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Male sex pheromone release and female mate choice in a butterfly

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Summary

In butterflies female mate choice is influenced by both visual and olfactory cues, the latter of which are important at close range. Males of the green-veined butterfly, *Pieris napi*, are known to release citral (mixture of geranial and neral, 1:1), but its role(s) and conditions of release are not known. Here, we show that male *P. napi* release citral when interacting with conspecific males, conspecific females, heterospecific males and also when alone. The amount of citral released correlated strongly with male flight activity, which explained more than 70% of the variation. This suggests that males do not exercise control over turning release on or off, but rather that citral is emitted as a passive physical process during flight. Electroantennogram experiments showed that female antennal response was ten times more sensitive to citral than male response. Females expressed acceptance behavior when exposed to models made with freshly excised male wings or those treated with citral following chemical extraction, but not to ones with extracted wings only. Hence, these behavioral and electrophysiological tests provide strong evidence that citral is a signal from the male directed to the female during courtship, and that it functions as a male sex pheromone.

Key words: terpenes, geranial, neral, androconia, Pieridae, courtship, aphrodisiacs, species specificity.

Introduction

Male butterflies of many species emit scents, many of which are also perceptible to humans. Usually the scents are produced in scent glands and are emitted either from transformed scales, androconia, which can be aggregated into so-called sex brands on the dorsal or the ventral part of male wings as in many pierids and nymphalids (Tinbergen et al., 1942; Rutowski, 1980; Scoble, 1992; Rauser and Rutowski, 2003), or from eversible hairpencils at the tip of the abdomen, as in many danaids (Brower et al., 1965; Meinwald et al., 1969; Pliske and Eisner, 1969; Pliske, 1974; Pliske et al., 1976). There are at least five conceivable functions that could explain the widespread occurrence of male scents in butterflies. First, they could function as male sex pheromones during courtship, facilitating a female's acceptance of a courting male. Second, they could provide the female with reliable information about male quality and so have a role in influencing female mate choice (cf. Rutowski, 1984; Dussourd et al., 1991; Iyengar et al., 2001). Third, they could function as species-specific identifying cues allowing females to distinguish between conspecific and non-conspecific males. Fourth, they could function as sex-specific signals facilitating sexual identification during courtship. Fifth, male scents might function as intrasexual signals between males, making them curtail courtship or engage in contest behavior in species in which males defend mating territories (cf. Kemp and Wiklund, 2001). It should be pointed out that these functions are not mutually exclusive, and in particular, the first four could all be relevant to a female when courted by a male butterfly, allowing simultaneous sex and species identification, as well as a sexual stimulus to show acceptance behavior under the influence of a male sex pheromone. In view of strong selection against interspecific matings (Andersson, 1994), male sex pheromones that effectively release a female's acceptance behavior should be simultaneously species-specific and sex-specific.

Because scent scales appear largely confined to the male sex, it seems reasonable to assume that male scent plays a role during courtship, and observations on the behavioral idiosyncracies of courtship in different species reveal that males actively use their scent-disseminating devices when courting a female. In the grayling butterfly, *Hipparchia semele*, the male sex brand is located on the dorsal surface of the forewings, and Tinbergen et al. described how the male courtship culminates in a 'bow' during which he captures the female's antennae between his forewings, whereby they are brought into direct physical contact with the male scent-producing organ (Tinbergen et al., 1942). Likewise, in the queen butterfly, *Danaus gilippus berenice*, Brower et al. described how the male performs a courtship flight just above the female during which he everts his hairpencils and douses

the female with love dust from above (Brower et al., 1965). In the monarch butterfly, *Danaus plexippus*, Pliske described how, in a similar manner, male monarchs first pursue females in the air, then proceed with aerial 'hairpencilling' before performing an aerial takedown just prior to copulation (Pliske, 1974).

In the green-veined white butterfly, *Pieris napi*, males emit a strong scent of citral [a mixture of neral (2*Z*)-3,7-dimethylocta-2,6-dienal) and geranial (2*E*)-3,7-dimethylocta-2,6-dienal] (Bergström and Lundgren, 1973). This scent is emitted when males are interacting in flight with conspecific males (Andersson et al., 2000), but it is not known whether males emit citral also when interacting with conspecific females. Therefore, our first objective was to test during which interactions males release citral, by staging interactions with conspecific males, virgin or mated conspecific females, and with heterospecific butterflies. Moreover, it is not known whether males are able to turn the release on or off, and therefore we carefully assessed the time that males spent in flight when recording male release of citral.

