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Sexual cooperation and conflict in butterflies: a male-transferred anti-aphrodisiac reduces harassment of recently mated females

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Sexual selection theory predicts that the different selection pressures on males and females result in sexual conflict. However, in some instances males and females share a common interest which could lead to sexual cooperation. In the pierid butterfly *Pieris napi* the male and the recently mated female share a common interest in reducing female harassment by other males soon after mating. Here we show that *P. napi* males transfer an anti-aphrodisiac to the female at mating, methyl-salicylate (MeS), which is a volatile substance which mated females emit when courted and which makes males quickly abandon them. A ^{13}C-labelling experiment demonstrated that only males synthesize MeS. The effect of this anti-aphrodisiac is so strong that most males will refrain from mating with virgin females to whom MeS has been artificially applied. In *P. napi*, males also transfer nutrients to females at mating. This increases female fecundity and longevity and so females benefit from remating. Hence, sexual cooperation gradually turns to conflict. Future research is required to reveal which sex controls the gradual decrease in the MeS titre which is necessary for allowing mated females to regain attractiveness and remate.

Keywords: *Pieris napi*; sexual selection; pheromones; methyl salicylate; solid-phase micro-extraction (SPME); ^{13}C labelling

1. INTRODUCTION

Butterfly mating systems vary from monogamy to polygamy (Burns 1968; Ehrlich & Ehrlich 1978; Drummond 1984; Wiklund & Forsberg 1991). 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 which allows females to increase their reproductive output (Boggs & Gilbert 1979; Wiklund *et al.* 1993, 1998; Kaitala & Wiklund 1994; Karlsson 1998). Regardless of female remating 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 (Bissoondath & Wiklund 1997), 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 typical posture when courted, spreading their wings horizontally and lifting their abdomen vertically; a posture which is 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/or unreceptivity, there is abundant evidence that males are able to 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, males' capability for inducing female unreceptivity and unattractiveness should be selected for. In line with this expectation, Gilbert (1976) suggested that males in the butterfly *Heliconius erato* (Nymphalidae) transfer an anti-aphrodisiac (Happ 1969) to females at mating which reinforces female monogamy. However, although his reasoning was lucid, Gilbert (1976) himself admitted that 'The evidence for the antiaphrodisiac function of the odor is more anecdotal but highly suggestive' (p. 419) and, lacking chemical identification of the male-contributed odour, experimental confirmation of his hypothesis was not possible. Here we show that male butterflies of *Pieris napi* (Pieridae) synthesize a volatile substance, methyl-salicylate (MeS), which they transfer to females at mating and that this substance makes females unattractive and unlikely to be mated.

2. MATERIAL AND METHODS

(a) *Attractiveness of virgin and mated females: bioassay*

In order to assess whether virgin and mated females differed in their attractiveness to males, we measured the duration of male aerial courtship of females. Virgin females were courted on the day they eclosed from the pupa and recently mated females were courted within 30 min after copulation termination. Courtship duration was measured for eight individual females using a set of five different virgin males. Each male was allowed a maximum of ten courtship bouts; hence, the attractiveness of each female was measured as the mean courtship duration of five males.

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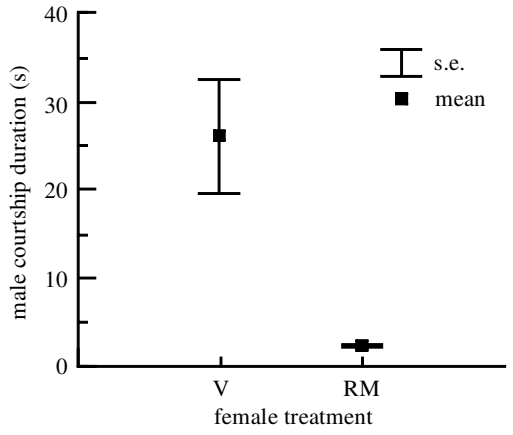

Figure 1. Male *P. napi* courted virgin (V) females much longer than they courted recently mated (RM) females ($x_V = 25 \pm 4$ versus $x_{RM} = 3 \pm 1$ s) ($p < 0.001$ and $F = 73.27$).

