

Sexual conflict and anti-aphrodisiac titre in a polyandrous butterfly: male ejaculate tailoring and absence of female control

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Males of the green-veined butterfly *Pieris napi* synthesize and transfer the volatile methyl salicylate (MeS) to females at mating, a substance that is emitted by non-virgin females when courted by males, curtailing courtship and decreasing the likelihood of female re-mating. The volatile is released when females display the 'mate-refusal' posture with spread wings and elevated abdomen, when courted by conspecific males. Here, we assess how the amount of MeS released by courted females changes over time since mating, and whether it is influenced by the frequency with which females display the mate-refusal posture. We also assess whether males tailor the anti-aphrodisiac content of ejaculates with respect to the expected degree of sperm competition, by comparing how males allocate MeS proportionately to first and second ejaculates in relation to ejaculate mass. The results show that females housed for 5 days in individual cages where they were able to fly and oviposit normally, released similar amounts of MeS. However, females housed together for the same period of time, causing them to frequently display the mate-refusal posture, released significantly lower levels of MeS than the individually housed females. This indicates that female display of the mate-refusal posture depletes their anti-aphrodisiac stores, and suggests that females are unable to voluntarily control their release of the anti-aphrodisiac. A comparison of relative proportion of MeS transferred by males in their first and second ejaculates showed that proportionately more MeS was allocated to the first ejaculate, in accordance with the idea that these are tailored to delay female re-mating.

Keywords: Pieridae; pheromone; methyl salicylate; solid-phase micro-extraction; sexual selection; anti-aphrodisiac

1. INTRODUCTION

Butterfly mating systems vary from monogamy to polygamy (Drummond 1984; Wiklund & Forsberg 1991; Gage 1994). In butterflies, the mass of the ejaculate transferred from the male to the female at mating increases with female polygamy. There is evidence that the ejaculate functions as a nuptial gift, allowing females to increase their reproductive output (Boggs & Gilbert 1979; Wiklund *et al.* 1993, 1998; Kaitala & Wiklund 1994; Karlsson 1998). Regardless of female re-mating frequency, male butterflies seem incapable of forcing matings on females, and female receptivity is a prerequisite to successful male courtship (Svärd & Wiklund 1989). Hence, although last male sperm precedence is the rule in butterflies, as in most insects (Parker 1970; Drummond 1984; Bissondath & Wiklund 1997; Wedell & Cook 1998), males gain little from courting unreceptive females. In butterflies, female receptivity is usually signalled by a combination of visual and olfactory signals, visual stimuli being more important at a distance and olfactory stimuli being more important close up (Silberglied 1984). In the Pieridae, both virgin and mated females exhibit an identical posture when courted, usually referred to as the 'mate refusal' posture (Obara 1964), spreading the wings horizontally and lifting the abdomen

vertically, a posture well designed for emitting chemical stimuli close to the location of the hovering courting male (Wiklund & Forsberg 1985; Forsberg & Wiklund 1989). Although there is little information on the actual chemicals involved in the signalling of female receptivity and unreceptivity, there is abundant evidence that males distinguish between receptive virgin females and unreceptive mated females. Virgin females are courted intensively, but unreceptive mated females are quickly abandoned (Wiklund & Forsberg 1985; Forsberg & Wiklund 1989). Because a recently mated female gains from becoming unattractive, and mating males gain from inducing female monogamy, sexual selection should select for male capability to induce female unreceptivity and unattractiveness. Hence, Gilbert (1976) suggested that males of *Heliconius* butterflies transferred anti-aphrodisiacs to females at mating. Recently, Andersson *et al.* (2000, 2003) demonstrated the existence of such a male-contributed anti-aphrodisiac system in *Pieris napi*, *P. brassicae* and *P. rapae*. In these three *Pieris* species, males synthesize identified volatiles which are transferred to females at mating, and which render females unattractive to other males, thereby substantially lowering the female rate of re-mating. Although all three *Pieris* species use the same principal system of male synthesis and transfer of an anti-aphrodisiac, the chemical identity differs between species, with *P. napi* using methyl salicylate (MeS), *P. brassicae* using benzyl cyanide and

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P. rapae using a blend of MeS and indole as anti-aphrodisiacs (Andersson *et al.* 2000, 2003).

