Structure, ratios and patterns of release in the sex pheromone of an aphid, *Dysaphis plantaginea*

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Summary

Insect communication is primarily *via* chemicals. In Aphidinae aphids, the structure and ratio of iridoid (monoterpenoid) chemicals are known to be important components of the sex pheromone. However, for enhanced species specificity, it has been suggested that release of sex pheromone might be restricted to a narrow time period within the diel cycle. Here, we determine the structure, ratios and release patterns of iridoid chemicals produced by a serious global pest, the rosy apple aphid, *Dysaphis plantaginea*. Volatiles were collected from batches of oviparae (sexual females) and chemicals identified by gas chromatography, mass-spectrometry and microscale NMR spectroscopy. (1*R*,4a*S*,7*S*,7a*R*)-Nepetalactol and (4a*S*,7*S*,7a*R*)-nepetalactone were detected in a 3.7:1 ratio. To investigate timing of release, we constructed a sequential sampling device that allowed volatile chemicals to be captured hourly from 95 same-aged oviparae over 20 consecutive days. Release patterns of the two sex pheromone components show that *D. plantaginea* oviparae release high levels of the two components during photophase and low levels during scotophase. Release of the two components increased significantly during the first 3 h of photophase and thereafter remained at a high level until the onset of scotophase. The ratio of (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol to (4a*S*,7*S*,7a*R*)-nepetalactone released did not change significantly between days two to 14 of the adult stadium, but from the 15th day onward there was a significant decrease in the relative amount of (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol. Pheromone release was greatest on the eighth day of the adult stadium, with up to 8.4 ng of pheromone released per ovipara per hour. This is the first report on the full structural identification and ratios of volatile iridoid components collected from *D. plantaginea* oviparae and is also the most detailed temporal study on sex pheromone release from any aphid species. The lack of a temporally narrow and distinct period of very high sex pheromone release suggests that alternative mechanisms or factors for species recognition and isolation may be important. Findings are discussed broadly in relation to the biology of the aphid.

Key words: *Dysaphis plantaginea*, nepetalactol, nepetalactone, oviparae, periodicity, pheromone ratios, rosy apple aphid, sex pheromone.

Introduction

For many species of insect, both chemical ratios and timing of release within the circadian cycle are important elements of sex pheromone signalling. In aphid species studied so far, all of which belong to the subfamily Aphidinae, sex pheromone is known to comprise iridoid (monoterpenoid) components. (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (1) and (4a*S*,7*S*,7a*R*)-nepetalactone (5) were first determined as components of vetch aphid (*Megoura viciae*) sex pheromone (Fig. 1) (Dawson et al., 1987; Dawson et al.,1989). Since then, nepetalactol (1 or 2) and nepetalactone (5 or 6) have been found either singly or as a mixture in the sex pheromone of numerous other species of aphid (Table 1). However, because analyses used achiral methods, most identifications did not confirm which enantiomers were present nor whether the component was a mixture of enantiomers, hence 1 or 2 and 5 or 6. In one species, *Phorodon humuli*, the sex pheromone comprises two different nepetalactol diastereoisomers (3 and 4) (Campbell et al., 1990).

When sampled by air entrainment, aphid oviparae (sexual females) have been shown to release the two iridoid pheromone components in a ratio that is relatively species-specific (Table 1); however, there are some studies that indicate that ratios change with aphid age (Hardie et al., 1990; Jeon et al., 2003; Goldansaz et al., 2004). Males generally show strongest behavioural responses towards their typical conspecific ratio but some laboratory studies have reported response to a broad range of synthetic ratios and even to a pheromone ratio released by a different species of aphid (Petterson, 1971; Marsh, 1975;
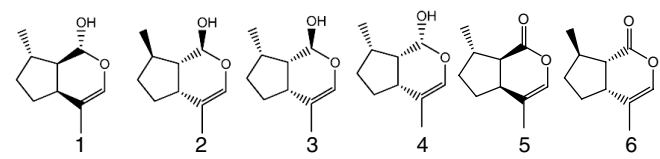
Fig. 1. Chemical structures. 1, (1R,4aS,7S,7aR)-nepetalactol; 2, (1S,4aR,7R,7aS)-nepetalactol; 3, (1S,4aR,7S,7aS)-nepetalactol; 4, (1R,4aR,7S,7aS)-nepetalactol; 5, (4aS,7S,7aR)-nepetalactone; 6, (4aR,7R,7aS)-nepetalactone.