(b) *Attractiveness of virgin and mated females: sampling technique*

To assess whether the difference in female attractiveness to males could be mediated by some volatile substance we used a solid-phase micro-extraction (SPME) (see §2(c)) technique and analysed all SPME samples on a gas chromatograph linked to a mass spectrometer (GC–MS). To identify the odour emitted by virgin and mated female and male *P. napi*, we used a glass cylinder (height 18 cm, diameter 8 cm and volume = 0.9 m^3) with three small openings and one SPME fibre holder placed in each of the three openings. The odour emitted by virgin females was sampled in eight replicates with five unmated females in the container each time, ten replicates with five mated females in the container each time and five replicates with five males in the container each time. To identify the odour from spermatophores, three spermatophores were placed in a small vial with the SPME syringe placed a few millimetres above. The volatile compounds released from the spermatophores were trapped for 1 h. Nine replicates were made.

(c) *Analytical methods*

The sampling method used was SPME with a 100 µm polydimethylsiloxane fibre (Supelco™, Sigma-Aldrich Sweden AB, Stockholm, Sweden) (Zhang & Pawliszyn 1993; Borg-Karlson & Mozuraitis 1996). The SPME fibre was cleaned before each sampling by heating in the GC injector (250 °C for 10 min) with a helium gas flow. The analyses were conducted on a Varian 3400 gas ghromatograph (Varian AB, Solna, Sweden) linked to a Finnigan SSQ 7000 mass spectrometer (ThermoQuest AB, Kungens Kurva, Sweden). A DB-WAX column (internal diameter 0.25 mm, film thickness 0.25 µm and length 30 m) was used with temperature programming of 40 °C (1 min) − 3 °C min^{-1} − 110 °C − 10 °C min^{-1} − 200 °C (30 min), the injector temperature at 225 °C (splitless injection at 45 s) and helium as the carrier gas at a pressure of 10 psi. Identification of the compounds was made by comparison of the retention times and mass spectra with authentic reference samples.

(d) *^{13}C-labelling experiments*

The GC–MS analyses showed that mated females emitted MeS when courted by males. To assess whether this substance could be transferred by the male to the female at mating, we dissected out spermatophores from newly mated females and analysed the odours emitted by five spermatophores by using the combined GC–MS and SPME techniques described above. We performed a labelling experiment in order to assess further whether both males and females or only one sex synthesized MeS. Both male and female *P. napi* larvae were given a meal of leaves to which labelled L-phenylalanine (0.3 µg of 99% six-ring ^{13}C) had been applied—the larvae were fed this meal in the final instar when weighing 100–150 mg, approximately one to two days prior to pupation. When the adult butterflies eclosed the labelled males were mated with non-labelled females and vice versa. All females were sampled as described in §2(b) and analysed by GC–MS.