The unattractiveness of recently mated females benefits both the male and the female (Forsberg & Wiklund 1989). However, this mutual interest between a female's first mate and the female could turn into conflict over time if the female could later benefit from mating a second time. In this case, a high titre of anti-aphrodisiac could reduce a female's chance of attracting a new mate: recently mated *P. napi* females under natural conditions are usually abandoned by courting males within a second or two (Forsberg & Wiklund 1989). To assess the degree to which males are sensitive to small differences in the amount of MeS released by females, and to study how a female's attractiveness changes over time since mating, we studied the duration of male courtship of female *P. napi* in the laboratory in relation to the time passed since mating.

Given that female attractiveness to males is strongly related to the amount of MeS they emit when courted, this raises the question of whether females can exercise any control over their release of anti-aphrodisiacs, or whether the amount released is directly proportional to the titre in their stores. To answer this question it is necessary to establish whether the anti-aphrodisiac breaks down over time, if the anti-aphrodisiac is released during normal female behaviour such as flight and egg-laying leading to a decreased titre, or if anti-aphrodisiac titre decreases as a result of females performing the mate-refusal posture. To answer this question we compared the release of MeS from females on two occasions, on the day after mating and 6 days after mating, for females that (i) were kept inactive and hence had no opportunity to release the anti-aphrodisiac, (ii) were allowed to feed and lay eggs but not to adopt the mate-refusal posture, and (iii) were allowed to feed, lay eggs and adopt the mate-refusal posture.

A third objective was to see whether males practise strategic anti-aphrodisiac transfer in a way similar to strategic sperm allocation in *P. rapae* (Wedell & Cook 1999). In butterflies males transfer two types of sperm: fertilizing (eupyrene) sperm and non-fertilizing (apyrene) sperm, with apyrene sperm representing up to 90% of the total sperm number (Cook & Wedell 1996). Although the function of apyrene sperm has been under considerable debate, Cook & Wedell (1999) have demonstrated that they function as cheap filler sperm and delay female re-mating. Wedell & Cook (1999) further demonstrated that males of *P. rapae* tailor their ejaculates in response to sperm competition risk and intensity, by showing that a male's first ejaculate is designed to delay female re-mating and contains a higher proportion of apyrene sperm, whereas a male's second ejaculate is designed for sperm competition and contained a higher proportion of eupyrene sperm. The degree of polyandry is very similar in *P. napi* and *P. rapae*, the number of spermatophores in wild-caught females being 2.03 and 2.13, respectively (Svärd & Wiklund 1989), and so a similar reasoning when applied to strategic anti-aphrodisiac allocation should lead to the prediction that a male's first ejaculate should be designed for delaying female re-mating (and so contain a higher proportion of anti-aphrodisiac), whereas delaying an already mated female's re-mating should be less imperative (and so a male's second ejaculate should be expected to contain proportionately less anti-aphrodisiac). To answer this question

we allowed female *P. napi* to mate with males of one of two histories: (i) virgin males that transfer large first ejaculates; or (ii) males that had mated the previous day (that transfer small ejaculates), and then comparing female release of MeS in relation to the size of the ejaculate received.

2. METHODS

To assess the degree to which males are sensitive to small differences in the amount of MeS released by females, and how a female's attractiveness changes over time since mating, we studied the duration of male courtship of female *P. napi* in the laboratory in relation to the time passed since mating. The experiment was performed in a 50 cm × 50 cm × 50 cm butterfly cage in which a single female and a single male was admitted. Male *P. napi* perform aerial courtship during which they approach the female and spend a variable amount of time hovering a few centimetres behind the sitting female. When approached, the female initially performs the mate-refusal posture, and when the courted female is a virgin she usually terminates the display while the male is still hovering behind her, folding her wings and acquiescing: a posture that signals mate acceptance. When the approached female has already mated, she also adopts the mate-refusal display and continues displaying until the male disappears. Courtship duration was measured to the nearest second by a stopwatch and was defined as the time from the female's initial adoption of the mate-refusal display until the female closed her wings. The attractiveness of each of eight females was assessed on seven occasions, first when the female was virginal and then later the same day, and subsequently 1, 2, 6, 7 and 12 days after mating. On each occasion the attractiveness of each female was calculated as the mean of the courtship duration by five different males that were allowed to interact with the female, except for the virgin females whose attractiveness was calculated as the mean courtship duration by one or more males until the female mated.