Dawson et al., 1990; Hardie et al., 1990; Steffan, 1990; Lilley and Hardie, 1996). A similar lack of pheromone specificity has also been reported from field trapping, as a given ratio sometimes catches many species of male aphid (Boo et al., 2000; Goldansaz et al., 2004). If nepetalactol and nepetalactone are ubiquitous aphid sex pheromone components, as has been suggested (Dawson et al., 1990), ratio combinations are limited and additional mechanisms for species isolation are likely to exist. Numerous factors have been proposed and these include the use of different blends of diastereoisomers or enantiomers of nepetalactol and nepetalactone (Campbell et al., 1990; Hardie et al., 1997), the presence of unidentified additional constituents (Guldemond et al., 1993; Lilley and Hardie, 1996), interactions with host-plant volatiles (Petterson et al., 1970a; Campbell et al., 1990; Hardie et al., 1994b; Losël et al., 1996), differences in oviparae colour and species-specific movements (Steffan, 1990), genitalia incompatibility, spatial and seasonal separation of populations (Hardie et al., 1990), and circadian or diel separation of pheromone release (Guldemond and Dixon, 1994; Thieme and Dixon, 1996).

It is known for Lepidoptera (butterflies and moths) that the timing of sex pheromone release within the daily light:dark cycle can be an important component for effective communication that also parallels appropriate behavioural responsiveness in the conspecific receiver. There are typically species-specific peaks in pheromone release during the light:dark cycle; however, exact onset and duration is modulated by age and abiotic factors (Dreisig, 1986; Delisle and McNiel, 1987; McNeil, 1991; Rosén, 2002; Silvegren et al., 2005; Mazor and Dunkelblum, 2005). Some have reported that aphids release sex pheromone only during the photophase (Marsh, 1972; Eisenbach and Mittler, 1980; Eisenbach and Mittler, 1987) and in sympatric situations there is some evidence that pheromone release during the photophase is divided according to the aphid sub-species (Guldemond and Dixon, 1994; Thieme and Dixon, 1996). However, in aphid studies so far, inferences about sex pheromone release throughout the day have been indirect and categorical and are drawn from observations of female calling behaviour and/or male responses (e.g. Marsh, 1972; Eisenbach and Mittler, 1980; Eisenbach and Mittler, 1987; Guldemond and Dixon, 1994; Thieme and Dixon, 1996). Although technically challenging, direct measurements of sex pheromone chemicals are continuous and quantitative. Such a direct approach has recently been attempted by Jeon et al. (Jeon et al., 2003) but measurements were limited to a single day and resolution was at 2 h intervals. With a greater understanding of sex pheromone release, physiological and evolutionary aspects of aphid biology can be addressed, implications for natural enemies who exploit this pheromone can be evaluated, and improvements to aphid management approaches facilitated (Hardie et al., 1991; Hardie et al., 1994a; Powell et al., 1993; Powell et al., 1998; Gabryś et al., 1997; Boo et al., 1998; Zhu et al., 1999).