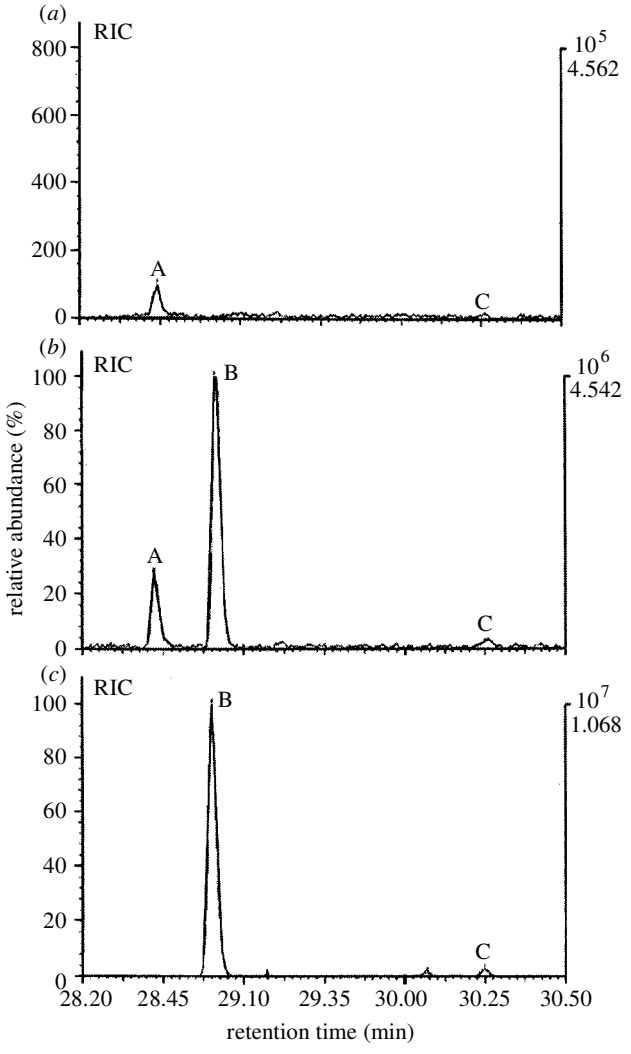

Figure 2. GC–MS analyses showed that both mated females and spermatophores emitted MeS, substance B, whereas virgin females did not (the figure depicts the total ion chromatogram section with the retention time, i.e. 28.20–30.50 min). Both (*a*) virgin and (*b*) mated females emitted *E,E*-α-farnesene, substance A. Moreover, both virgin and mated females and (*c*) spermatophores all emitted geranyl-acetone, substance C. A bioassay (see figure 4) showed that MeS renders the females unattractive to males and, hence, functions as an anti-aphrodisiac. The observation that the spermatophores but not the virgin females emit MeS indicates that the anti-aphrodisiac is contributed by the males.

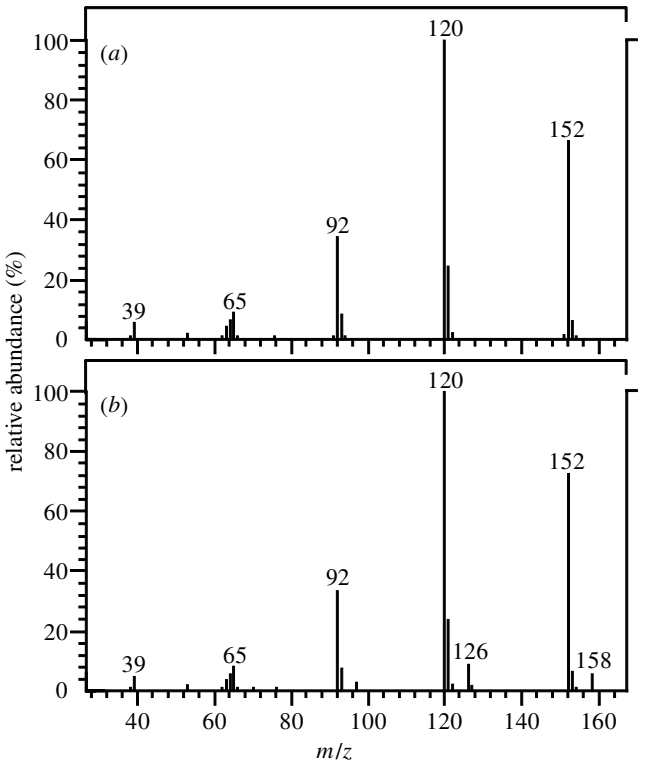

Figure 3. The anti-aphrodisiac MeS emitted by courted females is derived from MeS transferred to the female by the male at mating. In an experiment, male and female *P. napi* larvae were given a meal of leaves to which labelled L-phenylalanine (0.3 µg 99% six-ring ^{13}C) had been applied—the larvae were fed this meal in the final instar when weighing 100–150 mg approximately one to two days prior to pupation. When the adult butterflies eclosed the labelled males were mated with non-labelled females and vice versa. All females were sampled as in the bioassay and analysed on a GC–MS. (*a*) Labelled females (mated with non-labelled males) yielded an MeS spectrum identical to synthetic MeS. (*b*) Non-labelled females (mated with labelled males) released MeS with a spectrum containing two additional fragments, 126 and 158. These are both 6 mass-units larger than the 120 and 152 fragments in the mass spectrum of synthetic MeS. The molecular weight 152 fragment and the largest fragment 120 both contain the ring structure, which obviously originates from phenylalanine as is indicated by fragments 126 and 158.

(e) *Laboratory and field experiments: testing the effect of MeS as an anti-aphrodisiac*

In order to test whether MeS functions as an anti-aphrodisiac, we performed two experiments, one in the laboratory and one in the field. We tested whether virgin females painted with MeS dissolved in hexane were less attractive than virgin females painted with only the solvent hexane in both experiments. To ascertain whether the amount of MeS applied to virgin females was physiologically and/or behaviourally relevant, we painted five virgin females with 2 µl MeS (1/100 in hexane using *ca.* 20 ng per female). The odour was sampled by SPME and analysed by GC–MS in the same way as the five mated females in a glass cylinder (see § 2(b)). The amount of MeS in the chromatograms was compared to the amount of MeS in the chromatograms received from the SPME analysis of the mated females. The painted females emitted similar amounts of MeS as the mated females.