We compared MeS release by three groups of *P. napi* females to answer two questions. First, to assess whether females can control their release of anti-aphrodisiacs, or whether the amount of anti-aphrodisiac released is directly proportional to the titre in their stores. Second, to assess whether the anti-aphrodisiac breaks down with time or whether its titre decreases in proportion to the frequency of female displaying the mate-refusal posture. Each group consisted of five females, and each female was allowed to mate with a virgin male. Females were analysed (see § 2a) and concentration of MeS was assessed on two occasions, first on the day following mating (day 1) and later after 5 days (day 6). The three treatment groups were maintained as follows: (i) females were kept individually in a dark box at 5 °C (these females remained inactive throughout the 5 day period); (ii) females were maintained individually in 11 plastic jars with a gauze top and allowed to fly, feed on nectar, and lay eggs on their natural host plant *Alliaria petiolata* (these females were never observed to adopt the mate-refusal posture); or (iii) five females were kept together in a 11 plastic jar with a gauze top and allowed to fly, nectar and oviposit (these females were observed to perform the mate-refusal posture with great frequency: females perform this behaviour when approached physically by either male or female conspecifics).

To assess whether male *P. napi* tailor their ejaculates in accordance with predictions from sperm competition theory and transfer proportionately more MeS in their first ejaculates, we compared the amount of MeS transferred by male *P. napi* in their first and second ejaculates, respectively, as follows: nine virgin males were

first mated with virgin females and the following day the nine males were allowed to mate a second time with a new set of virgin females. The females were analysed (see § 2a) and concentration of the anti-aphrodisiac MeS was assessed as described below.

(a) Sampling technique

To assess the differences in concentration of the anti-aphrodisiac among individual females in the groups of females, we used solid-phase micro-extraction (SPME) technique (see § 2b), and analysed all SPME samples on a coupled gas chromatograph–mass spectrometer (GC–MS). To identify the odour emitted by females, we used a glass cylinder (height: 18 cm, diameter: 8 cm, $V = 905 \text{ cm}^3$) with one small opening and one SPME fibre holder placed in the opening, the sample time was 1 h for the fibre. One female was kept together with two males in the jar each time, and the total time for the female displaying the mate refusal posture was measured, as a measurement of female releasing the volatile anti-aphrodisiac when courted by the two males.

(b) Analytical methods

Polydimethylsiloxane-divinylbenzene fibres (65 μm , Supelco, Stockholm, Sweden) were used to sample the volatiles from live butterflies (Borg-Karlson & Mozuraitis 1996). The SPME fibres were desorbed before each sampling by heating in the GC injector (250 °C for 10 min) with a He gas flow. Analyses were conducted on a Varian 3400 GC coupled to a Finnigan SSQ 7000 MS. A DB-WAX column (internal diameter: 0.25 mm, film thickness: 0.25 μm , length: 30 m) was used, programmed from 40 °C (held for 1 min) then 5 °C min^{-1} to 110 °C then with 10 °C min^{-1} to 200 °C (and held for 10 min), injector temperature 225 °C (splitless injection, 45 s) and He carrier gas 69 kPa. Identification of the compounds was made by comparison of retention times and mass spectra with authentic reference samples; MeS (98% (GC), Lancaster).

3. RESULTS

Females were courted for a longer time before mating than after mating (figure 1; ANOVA: $p < 0.001$, $F = 73.27$). One day after mating the females were significantly less attractive than ‘just mated’ females tested within hours of copula termination (figure 1; ANOVA: $p < 0.001$, $F = 12.77$), but there was no difference in attractiveness between ‘just mated females’ and females 2 days after copula termination (figure 1; ANOVA: $p = 0.85$, $F = 0.03$). However, female attractiveness gradually increased with time because mating and females were more attractive six, seven and 12 days after mating when compared with ‘just mated females’ (figure 1; ANOVA: $p < 0.001$, $F = 15.31$, $p < 0.001$, $F = 38.39$, $p < 0.01$, $F = 7.96$).