The rosy apple aphid [Dysaphis plantaginea (Passerini); Homoptera, Aphididae] is the most serious pest of apple in Europe (Blommers, 1994). It is a host-alternating species that, over the summer, reproduces parthenogenetically on herbaceous Plantago lanceolata L. (Plantaginaceae). In early autumn, a sexual generation is produced. First to develop are the gynoparae (winged females), who return to the apple tree, where they lay the oviparous stage. Once mature, the oviparae release a sex pheromone that approximately corresponds with the

Table 1. *Ratios of nepetalactol (1 or 2) to nepetalactone (5 or 6) [or *nepetalactol diastereoisomers (3 and 4)] found in entrainments collected from oviparae of different aphid species*

Common name	Species name	Ratio (ol:one)	Reference
Cabbage aphid	Brevicoryne brassicae	0:1	(Gabry et al., 1997)
Black-berry cereal aphid	Sitobion fragariae	0:1	(Hardie et al., 1992)
Grain aphid	Sitobion avenae	(trace) 0:1	(Lilley et al., 1995)
Spiraea aphid	Aphis spiraecola	1:2[a]	(Jeon et al., 2003)
Peach aphid	Tuberocephalus momonis	1:4	(Boo et al., 2000)
Vetch aphid	Megoura viciae	1:5–1:12 (age effect) 1:6	(Hardie et al., 1990) (Dawson et al., 1990)
Spiraea aphid	Aphis spiraecola	1:6–1:8 (age effect)[b]	(Jeon et al., 2003)
Black bean aphid	Aphis fabae	1:29	(Dawson et al., 1990)
Pea aphid	Acyrthosiphon pisum	1:1	(Dawson et al., 1990)
Peach-potato aphid	Myzus persicae	1.5:1	(Dawson et al., 1990)
Rosy apple aphid	Dysaphis plantaginea	3.7:1 3.7:1–3.3:1 (age effect)	Current study
Potato aphid	Macrosiphum euphorbiae	4:1–2:1 (age effect)	(Goldansaz et al., 2004)
Greenbug	Schizaphis graminum	8:1	(Dawson et al., 1988)
Currant aphids	Cryptomyzus spp.	30:1	(Guldemond et al., 1993)
Bird cherry-oat aphid	Rhopalosiphum padi	1:0	(Hardie et al., 1994b)
Damson hop aphid	Phorodon humuli	1*:0	(Campbell et al., 1990)

All species belong to the sub-family Aphidinae. [a]Field-collected oviparae; [b]laboratory reared oviparae.

development of winged males on *P. lanceolata*. Males are attracted and mate with the oviparae, who then lay cold-hardy overwintering eggs on the apple tree. Further details of aphid life cycles can be found in Dixon (Dixon, 1998). In the present study, we determine a typical ratio of nepetalactol and nepetalactone for *D. plantaginea*, confirm the enantiomeric form of the iridoids and present uninterrupted hourly sampling data on the pheromone released from a group of same-aged oviparae over a 20-day period. A simple and inexpensive sequential sampling device was developed to examine release periodicity through consecutive diel cycles and also to investigate the stability of ratios throughout the day and over increasing aphid age. This is the first report on the full identification and ratio of the two major components of *D. plantaginea* sex pheromone and is also the most detailed temporal study on sex pheromone release from any species of aphid.

Materials and methods

Aphids and plant material

All apple material, *Malus silvestris* Mill. (Rosaceae), and aphids were collected from an orchard at Leckford Fruit Farm (Leckford Estate, Leckford, Hampshire, UK). Gynoparae (winged females) and oviparae (sexual females) of *D. plantaginea* were collected during the autumn of 2003 and 2004.

In 2003, an excised branch and detached leaves heavily infested with mixed-aged oviparae of *D. plantaginea* and uninfested plant material were collected from apple, cv. Braeburn overgrafted onto Katy on a MM106 rootstock. In addition, a same-aged cohort of oviparae (*N*=35) were reared outdoors from gynoparae of *D. plantaginea* that were enclosed overnight by mesh on a branch of potted apple, cv. Cox's Orange Pippin on MM106 rootstock. Samples collected in 2003 from the oviparae were used to determine the ratio of the two major components of *D. plantaginea* sex pheromone. Samples from the detached leaves were used for full structural identification.