If male *P. napi* emit citral when courting females, this suggests that it might function as a male sex pheromone. In *P. napi*, as in other pierids such as *Pieris rapae*, *Pieris brassicae* and *Anthocharis cardamines*, unreceptive females react to male courtship by spreading their wings laterally and lifting their abdomen up in the air, performing the so-called 'mate-refusal posture' (Obara, 1964; Wiklund and Forsberg, 1985; Forsberg and Wiklund, 1989). This female behavior appears to have two functions. First, it makes it physically difficult for a courting male to couple with the female. Second, females use this posture to disseminate volatiles that can be perceived easily by the courting male; in many pierids males appear capable of distinguishing between virgin and mated females, and in *P. napi*, *P. rapae* and *P. brassicae* different volatiles are disseminated by virgin and mated females when exhibiting the mate-refusal posture (Andersson et al., 2000; Andersson et al., 2003). Both virgin and mated females initially exhibit the mate-refusal posture when approached by a male, but when a female accepts a courting male she signals her receptivity by closing her wings and acquiescing (Forsberg and Wiklund, 1989). When perceiving this signal the courting male alights next to the female, bends his abdomen sideways to make genital contact, couples with the female, and usually carries the female away in a short post-nuptial flight.

Our second objective was to compare the sensitivity of female and male antennal responses to citral to see whether females were more sensitive, which would suggest that citral increases sexual receptivity in females. Thus, we tested the antennal response of females to biologically relevant concentrations of citral by electroantennography (EAG).

Our third objective was to assess whether the male-produced volatile citral functions as a male sex pheromone. We tested this hypothesis by subjecting virgin females to artificial courtship by a male model to which citral had been applied, and assessed whether females as a response exhibited male acceptance behavior or not. As a control, we subjected females to artificial courtship with an odorless male model.

Materials and methods

Male release of citral and analytical methods

To test under what circumstances male *P. napi* release citral, the volatiles emitted from the butterflies were sampled for 60 min using the solid phase microextraction (SPME) technique (Borg-Karlson and Mozuraitis, 1996; Andersson et al., 2000). The butterflies were kept in a glass cylinder (height 11 cm, diameter 4.5 cm) that was sealed with aluminium foil. Prior to the experiments, the glass cylinder was kept overnight at 150°C. The emission was collected for three replicates of either a single *P. napi* male, one *P. napi* male and one *P. aegeria* male, two conspecific *P. napi* males, one *P. napi* male and a virgin *P. napi* female and finally one male and a mated female of *P. napi*. The butterflies used in the experiment were the offspring of wild females collected on the campus of Stockholm university, and had been reared as larvae on the natural host plant *Alliaria petiolata* under direct development conditions at 23°C in a 20 h day-length. After eclosion, males were individually marked and brought to a refrigerated room (8°C), where they remained until the experiment. The total time (s) that males (2–7-days-old) spent flying during the 60-min assay was recorded. Each male was used only once.

To compare the amount of citral released by males with the amount released by the male models used in the bioassays, we dissolved citral in hexane (v/v) at five concentrations (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}). Each solution (100 μl) was applied on filter papers with a diameter of 1.6 cm that were fixed to a pipette tip, and placed in an identical glass cylinder as described above. Volatiles were collected using SPME during 1 h. We used the polydimethylsiloxane-divinylbenzene fibers (65 μm) (Supelco, Stockholm, Sweden). The SPME fibers were cleaned before each sampling by heating in the gas chromatograph injector (at 250°C for 10 min), using He as gas flow, and the background was measured by one gas chromatography (GC)-mass spectrometry (MS) run before starting the collection of volatiles. A Varian 3400 gas chromatograph connected to a Finnigan SSQ 7000 MS (70eV) was used for the analyses. A DB-1 column (0.25 mm i.d., 0.25 μm film thickness, 30 m length; J&W Scientific, Folsom, CA, USA) was programmed at 40°C for 1 min then increased to 220°C for 12 min (5°C min^{-1}), with an injector temperature at 215°C (splitless injection, 45 s) and He as the carrier gas at 69 kPa. Compounds were identified by comparing retention times and mass spectra with reference samples.

