hoc Bonferroni, $p = 0.03$) or virgin females (post-hoc Bonferroni, $p = 0.011$). Males also provide females mated to mated males with more apyrene sperm than to virgin females (post-hoc Bonferroni, $p = 0.009$). However, there is no effect of female mating status on the proportion of eupyrene sperm transferred (ANOVA: $F_{2,45} = 2.299$, $p > 0.1$; figure 1*d*).

(b) *Female quality*

Females mated to virgin males display longer periods of sexual unreceptivity (3.97 ± 0.27 days) than females mated to non-virgin males (2.89 ± 0.25 days; ANOVA: $F_{1,71} = 8.928$, $p = 0.0039$). This may be due to virgin males producing larger spermatophores than mated males (figure 1*a*). However, the duration of the female refractory period was also influenced by female body weight; larger females remate sooner. This was true for both females mated to virgin ($r = -0.34$, $p = 0.042$, $n = 37$) and non-virgin males ($r = -0.43$, $p = 0.008$, $n = 36$; figure 2). Moreover, virgin males, irrespective of female mating status, provide heavier females with more eupyrene sperm (multiple $r = 0.41$, $p = 0.0034$, $n = 64$; male weight partial $r = 0.22$, $p = 0.09$; female weight partial $r = 0.27$, $p = 0.034$; figure 3). Bigger males produce heavier spermatophores, but there is no effect of female body weight on spermatophore size (multiple $r = 0.25$, $p > 0.1$, male weight partial $r = 0.28$, $p = 0.0458$; female weight partial $r = -0.10$, $p > 0.4$) or the number of apyrene sperm transferred (multiple $r = 0.19$, $p > 0.3$, male weight partial $r = 0.01$, $p > 0.9$; female body weight partial $r = 0.18$, $p > 0.1$). There was no significant effect of either male or female body weight on spermatophore weight (ANCOVA: male weight $F_{2,43} = 1.268$, $p > 0.2$; female weight $F_{2,43} = 3.223$, $p = 0.080$), eupyrene (ANCOVA: male weight $F_{2,43} = 2.628$, $p > 0.1$; female weight $F_{2,43} = 0.677$, $p > 0.4$) or apyrene sperm number (ANCOVA: male weight $F_{2,43} = 2.166$, $p > 0.1$; female weight $F_{2,43} = 0.332$, $p > 0.5$) for previously mated males.

4. DISCUSSION

Male *P. rapae* assess female mating history and adjust the number of sperm ejaculated accordingly. During mating the male's genitalia enters through the female's copulatory opening and forms the spermatophore in the receptacle (bursa copulatrix). In most Lepidoptera, whilst the sperm migrate to the spermatheca, spermatophore residues from previous matings remain in the female's bursa throughout her life (Drummond 1984). The mating male may directly determine the mating history of the female, being in genital contact with potential spermatophore residues during spermatophore formation. Virgin males produce significantly larger spermatophores than mated males but provide fewer sperm (Cook & Wedell 1996). This is also reflected in the size of spermatophore residues (Wedell & Cook 1998). Males may therefore have the opportunity to assess not only the mating status of the female, but also that of her previous mate. Small residual spermatophores in the bursa (originating from mated males) indicate the presence of high sperm numbers, whereas large spermatophore residues (originating from virgin males) indicate fewer sperm to compete with. Males may therefore be able to determine not only that their sperm will be in competition, but also the intensity of sperm competition in terms of many or few sperm. Males of polyandrous species do not ejaculate all available sperm in one mating (Wedell & Cook 1999), instead retaining sperm in a storage organ (duplex), enabling them to tailor the number ejaculated in relation to female mating history.

Recent theoretical models predict the optimal ejaculate expenditure by individual males in relation to their

assessment of the risk of sperm competition occurring (Parker 1990*a*,*b*, 1993; Parker *et al*. 1997; Ball & Parker 1998). The models assess situations with a fair raffle, where fertilization probability increases with the proportion of the male's sperm in competition (e.g. sperm mixing), and a loaded raffle, where males have unequal probability of success in competition (e.g. a first- or second-male sperm precedence). In general, these models predict that, across species, ejaculate expenditure should increase with increasing probability of sperm competition, but decrease with degree of unfairness of the raffle, and if males occupy roles non-randomly, males in favoured roles should produce fewer sperm. Within a species, the number of sperm ejaculated also critically depends on the information available to the two competitors. Males should ejaculate more sperm when the risk of sperm competition is higher. Irrespective of the probability of being the first or second male to mate with the female, males should spend equal effort on sperm production in a fair raffle, assuming that roles are occupied randomly. In a loaded raffle, if males occupy roles non-randomly, the male most likely to occupy the unfavoured role should compensate by increasing his ejaculate expenditure (e.g. Stockley & Purvis 1993; Gage *et al*. 1995). In a two-male competitive situation where both males have perfect knowledge, ejaculate expenditure should increase with increasing sperm competition risk (Parker *et al*. 1997; Ball & Parker 1998). If males can distinguish high- and low-risk females (i.e. mated from virgin females), they should increase their ejaculate expenditure when mating with non-virgin females (Parker *et al*. 1997). *P. rapae* males seem capable of distinguishing female mating history, with mated males providing more sperm to females previously mated to a mated male. Similarly, in the moth *Plodia interpunctella*, males produce bigger ejaculates when the level of sperm competition is higher (Cook & Gage 1995).