In 2004, gynoparae (*N*=125) were collected in the apple orchard cv. Cox's Orange Pippin on MM106 and were placed onto the leaves of a fresh branch of apple (cv. Cox's Orange Pippin, 45 cm long) standing in a pot of distilled water. This branch and gynoparae were then enclosed in mesh and placed at the onset of scotophase in a controlled environment (CE) room that mimicked outside conditions [L:D 12 h:12 h; photophase 09.00–21.00 h BST (British summer time), 16±0.5°C; scotophase 21.00–09.00 h BST, 12±0.5°C]. After 24 h, the gynoparae were removed, leaving a cohort of same-aged oviparae (*N*=95). The mesh bag was replaced and the branch with oviparae returned to the CE room. When adult, these oviparae were used in the temporal study.

Collection of pheromone for identification and ratios (aphids collected in 2003)

All volatiles were trapped onto Porapak Q 50/80 (50 mg; Supelco, Bellefonte, PA, USA) held in glass tubing (5 mm o.d.) by two plugs of silanised glass wool. The Porapak Q was conditioned by washing with redistilled diethyl ether (5 ml) and heating at 132°C for 2 h under a stream of nitrogen. After the air entrainment, the volatiles were eluted from the Porapak with redistilled diethyl ether (750 μl) and samples were stored in a freezer (–22°C).

The infested apple branch and detached leaves were placed in two glass vessels with volumes of 2 litres and 500 ml, respectively. Air that had been purified by passage through an activated charcoal filter (BDH, Poole, Dorset, UK; 10–14 mesh, 50 g) was pushed into (800 ml min⁻¹) and pulled out of (700 ml min⁻¹) the vessels. The difference in air volume passed outwards at seals that were not airtight. Volatiles were entrained onto Porapak Q for 4 days. The same procedure was performed on an uninfested branch and leaves.

The same-aged adult oviparae (*N*=35) reared on the potted Cox's plant were moved onto a single leaf that was enclosed by a cleaned cylindrical glass vessel that had a slot for the petiole (length 8 cm, depth 5 cm). This slot was plugged with a ball of silanised glass wool, which prevented aphids from escaping but allowed excess air volume to pass outwards. For comparison, a second vessel enclosed an uninfested leaf. Air that had been purified by passage through an activated charcoal filter (BDH, 10–14 mesh, 50 g) was pushed into (400 ml min⁻¹) and pulled out of (350 ml min⁻¹) both vessels. Volatiles were entrained onto Porapak Q for 24 h.

The sequential sampling machine

The mains power source (240 V AC) was split, and half was transformed to power the 24 V DC circuit. Twelve 24 h timers (ETU2000; Timeguard Ltd, London, UK) and 240 V AC DPDT power relays (Maplin Electronics, Wombwell, Yorkshire, UK) were wired as pairs, in parallel, into the 240 V circuit. Twelve 24 V two-way solenoid valves (Valeader Pneumatics Ltd, Cambridge, UK) were wired in parallel into the 24 V DC circuit. Since relays interrupted power to individual solenoid valves, the valves were normally in a closed position. However, when activated by its respective timer, the relay would complete the circuit for an individual valve and thus cause it to open. When in an open state, each valve allowed air to be pulled through one of the 12 sampling lines that it regulated; by using flow-meters, flow through each arm was confirmed to be equal. The electronic components were arranged inside a wooden box and timers surrounded a central piece of glassware (the distribution chamber) (Fig. 2). The distribution chamber was a tube of glass (12 mm i.d.) sealed at one end with six paired side arms, each with a PTFE tap (4 mm hole). The open top end of the chamber was the inlet through which volatiles would be drawn and, depending on which valve was activated, the volatiles would be pulled out through one of the side arms.