Electrophysiological recordings

We performed an EAG experiment to assess whether there was a difference between male and female antennae with respect to their sensitivity to citral. Each insect was placed in a Plexiglass holder so that only the head with the antennae was exposed. The animal was strapped to the holder with tape and the head was immobilized with wax. One antenna was fastened

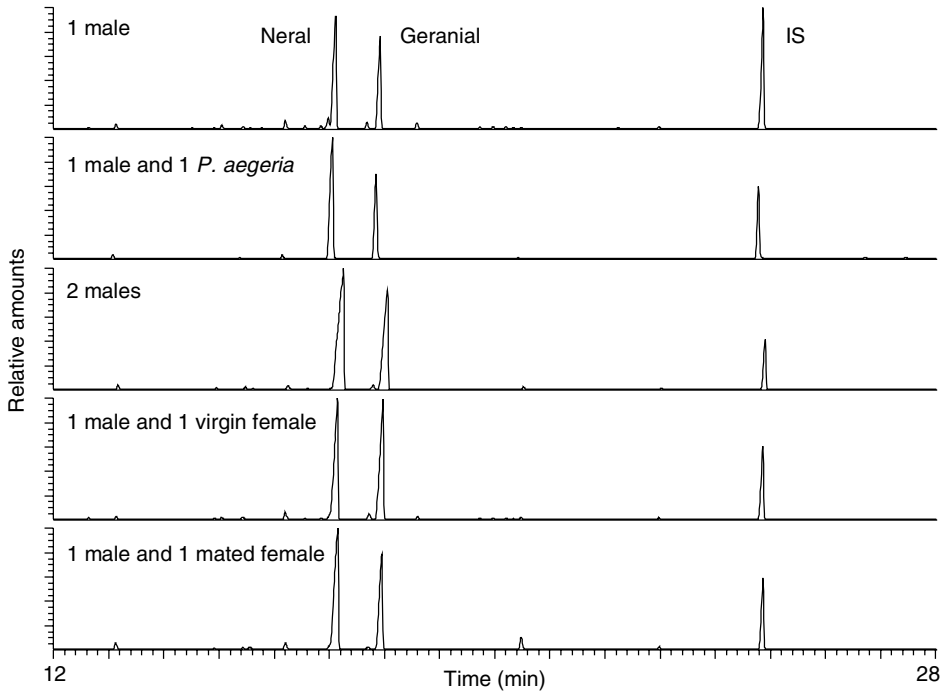

Fig. 1. Five representative GC-MS chromatograms showing the emission from one single *Pieris napi* male, one *P. napi* male interacting with one male of *Pararge aegeria,* two *P. napi* males, and one *P. napi* male and one virgin *P. napi* female, and one *P. napi* male interacting with one mated *P. napi* female. Volatiles were collected by SPME for 1 h. The two main compounds were neral and geranial, with approximate proportions of 1:1. IS, the internal standard, n-pentadecane.

to the wax with hooks of wolfram thread and immobilized with wax along the edges, and the other antenna was totally covered with wax. The glass capillary microelectrodes were filled with Ringer's solution (150 mmol l^{-1} NaCl, 3 mmol l^{-1} $CaCl_2$, 3 mmol l^{-1} KCl, 10 mmol l^{-1} Tes buffer, pH 6.9). The recording electrode was placed into the cut hole in the tip of the antenna and the reference electrode into the haemolymph of the first or second flagellar segment. The signal was amplified 1000 times and visualized on an oscilloscope (Yokogawa DL 1200, Tokyo, Japan) and recorded with the Syntech EAG software (Syntech, Hilversum, The Netherlands). The compound used for stimulation of the antenna, citral (neral + geranial, 1:1; Sigma-Aldrich) ⩾95.0% purity by GC, was dissolved in hexane and first applied on a piece of filter paper (100 μl, diameter 15 mm) in the dilutions (v/v) 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}, corresponding to 90 μg–0.9 ng. The filter paper was placed in a glass tube (10×150 mm) and placed in the setup so that it was subtended in a 45° angle to the antenna at a distance of 20 mm. Air stream (500 ml min^{-1}) blown through the tube for 0.5 s provided puffs of stimulus over the antennal preparation.