Male *P. rapae* vary the number of sperm provided to females depending on her mating history and body size. However, virgin males allocate their sperm differently from that of previously mated males. Males provide large spermatophores on their first mating when they may have a higher probability of mating with virgin females (Cook & Wedell 1996). Large spermatophore size induces longer periods of sexual unreceptivity (figure 2), during which time females lay eggs exclusively fertilized by that male. Larger spermatophores result in more eggs sired since larger spermatophores induce longer refractory periods. Virgin males thus have the favoured role: they have a high probability of mating with virgin females and induce longer refractory periods, resulting in many fertilized eggs. Mated males, on the other hand, have a higher probability of mating with non-virgin females, induce shorter refractory periods and may compensate by increasing sperm numbers resulting in higher fertilization success in sperm competition (Wedell & Cook 1998).

Previously mated males tailor their spermatophores, reserving eupyrene sperm for future matings, even when remating within an hour of their first copulation (Wedell & Cook 1999), and provide more sperm to females previously mated to mated males (figure 1). This may result in maximization of their fertilization success. Although the function of apyrene sperm remains elusive for most butterfly species, the fact that mated males provide females previously mated to non-virgin males with more apyrene sperm than virgin females, suggests they play a role in sperm competition (Silberglied *et al*. 1984; Cook & Wedell 1999). There is a second male sperm priority in *P. rapae*. However, mated males have higher fertilization success when competing with virgin males' ejaculates, even when mating in the first role. When competing with the ejaculate of a mated male, there is a strong second male advantage (Wedell & Cook 1998), suggesting that increasing sperm numbers result in higher fertilization success. However, the mechanism whereby the second male achieves high paternity is not known, it may be due to high sperm numbers or, in part, sperm displacement and female post-copulatory choice (Sakaluk & Eggert 1996).

Recent models examining males' optimal ejaculate expenditure predict that males should at times decrease sperm number under high intensity of sperm competition (Ball & Parker 1996, 1997; Parker *et al*. 1996). In *P. rapae*, mated males instead increase the number of sperm ejaculated under high sperm competition intensity (i.e. when mating to females previously mated to mated males). However, until an explicit model is constructed for the *P. rapae* case, it is not yet certain that males should show an analogous reduction in expenditure; much will depend on the frequencies of the different mating circumstances. The further reason for this apparent discrepancy may be explained by the mechanism of sperm competition. The original models predicting the optimal sperm number in relation to sperm competition intensity were developed for external fertilizers where males gain equal proportion of fertilizations according to the raffle principle (Parker *et al*. 1996), whereas in *P. rapae* there is a strong mating order effect (Wedell & Cook 1998). It is also likely that induction of a refractory period influences males' ejaculate expenditure. High numbers of eggs fertilized can be achieved by inducing a refractory period and not only by males' success in sperm competition.

Female mass affects female remating behaviour: heavier females remate sooner. This may be because heavier females have more of their eggs mature and ready to be oviposited. Males provide more sperm to larger females, either because they may be more fecund or because sperm numbers may be related to spermathecal volume if larger females have bigger sperm storage (Gage 1998). Alternatively, virgin males may respond to the higher risk of sperm competition by increasing eupyrene sperm number, since larger females remate sooner. This differs from the bushcricket *Kawanaphila nartee*, where female body size also covaries with risk of sperm competition, but males provide fewer sperm to larger females (Simmons & Kvarnemo 1997). In *K. nartee*, although fertilization is internal, sperm mix and are used numerically in a fair raffle (Simmons 1995), possibly explaining the difference between these two species.

It has previously been shown that male *P. rapae* respond to increased probability of sperm competition by ejaculating more sperm on their second mating (Cook & Wedell 1996). Here we show that males also adjust the number of sperm in relation to direct sperm competition by increasing the number of sperm under high sperm competition intensity (i.e. when mating to females

previously mated to non-virgin males that provided many sperm). Virgin males do not adjust their ejaculate in relation to female mating history, but provide heavier females with more sperm. Virgin males may be responding to the higher risk of sperm competition by providing more sperm to heavier females. Although virgin males induce longer refractory periods in females than mated males, heavier females remate sooner. Alternatively, larger females may require more sperm to fertilize all her eggs. It is clear from this study that males are sensitive to factors affecting sperm competition risk and intensity, tailoring their ejaculates as predicted by recent sperm competition models in order to maximize their lifetime reproductive success.

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