In the laboratory experiment (experiment A), we painted nine live virgin females with 2 µl MeS (1/100 in hexane using *ca.* 20 ng per female which was found to be a biologically relevant amount; see above) and assessed their attractiveness by using the previously described bioassay (see § 2(a)). As a control, five virgin females painted with hexane were courted by five males. In the field experiment (experiment B), 26 newly eclosed males were released in the area, after which females were released one at a time. Ten females were painted with 2 µl MeS (1/100 in hexane using *ca.* 20 ng per female) and eight females were painted with hexane. The females were monitored individually and the time for each encounter with a male was measured. Each female was followed until mating or until she had been courted five times by males.

3. RESULTS

The results showed that, on average, virgin females were courted eight times as long as recently mated females, suggesting that males could distinguish between the two and showing that virgin females were more attractive (figure 1). The GC–MS analysis made on the odours emitted by virgin and mated females showed that the main difference was that only mated females emitted MeS (figure 2). The GC–MS analysis on the spermatophores which had been dissected from recently mated females showed that MeS was emitted from spermatophores as well (figure 2), hence indicating that the substance MeS is contributed to the female by the male.

This inference was shown to hold true in an experiment where male and female larvae were fed L-phenylalanine with all six carbons in the aromatic ring labelled with ^{13}C (L-phenylalanine is a biosynthetic precursor to MeS in tobacco) (Lee *et al.* 1995). After labelled males had been mated with non-labelled females and vice versa, only non-labelled females emitted MeS containing the ^{13}C aromatic ring structure (figure 3). Hence, our results unequivocally demonstrate that the MeS emitted by courted females is derived from MeS transferred to the female by the male at mating. We have also shown that L-phenylalanine can be used as the precursor to MeS in the butterfly system *P. napi* and not only in plants.

Large amounts of neral and geranial (citral) and their corresponding alcohols were present in the fragrance of the active males (Bergström & Lundgren 1973), but no trace of MeS. The function of the odour of the citral emitted by male *P. napi* is presently unknown. It is well known that many male butterflies have transformed scales, known as androconia (Bergström & Lundgren 1973), the function of which is to emit aphrodisiaca and thereby increase the likelihood that a courted female will mate (Tinbergen *et al.* 1942; Rutowski 1980). Although we have not identified the source of where citral is emitted, the male androconia, which are spread all over the wings of *P. napi* (Warren 1961, 1963), are likely candidates. This possibility is also supported by the observation that we did not find citral in the fragrance from mated females which demonstrates that there is no transfer from the male to the female during mating.

To test whether MeS functions as an anti-aphrodisiac, we performed two experiments, one in the laboratory and one in the field. The results showed that this was indeed so; females painted with MeS were courted for a

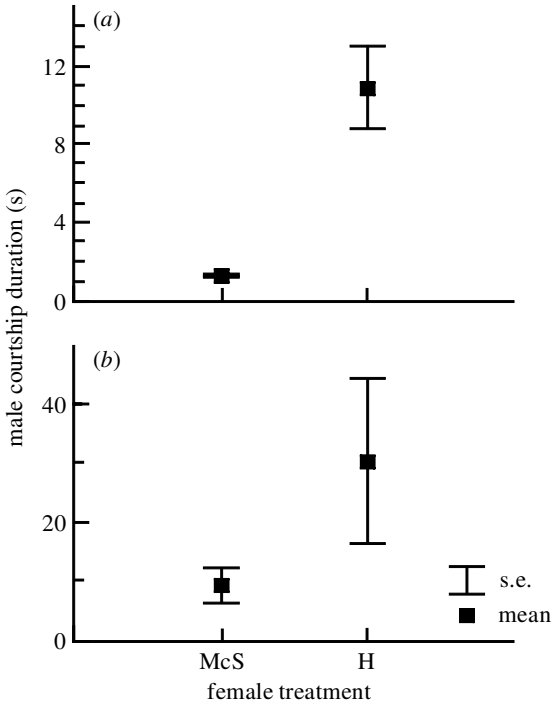

Figure 4. Males courted virgin females which had their abdomen painted with MeS for a shorter duration than virgin females painted with the solvent hexane (H) in both (*a*) cages in the laboratory ($x_{McS} = 1 \pm 0.5$ versus $x_H = 11 \pm 2$ s) ($p > 0.001$ and $F = 90.75$), and (*b*) in a field experiment ($x_{McS} = 10 \pm 5$ versus $x_H = 30 \pm 10$ s) ($p = 0.017$ and $F = 6.10$). This result indicates that MeS functions as an anti-aphrodisiac which curtails male courtship.

significantly shorter duration than virgin females painted with hexane in both the laboratory and the field (figure 4). Moreover, the field experiment also showed that virgin females painted with MeS were significantly less likely to be mated compared to virgin females painted with hexane. All of the seven females which were painted with hexane were mated, whereas only two out of the ten virgin females painted with MeS were mated (Fisher's exact test, $p = 0.0023$).