There was no difference in concentration of MeS between day 1 and day 6 for females that had spent the time period inactive at 5 °C (group 1) or actively flying, feeding and laying eggs at 25 °C (group 2). This indicates that MeS does not break down with time *per se*, and, moreover, that MeS titre remains intact over time also when females are actively flying and laying eggs. By contrast, there was a significant difference in concentration of MeS between day 1 and day 6 for females that had spent the time period actively flying, feeding, laying eggs and frequently adopting the mate-refusal posture (group 3) (Wilcoxon matched pairs test: $N = 8$; $Z = 2.52$; $p = 0.012$; figure 2). This indicates that the display of the mate-refusal posture and concomitant release of MeS lowers the titre in the female’s

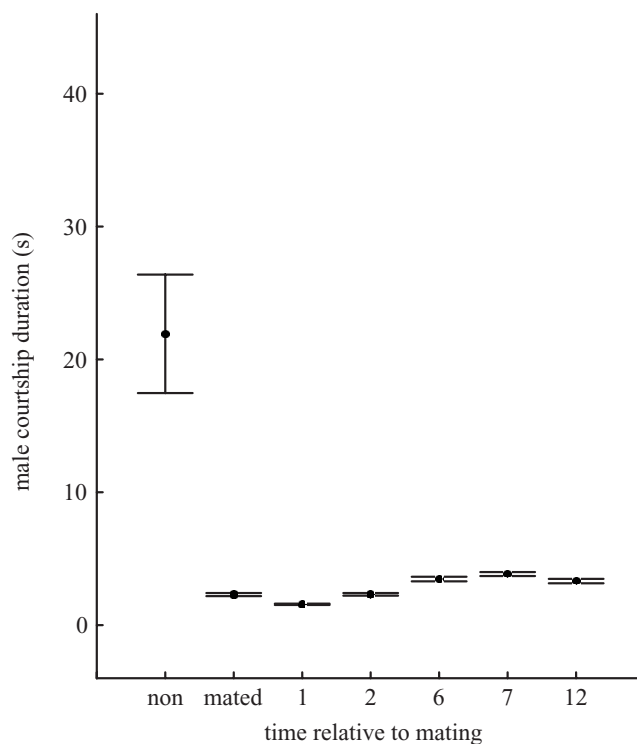


Figure 1. Duration of male courtship of eight females before mating (non) and mated females (mated); mated females were courted within 1 h after copula termination and later 1, 2, 6, 7 and 12 days after mating. Circles, mean; bars, \pm s.e.m.

stores, and that the amount of anti-aphrodisiac released by a female decreases in proportion to the frequency of female signalling the mate-refusal posture.

There was a significant difference in concentration of MeS between females mated with males that have mated for the first time and females mated with males that have mated for the second time (Wilcoxon matched pairs test: $N = 9$; $Z = 2.67$; $p = 0.008$; figure 3). Moreover, the difference in the amount of MeS released by females having received a male’s first ejaculate was, on average, 2.5 ± 0.5 (mean \pm s.e.m.; $n = 9$) times higher than that released by females that had received a male’s second ejaculate when the male re-mated on the day following his first mating. When comparing the mass of first and second ejaculates transferred by male *P. napi* when mating on two consecutive days, the mass of the first ejaculate was, on average, 1.5 ± 0.1 ($n = 10$) times larger (Bissondath & Wiklund 1996). A statistical comparison between the arcsine-transformed values of these proportions shows that the amount of anti-aphrodisiac in relation to ejaculate mass is higher in a male’s first ejaculate than in a male’s second ejaculate ($t = 2.16$, d.f. = 17, $p < 0.05$).