We tested the antennal responses of three males and three females to five different concentrations of citral. Stimulation with the dilution series of citral started with the lowest concentration to avoid saturation of the receptors. Each dilution was represented twice in a series and there was a 2-min pause between stimulations. An EAG standard technique was employed for recording the summated receptor potential (Schneider, 1957; Kaissling, 1971). This entails EAG-registration as the response given as an EAG-amplitude started off by the odor stimulation minus the control stimulus (air over antenna). The registration was then compared with the test signal of 1 mV. After every third sample, one reference compound (hexane) and one control stimulus (filter paper) were run to make it possible to account for the variations owing to the physiological state of the animal throughout the test run.

Male sex pheromone experiment and behavioral assays

To test female propensity to exhibit mate acceptance or refusal behaviors when subjected to artificial courtship by male models scented with citral or no scent, we reared approximately 100 *P. napi* from offspring of five wild-caught females from the vicinity of Stockholm. The larvae were reared on the natural host plant *A. petiolata*, and when the adult butterflies emerged they were sexed and transferred to a refrigerated room (8°C). Females were held for 2–5 days before the experiment, whereas 10 males were killed by freezing at –25°C. Wings from the newly killed males were cut at the base and separated from the body, and the two wing pairs from each male were extracted by two immersions (each lasting 1 h) in 10 ml diethyl ether at 20°C. The extracted wings were air dried for 24 h. The extracted wings were then sampled by SPME for 1 h and analyzed with a gas chromatograph connected to a mass spectrometer (GC-MS; see analytical methods). Male wings that were not extracted were also sampled the same way for 1 h. Analysis showed that wings that had not been extracted released significant amounts of citral. Wings that had been extracted did not release detectable amounts of citral, and will heneceforth be referred to as 'extracted odorless wings'.

A single female was released into a 0.5 m×0.5 m×0.5 m flight cage; the cage had a transparent plastic top and was located in a greenhouse. The experiments were performed at

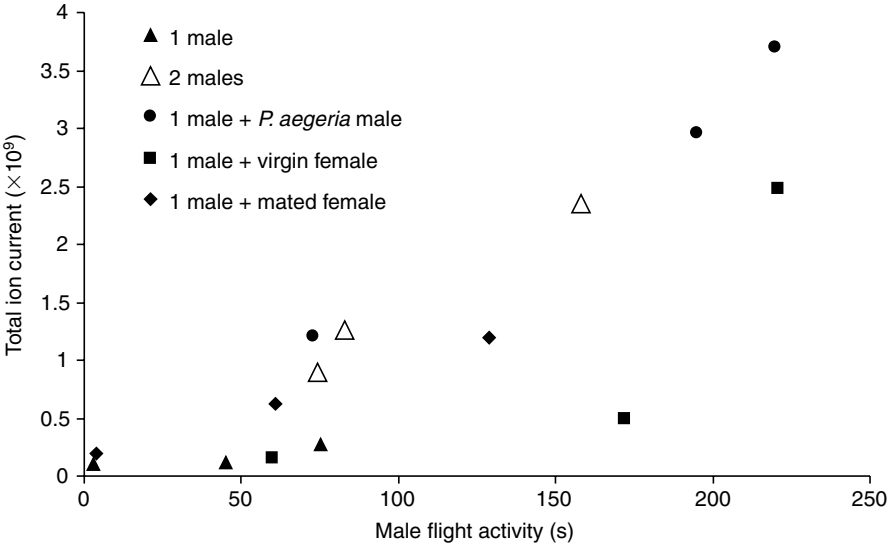

Fig. 2. Activity-dependent emission of neral from one single *Pieris napi* male, one *P. napi* male interacting with one male of *P. aegeria,* two *P. napi* males, one *P. napi* male and one virgin *P. napi* female, and one *P. napi* male interacting with one mated *P. napi* female. It can be observed that the greater the flight activity of the males (measured in s), the greater the emission of citral.