Hourly sampling of pheromone release for the temporal study (aphids collected in 2004)

In preliminary trials conducted using the same CE room conditions as described above, oviparae took 12 days to develop to the adult stadium. When the experimental cohort of 95 oviparae was 12 days old, they were transferred onto a fresh branch of apple (cv. Cox's Orange Pippin) standing in distilled water. This ovipara-infested branch was then enclosed in a borosilicate glass vessel with ground glass base (height 30.8 cm, diameter 11.1 cm) that had a threaded outlet at the top and a threaded inlet near the base (Stewart-Jones and Poppy, 2006). The open end at the bottom of the vessel was closed using two

Glass distribution chamber with PTFE taps

Inlet tube for volatiles

Poropak Q sampling tube linked to solenoid valve

LED indicating solenoid open/closed state
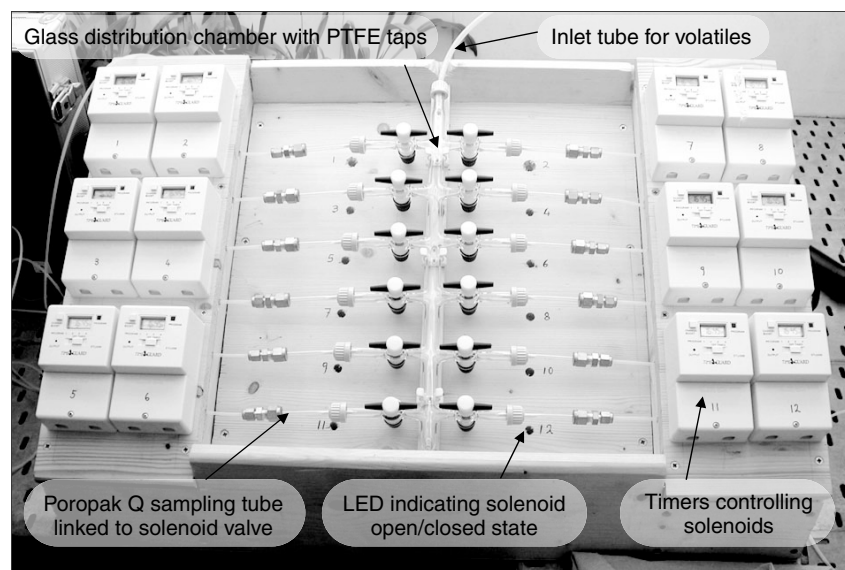
Timers controlling solenoids

Fig. 2. Photograph of the sampling device with 12 sampling tubes fitted to the side arms of the glass distribution chamber. Teflon tubing (6 mm) entering at the top of the glass chamber leads to the glass aeration chamber containing the oviparae.

semicircular aluminium plates that, when put together, left a small hole in the middle for the apple stem to pass through, and any gap was sealed with balls of PTFE tape. Air that had been purified by passage through activated charcoal (BDH, 10–14 mesh, 50 g) was then pushed in at the inlet ($1.2\,l\,min^{-1}$) and the aphids were allowed to settle on the new branch in the chamber for 24 h. Excess air volume passed outwards through the gap loosely sealed by PTFE tape.

The sequential hourly sampling of headspace started at the beginning of the second day of the adult stadium at the onset of scotophase (21.00 h BST). Timers were synchronised with the CE room, and each was programmed to open their respective solenoid valve for 1 h at two different times 12 h apart (e.g. solenoid 1 09.00–10.0 h BST and 21.00–22.00 h BST). Sampling tubes therefore had to be changed once every 12 h before the sequence started again. PTFE taps were closed for changeover of tubes so that the sample being collected was not disrupted.

The sampling machine was connected to the outlet of the glass vessel by a short length of PTFE tubing (6 mm i.d.), and the air was pulled out of the vessel at a rate of $1\,l\,min^{-1}$ through one of the 12 sampling tubes containing Porapak Q positioned on the distribution chamber. This system of greater inlet flow than outlet sampling, although causing a loss of 1/6th of the headspace (corrected for in quantitation), ensured that the glass chamber remained uncontaminated by outside sources. Samples were captured onto Porapak Q, eluted with redistilled diethyl ether (750 μl), and an internal standard of dodecane (400 ng) was added to each. Samples were reduced to 40 μl under a stream of purified nitrogen before analysis.