4. DISCUSSION

Our results clearly demonstrate that, virgin female *P. napi*, which are strongly attractive to males, are rendered strongly unattractive immediately after mating and that the rapid change in female attractiveness is mediated by the anti-aphrodisiac MeS, which is synthesized by males and transferred to the females at mating. Previous studies have demonstrated that female *P. napi* are most unattractive to males soon after mating and that courtship duration increases with time elapsed since mating (Forsberg & Wiklund 1989). These attractive, previously mated females can be severely harassed by courting males and may suffer two kinds of costs: first, by being forced to interrupt egg laying when discovered by males and, hence, paying a time-cost for being courted and, second, by being 'forced' to crawl down in the vegetation to escape particularly insistent males and, hence, paying a liable predation cost (Forsberg & Wiklund 1989). It therefore seems obvious that the anti-aphrodisiac confers a substantial selective advantage on females by making them efficient in curtailing male courtship and, thus, reducing male harassment.

The anti-aphrodisiac 'system' adopted by *P. napi* differs from that previously reported for *H. erato* by Gilbert (1976) in two ways. Gilbert (1976) suggested that males in the butterfly *H. erato* transfer an anti-aphrodisiac to females at mating which helps reinforce female monogamy. The basis for Gilbert's (1976) suggestion was that virgin and mated females of *H. erato* emit distinctly different odours and that the odour emitted from females is similar to the male odour; hence, the odour emanating from the anti-aphrodisiac seems to be similar to the fragrance emitted by the males themselves. In *P. napi*, males are also fragrant and give off a strong smell of citral when interacting with other males (or when handled by humans), whereas the anti-aphrodisiac transferred to the females at mating, MeS, is chemically completely different from the volatiles which make up the male odour. Second, *H. erato* is a monandrous species and so the anti-aphrodisiac functions so as to make females unattractive for the remainder of the mated female's life. In *P. napi*, where females benefit strongly from mating several times during their lifetime in terms of male-derived nutrients increasing female fecundity, the female's usage of the anti-aphrodisiac is more dynamic with respect to time, because recently mated females benefit from being able to curtail male courtship, whereas not-so-recently mated females benefit from remating and, in doing so, are dependent on becoming attractive to males once again.

Although female *P. napi* gain from remating through the nuptial gift transferred by the males, experiments have shown that females have a substantial refractory period after mating and only remate after an average of four to six days (Wiklund *et al.* 1983, 1998; Kaitala & Wiklund 1994). There are at least two reasons why females wait several days until they remate. First, the ejaculate mass transferred from male to female *P. napi* is extensive and can correspond to up to 23% of their body mass; this means that a new ejaculate can only be received by a female when the first one is digested (Forsberg & Wiklund 1989). Second, there is a time conflict between egg laying and mating—both activities require sunny weather and time spent in copulation interferes with egg laying (and the duration of copulation can be extensive in *P. napi*—matings involving recently mated males can last up to 21 h) (Kaitala & Wiklund 1995). Hence, the unattractiveness of recently mated females benefits both the male and the female (Forsberg & Wiklund 1989). This mutual interest between a female's first mate and the female turns into conflict as the female gradually becomes interested in remating. Which sex has the upper hand in this conflict depends on whether females can control their attractiveness (i.e. the release of MeS) or not. If females do not have complete control and when courted release whatever titre of MeS which remains, this means that the female's first mate exercises some control over the female remating frequency. However, if females do have complete control over MeS release, they should be able to realize a level of polyandry unthwarted by male interests. Further experiments are

needed in order to elucidate which of these alternatives is applicable.

The phenolic ester MeS is an example of a multifunctional compound which has been found to be active in a number of biological systems. The flower fragrance of certain Neotropical orchids contains MeS, which attracts oil-collecting, male, euglossine bees (Williams & Whitten 1983). It also acts in interplant communication and is released by insect-infested plants (Pettersson *et al*. 1994) and transported aerially between plants. In the presence of MeS the colonization density of aphids in crops is reduced (Pettersson *et al*. 1994) and it is also used for monitoring predators of the *Psylla* genus (Molleman *et al*. 1997). MeS is known to be formed from L-phenylalanine in plants (Lee *et al*. 1995). Our experiments with ^{13}C-labelled phenylalanine indicated that this also is valid in butterflies (Lepidoptera).

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