the beginning of October 2002, with an air temperature ranging between 15 and 20°C. Once the female had alighted on a wall in the cage, we presented the settled female with a 'male model' consisting of four wings from a male butterfly held together with soft forceps, as if the male was resting with only the ventral sides of the four wings visible. This male model was waved in front of the female to simulate the approach of a courting male, and was then brought into physical contact with the female, again simulating the manner in which male *P. napi* court female conspecifics when alighted in the vegetation (Forsberg and Wiklund, 1989). We used four different types of male models: (1) wings from newly eclosed males killed in the freezer 30 min prior to the assay, (2) extracted odorless wings with 10 μl of hexane applied to each of the two outer-wing surfaces, (3) extracted wings with 10 μl of citral solution (see specification above, diluted 1:100 in hexane; 90 μg) applied on each of the two outer-wing surfaces, and (4) extracted wings with 10 μl of citral solution (diluted 1:10 000 in hexane, 0.9 μg) applied on each of the two outer-wing surfaces. A total of 40 virgin females were subjected to the bioassay, and each female was subjected to a maximum of 10 artificial courtship bouts by one of the four male models described above; hence, 10 females were artificially courted by each of the four male models.

Results

Male release of citral

Regardless of the context, the airborne collections from *P. napi* males consisted of >95% neral and geranial, at a 1:1 ratio, in all assays (Fig. 1). When pooling the results from these 15 trials, a regression analysis showed that the amounts of neral and geranial released were strongly correlated with male flight activity (Fig. 2; neral: r^2=0.718, N=15, P<0.001; geranial: r^2=0.743, N=15, P<0.001). The emission of the two compounds from *P. napi* males in various interactions was in the same range as when 45 ng–45 μg of neral or geranial was applied on a filter paper and the emission was collected during 1 h (Figs 2 and 3).

Male and female antennal sensitivity to citral

The EAG recordings showed a marked sex difference in sensitivity to citral in *P. napi*; antennal response of females was approximately 10 times more sensitive to citral than that of males at most doses tested, and to elicit a response from males equal to that of females a dose 10 times stronger was required (Fig. 4). The doses applied on the filter paper used for EAG recordings were considerably higher than the doses in the behavioral test. However, when taking into account the short time during which the puffs of stimulus were provided during EAG recordings (0.5 s), the amount of citral presented to the antenna must be considered to be well within the dose–response curve and within the biologically relevant range (cf. Fig.·3).

Citral as a male sex pheromone

The females behaved in two different ways when approached by the male models: they either flew away or they sat still and folded their wings and acquiesced. The former behavior represents female rejection, and the latter signals female acceptance of the male's courtship (cf. Forsberg and Wiklund, 1989). When females were approached by extracted odorless male wings they invariably flew away and landed on the opposite side of the cage; this escape behavior was shown by all of the 10 females bio-assayed each of the 10 times each female was approached by the male model. Twenty-seven of 30 females also flew away when approached for the first time by wings from recently killed males, or with male models to which 0.9 μg or 90 μg of citral had been applied. However, all of these females acquiesced after between one and six courtship bouts. The remaining three females acquiesced immediately when approached by male models the first time. Females approached by wings of recently killed males or with extracted wings to which 90 μg citral had been added acquiesced after a

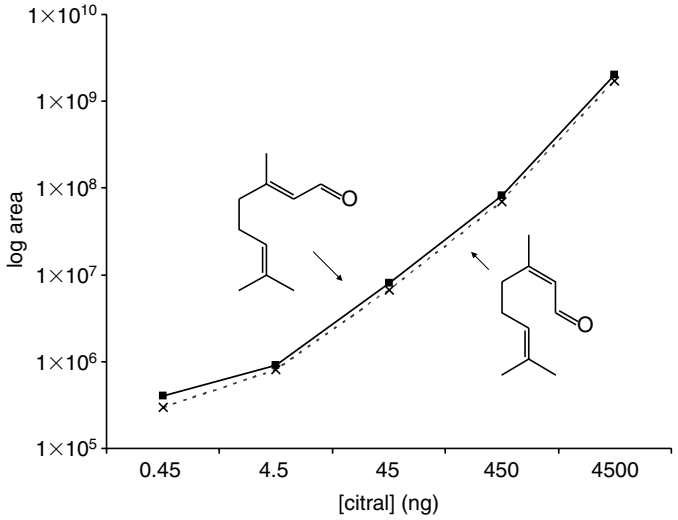

Fig. 3. The logarithmic calibration curve of citral, separated into geranial (solid line) and neral (dotted line), as assessed by SPME and GC-MS.