4. DISCUSSION

The observation that the amount of MeS released by females decreases significantly among females that actively adopt the mate-refusal posture, but not among females that do not adopt the posture, indicates that it is the displaying activity that depletes the stores of MeS (figure 2). The demonstration that female *P. napi* generally are most unattractive to males when recently mated, and later become more attractive in proportion to the time elapsed since

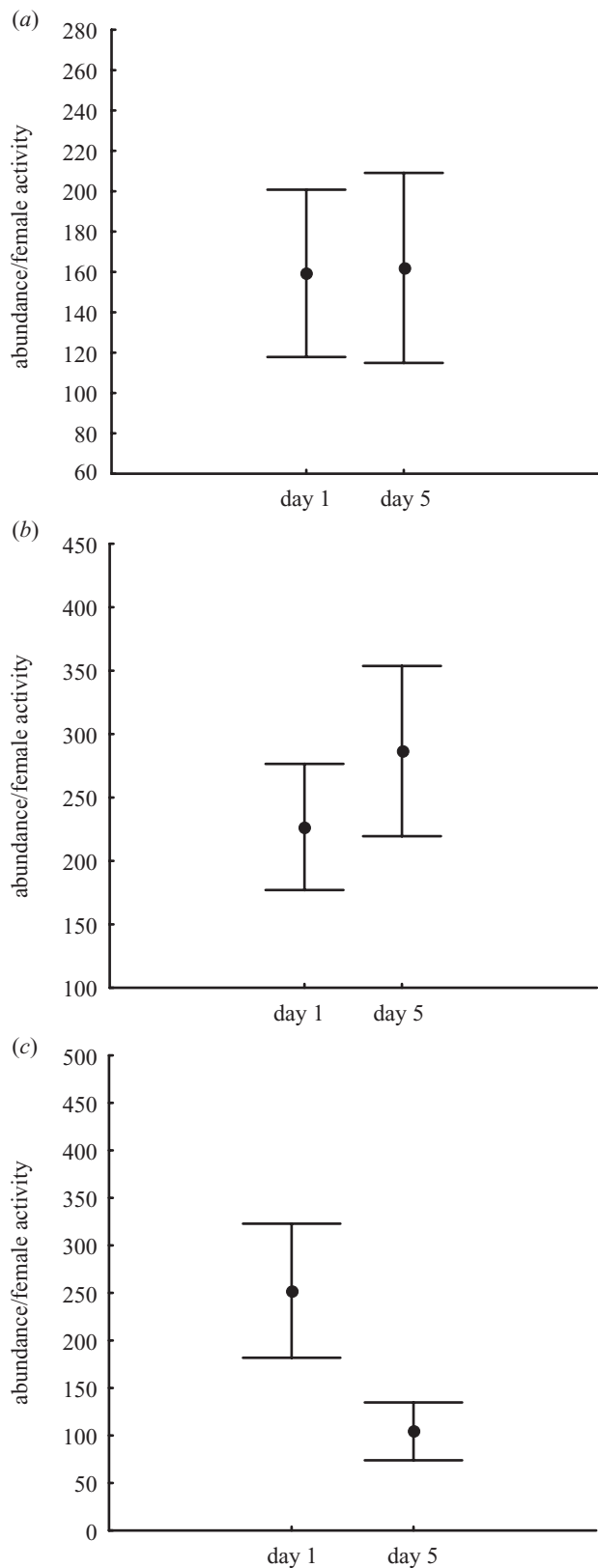


Figure 2. Amount of MeS released by females in relation to the duration of time females displayed the mate-refusal posture when bio-assayed on two occasions, on the day after mating (day 1) and later on day 5. (a) Group 1, which spent the whole time period inactive in darkness at 5 °C; (b) group 2, which spent the whole time period in individual 1 l jars being active but not displaying the mate-refusal posture; and (c) group 3, which spent the whole time period in the same 1 l jar being active and frequently displaying the mate-refusal posture. Circles, mean; bars, \pm s.e.m.