Laboratory calling behaviour

Pheromone-releasing plaques, or pseudorhinaria, are located on the hind tibia of aphids, and calling behaviour is characterised by the raising of hind legs and abdomen (Petterson, 1970b). In the laboratory, the number of oviparae displaying calling behaviour was recorded every hour on three different days. When the oviparae were 14, 18 and 21 days into the adult stadium, starting at 09.00 h (12 h of scotophase or 0 h of photophase), a count of how many oviparae were calling was made. Every hour, further counts were made until the onset of scotophase (21.00 h), and the last count was made 5–10 min into the scotophase using a red light.

Analysis of pheromone samples

Samples entrained from the infested branch and leaves in 2003 were analyzed (1 μl) by gas chromatography (GC) on non-polar (HP-1, 50 m×0.32 mm i.d.×0.52 μm film thickness; J%W Scientific, Falcon, CA, USA) and polar (HP-wax, 30 m×0.23 mm i.d.×0.5 μm film thickness) capillary columns, using a 6890 GC machine (Agilent Technologies UK Ltd, Stockport, Cheshire, UK) fitted with a cool on-column injector and a flame ionisation detector (FID). The oven was kept at 30°C for 1 min, heated at $5°C\,min^{-1}$ to 150°C and then $10°C\,min^{-1}$ to 250°C (220°C for the wax column), where it was maintained for 20 min. The carrier gas was hydrogen.

Samples entrained in 2003 from oviparae reared from gynoparae and a selection of samples collected in 2004 for the temporal study were initially analysed on a Hewlett-Packard 5890 Series II GC (Hewlett-Packard Co., Palo Alto, CA, USA) linked to a Hewlett-Packard 5971 Quadrupole Mass Selective Detector ionising by electron impact at 70 eV. The column used was a non-polar fused capillary column (HP-1MS, 30 m×0.25 mm i.d.×0.25 μm film thickness). The carrier gas was helium (constant 62 034 Pa) and oven temperature was held at 40°C for 2 min then programmed at $5°C\,min^{-1}$ to 150°C then $10°C\,s^{-1}$ to 250°C and held for 16 min. Samples (1 μl) were injected into a split/splitless injector (220°C, splitless 1 min) and data were captured and analysed by Enhanced ChemStation software (v. A.03.00; Hewlett-Packard Co.). After tentative identification of nepetalactol and nepetalactone, samples were also run on a Hewlett-Packard 5890 Series II GC fitted with split/splitless injector (220°C, splitless 30 s) and FID. This instrument was used for analysis of samples from the temporal study. The principal capillary column was non-polar (SOLGEL-1, 30 m×0.32 mm i.d.×0.25 μm film thickness, HP-1 equivalent; SGE, Melbourne, Victoria, Australia) and for confirmation a polar column was used (CARBOWAX, 30 m×0.32 mm i.d.×0.25 μm film; Alltech, Stamford, Lincolnshire, UK). The oven was kept at 40°C for 1 min, heated at $10°C\,min^{-1}$ to 250°C (220°C for Wax) and held for 2 min. The carrier gas was helium (constant $35\,cm\,s^{-1}$). Samples were injected (2 μl) in splitless mode and data were captured using a 35900 HPIB interface (Hewlett-Packard Co.) and analysed using HP 3365 Series 2 ChemStation (v. A.03.01).

Tentative identifications were confirmed to the enantiomeric pair by peak enhancement with authenticated samples of

(1*R*,4a*S*,7*S*,7a*R*)-nepetalactol and (4a*S*,7*S*,7a*R*)-nepetalactone on both non-polar (HP-1 or SOLGEL-1) and polar (HP-wax or CARBOWAX) columns. Quantities were initially determined using a multiple point external standard method but for the temporal study an internal standard was used. For the temporal study, when peaks were very small or not detected, samples were further concentrated using nitrogen before reinjection.