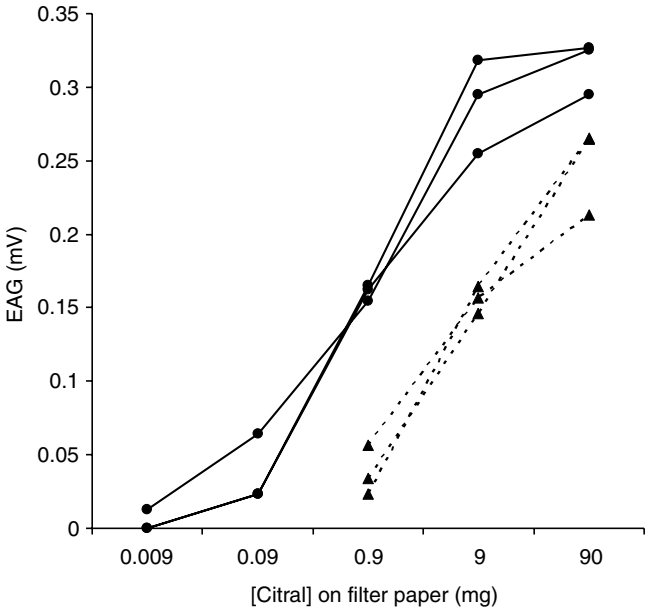

Fig. 4. Electroantennography dose–response curves to citral (neral and geranial, 1:1) for both sexes of *Pieris napi*, with the female response above (black lines) and the male response below (broken lines). The responses to different doses of citral for one individual are connected by a line.

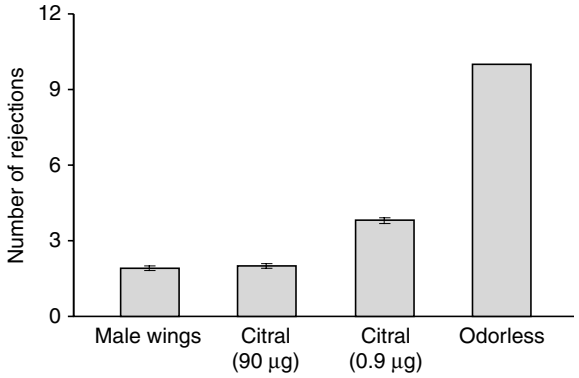

Fig. 5. Number of times females rejected male models when subjected to artificial courtship. All females subjected to courtship by male wings or extracted male models to which citral had been applied exhibited mate acceptance behaviour, a behavior that was never elicited by the extracted odorless male models. Values shown are means ± s.e.m.

mean of 2.9 and 3.0 courtship bouts, respectively, a difference that was not significantly different; females approached by extracted male wings to which 0.9 μg citral had been applied acquiesced after a mean of 4.8 courtship bouts, which was significantly different from female reactions to the two previous male models (Fig. 5; Kruskal–Wallis test: N=30; H_2,=7.966; P=0.018).

Discussion

Male release of citral

Male *P. napi* emitted citral not only when interacting with females, but also when interacting with conspecific males, with heterospecific males, and when alone (Fig. 1). Our results also demonstrated that the amount of citral emitted was strongly correlated with male activity, which explained more than 70% of variation in the amount of citral released (Fig. 2). This suggests that males do not exercise control over turning release on or off, but rather that citral is emitted in the normal course of male flight activity. If so, the fact that citral is emitted during male–male interactions does not call for an adaptive explanation. The observation that male antennae were less sensitive to citral than female antennae is in line with such a conclusion. Hence, the release of citral during male–male interactions does not necessarily serve any function, being merely a side effect of male flight activity.