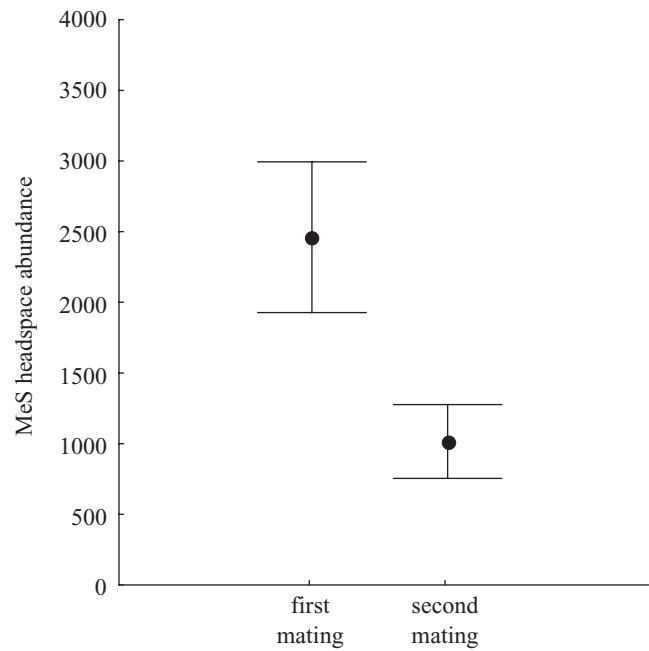


Figure 3. Total amount of MeS released by female *Pieris napi* after mating with a virgin male, receiving a male's first ejaculate; or mating with a non-virgin male that had mated the previous day, hence receiving a male's second ejaculate. Circles, mean; bars, \pm s.e.m.

mating, suggests that males are sensitive to small differences in titre of anti-aphrodisiac released by females (figure 1). This inference is based on the observation that, although the difference in duration of male courtship does not increase all that much during the 6 days following a female's first mating, most females do re-mate after between 4–5 days which indicates that their anti-aphrodisiac titre at that time does not discourage males from mating with these previously mated females (Kaitala & Wiklund 1994; Wiklund *et al.* 1998). Does this mean that females have control over the release of MeS, or is the amount released by females when courted out of their control and directly proportional to the amount in the female's stores? To answer this question it is necessary to know whether a female is receptive or not when a courted by a male. Because it is well established that female *P. napi* can increase their fecundity, egg size and longevity by mating multiply, and that females generally become receptive and re-mate between 3–7 days after their first mating (Wiklund *et al.* 1993, 1998; Kaitala & Wiklund 1994), the observation that females gradually become more attractive with time because mating seems to be in accordance with a female's best interests. However, although laboratory data clearly demonstrate multiple benefits of female polyandry (Karlsson 1998; Wiklund *et al.* 2001), spermatophore counts on wild-caught females show that some 12% of *P. napi* females mate only once in their lifetime and hence are effectively monandrous (Bergström *et al.* (2002), please note that this figure of 12% relates to spermatophore counts on females that show considerable wing-wear, hence excluding young females that may not have had time to re-mate). This variation in female propensity to re-mate is also displayed when female *P. napi* are kept under laboratory conditions and have constant access to courting males (Bergström & Wiklund 2002). Studies have indicated that

this variation has a heritable component, and that female *P. napi* display variation in propensity can be seen as variation in monandrous or a polyandrous lifestyle (Wedell *et al.* 2002). Therefore, it is difficult to be certain whether a female is receptive or not regardless of time elapsed since mating, which precludes the opportunity to assess whether females can control their release of anti-aphrodisiac.

In the absence of conclusive experiments, there is some evidence in accordance with the hypothesis that female release of MeS is a passive reflection of anti-aphrodisiac titre, and that the release of MeS is automatic when the female displays the mate-refusal posture. First, the mate-refusal posture comprises three distinct visual features: the lateral spreading of the wings, the raising of the abdomen (which prevents males from establishing genital contact), and the opening of the female genital valves (which presumably is the behaviour that allows the anti-aphrodisiac to be released). Although the mate-refusal display might be classified as ritualized, it is very dynamic and both the degree to which the abdomen is raised, and the degree to which the female genital valves are open, closely follow the position of a courting male with maximum abdomen raising and genital valve opening being performed only when the male is very close by. As soon as the male gets some 15 cm away from the female, she immediately lowers the abdomen and closes the genital valves. Hence, the raising of the abdomen and the opening of the abdomen seem to be two components of the same signal, and it appears that females do not ever show full mate-refusal display without simultaneously opening the genital valves.