Male and female antennal sensitivity to citral

Electroantennogram tests demonstrated that there was a pronounced sex difference in sensitivity to citral, with females being much more sensitive. Moreover, our results indicate a dose response and suggest that the level of male production of citral could influence mating success. It is relevant to add that the amount of citral emitted from living males corresponds to that emitted from between 0.45 and 4.5 μg citral applied to a filter paper, which, in turn, is intermediate with respect to the amount applied to the two male models used in the bioassay. However, it is worth observing that in *P. napi*, and in other pierids such as *Anthocharis cardamines*, the scent-emitting scales on the underside of the male wings are usually brought into direct contact with the female antennae during the sexual chase, suggesting that dose–response curves obtained in experimental studies in the laboratory are not necessarily

relevant for what is important under natural circumstances. Indeed, when receptive females of *P. napi* and *A. cardamines* are discovered by males when sitting in the vegetation, their mate acceptance takes longer than when females are pursued by males in flight prior to alighting in the vegetation (Forsberg and Wiklund, 1989; Wiklund and Forsberg, 1985). This shows that male sex pheromones in butterflies are used at close range and are not used for mate attraction over long distances (cf. Wyatt, 2003), and suggests that male courtship behaviors have been selected to bring scent-emitting structures into close contact with female olfactory receptors.

Although the male sex pheromone acts over short distances, it is conceivable that pheromone titer influences female mate choice, and so it is interesting to consider to what extent male sex pheromone production and titer varies over a male's lifetime. Biosynthesis of the male sex pheromone may also be relevant, especially if it is dependent on the larval host plant, because *P. napi* is oligophagous on several different species of Brassicaceae. Hence, further research should address the issue as to what extent larval host plant, male age and sex pheromone production and titer influence female mate choice.

Citral as a male sex pheromone

Experiments with male models showed that citral rendered females more prone to adopt mate-acceptance behavior, and hence functions as a male sex pheromone. In moths, sex pheromones are released by females and male mate location is largely governed by chemical cues. By contrast, mate location in butterflies is largely mediated by visual cues, and several studies have demonstrated color-based mate choice (Stride, 1958; Silberglied and Taylor, 1978; Fordyce et al., 2002; Ellers and Boggs, 2003; Sweeney et al., 2003; Robertson and Monteiro, 2005). However, there is accumulating evidence that these visual stimuli are accompanied by chemical signals that are important at close range (Rutowski, 1984; Silberglied, 1984; Pivnick et al., 1992; Schulz et al., 1993; Jiggins et al., 2001; Fordyce et al., 2002; Wiklund, 2003; Costanzo and Monteiro, 2007). Behavioral experiments have demonstrated that male wing scents function as a sex pheromone in several pierid butterfly species, including *Colias eurytheme* and *C. philodice* (Taylor, 1973; Silberglied and Taylor, 1978; Grula et al., 1980; Rutowski, 1980), *Eurema lisa* (Rutowski, 1977) and *Pieris melete* (Kan and Hidaka, 1997), as well as in two lycaenid butterflies, *Lycaeides argyrognomon* (Lundgren and Bergström, 1975) and *Zizeeria maha argia* (Wago, 1978). In *L. argyrognomon* the wing odor functions as a sex pheromone for receptive females. The main male wing odor has been identified as the sesquiterpene alcohol (–)-δ-cadinol (torreyol) (Lundgren and Bergström, 1975) but has not yet been tested as a sex pheromone. In *Z. maha argia* the chemical identity of the active component of the male scent has not yet been identified. In the satyrine butterfly *Bicyclus anynana*, recent research has demonstrated that both visual cues in the ultraviolet (UV)-range and olfactory cues have a strong influence on female mate choice (Robertson and Monteiro, 2005; Costanzo and Monteiro, 2007).

Recent research on pierid butterflies has demonstrated that males emit scents that are both sex- and species-specific; male *P. napi* smell strongly of citral and male *P. brassicae* emit substantial amounts of benzyl cyanide (Andersson et al., 2003). Our demonstration that female mate acceptance behavior of *P. napi* is mediated by citral that is emitted only by conspecific males is, to our knowledge, the first time an identified chemical substance has been convincingly shown to function as a male sex pheromone in a pierid butterfly. Because the male-specific substances present in the three congeneric *Pieris* species *P. napi*, *P. rapae* and *P. brassicae* are also species-specific, it is likely that these substances also function as species-specific signals. Male butterflies are generally incapable of forcing matings on females, and so female receptivity is a necessary prerequisite for mating. Thus, these species- and sex-specific compounds have a role in both recognition of an appropriate mate and in inducing receptive behavior in females.

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