The mate-refusal posture is not only exhibited by mated females, but also by virgin females when approached by a courting male. Andersson *et al.* (2000) have shown that the volatiles released by unmated females are fundamentally different from those released by mated females, and males react differently depending on what odour they perceive: when encountering a mated female that releases MeS the male abandons the female within seconds, but when perceiving the odour released by a virgin female the male continues courtship. When a virgin female is willing to mate she signals her receptivity by terminating the mate-refusal posture and closing her wings which almost invariably is followed by the male approaching the female, alighting beside her, and making genital contact. Andersson *et al.* (2000, 2003) have argued that the anti-aphrodisiac, by way of being synthesized by the male and being transferred by the male to the female at mating, functions as an honest signal which can economically be heeded by males because they are unable to force females to mate: hence a courting male approaching a mate-refusal signalling female releasing a strong odour of MeS may as well abandon the female and look for other females.

The crucial question is whether females can control their release of volatiles when displaying the mate-refusal posture or not: that is, are virgin females whose display behaviour invariably intensifies male courtship always receptive and willing to mate, and are previously mated females whose display behaviour usually curtails male courtship always unreceptive and unwilling to mate? One line of evidence that suggests the release of volatiles by females is an involuntary component of the mate-refusal display concerns the behaviour of virgin females that are unwilling to mate because they have been prevented experimentally

from feeding (Forsberg & Wiklund 1989). When such an unreceptive virgin female is released in the field she will invariably perform the mate-refusal posture when discovered by a male, but will not close their wings as a sign that she is receptive, which makes the male intensify his courtship until the female takes flight. During this flight the female will be followed arduously by the male, and the male pursuit can continue for several minutes, and is often punctuated by the female alighting, performing the mate-refusal posture, and then taking flight again (Forsberg & Wiklund 1989; C. Wiklund, personal observation). If it were the case that these virgin females could avoid releasing any volatile, it is conceivable that the courting male would mistake the female for a non-conspecific butterfly and curtail courtship. But, our experiments suggest that both virgin and non-virgin females invariably do release volatiles when performing the mate-refusal posture, suggesting that female release of volatiles during the display is automatic and hence out of their control.

This leads on to the issue of male–female conflict. If it is the case that females are unable to control their release of anti-aphrodisiacs, female opportunity to re-mate could be dependent on their titre of anti-aphrodisiac which, in turn, is dependent on the degree to which they have displayed the mate-refusal posture (which should be related to the abundance of males). In *P. napi*, as in most butterflies (Rutowski 1982), young virgin females are most attractive, and female attractiveness usually decreases with female age. Hence, because male *P. napi* appear to be very sensitive to the titre of MeS released by females, and because males are apt to curtail courtship rapidly when MeS titre is high, it is conceivable that mated females might encounter difficulty in re-mating. Hence, it is conceivable that some undetermined fraction of the 12% monandrous old females of *P. napi* are not voluntarily once mated, but that their monandrous status is brought about by their having received a large amount of MeS from their mate, in which case the male would have had the upper hand in the conflict over repeat matings that seems to be relevant for a potentially polyandrous species such as *P. napi*.

This further asks the question of whether males tailor their ejaculates. For reasons made clear by Wedell & Cook (1999), a male's first ejaculate should be tailored so as to maximize the duration of the time period that a female remains unreceptive, whereas a male's second ejaculate should be tailored so as to maximize efficiency in sperm competition. They showed that males of *P. rapae* did tailor their first and second ejaculates in accordance with theory with respect to proportion of eupyrene and apyrene sperm, with first ejaculates containing a significantly higher proportion of apyrene sperm which serve to delay onset of female receptivity. As a corollary a male's first ejaculate should contain a proportionately higher amount of anti-aphrodisiac compared with the second ejaculate, and the results demonstrate that males of *P. napi* do indeed allocate a higher proportion of MeS to the first ejaculate as compared to the second (figure 3). It appears that this has an effect on the mating system of *P. napi*, as females that have mated with recently mated males that transfer small ejaculates re-mate sooner than females that have mated with virgin males (Kaitala & Wiklund 1994). Hence, it appears that the olfactory signalling anti-aphrodisiac system in *P. napi* is a very fine-tuned one, with males reacting to small

differences in female release of the anti-aphrodisiac, setting the stage for a complicated interplay between males and females over mating decisions in the olfactory dimension.

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