Confirmation of nepetalactol enantiomer by derivatisation and nuclear magnetic resonance (NMR)

Full structural identification of the enantiomer of nepetalactol was achieved by microscale NMR spectroscopy after derivatisation. Air entrainment samples of *D. plantaginea* oviparae, containing 84 µg of nepetalactol (1) (GC approximation) were concentrated under a stream of purified nitrogen and dissolved in dichloromethane (0.5 ml) under nitrogen. A solution of (*S*)-(+)-α-methoxy-α-(trifluoro-methyl)phenylacetyl chloride (40 mg, 0.16 mmol) and pyridine (25 µl) in dichloromethane (0.5 ml), prepared under nitrogen, was added, together with a few crystals of dimethylaminopyridine, and the reaction stirred overnight. The solvent was then removed under a stream of nitrogen and the residue partially redissolved in 10% diethyl ether in petroleum ether (40–60°C boiling fraction). The insoluble material was discarded by decanting off the soluble portion. The solvent was then removed under a stream of nitrogen and the residue redissolved in deuteriochloroform for NMR analysis. ^{1}H, ^{13}C and ^{19}F NMR spectroscopy was performed using a Bruker 500 Avance NMR spectrometer (Billerica, MA, USA) with ^{1}H referenced to CDCl$_3$ (7.25 p.p.m.), ^{13}C to CDCl$_3$ (77.0 p.p.m.) and ^{19}F to CFCl$_3$ (0 p.p.m.). Quantitative ^{1}H NMR spectroscopy was performed using a pulse angle of 30°, an acquisition time of 5T1 (with T1 measured to be 2.5 s) and a delay of 5 s. The NMR analysis was compared to diastereoisomeric derivatives from the two enantiomers of synthetic nepetalactol (1 and 2), and full details are described elsewhere (Goldansaz et al., 2004).

Data analysis

Pheromone quantity data were ln (*X*+1) transformed, and percentages of components were arcsine $\sqrt{X}$ transformed. All data were checked for normality and homogeneity of variance using the Anderson-Darling and Levene's tests. Paired Student's *t*-tests were used to determine whether pheromone release was significantly increased during photophase relative to the preceding scotophase and whether there were significant decreases during scotophase relative to the preceding photophase. In order to test for peaks in sex pheromone release within the photophase, time periods of 0–3, 3–6, 6–9 and 9–12 h into photophase were ascribed, and mean release rate for each was calculated and subjected to analysis of covariance (ANCOVA) in which maximum pheromone release for that photoperiod was factored as the covariate to account for age influences. The same test was used to investigate scotophase changes in pheromone quantity. The effect of aphid age on percentage nepetalactol in the mixture was investigated by one-way analysis of variance (ANOVA). A two-way ANOVA tested for scotophase and photophase differences in ratios. *Post-hoc* Tukey's tests were used to identify where significant differences lay within the above. All data were analysed on Minitab 13.1 (Minitab Inc., State College, PA, USA), and differences were considered significant if $P \leqslant 0.05$.

Results
Identification of components and determination of ratios

Peak enhancement on achiral polar and non-polar columns with synthetic standards confirmed that nepetalactol (1 or 2) and nepetalactone (5 or 6) were present in volatile collections. Diastereoisomeric Mosher's ester derivatives were prepared separately from (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (1) and its enantiomer (1*S*,4a*R*,7*R*,7a*S*)-nepetalactol (2). These were analysed by NMR, and characteristic signals from the H-1 and H-3 proton resonances of two synthetic standards were seen (Fig. 3). The air entrainment sample of *D. plantaginea* oviparae was derivatised in an identical fashion and shown to contain H-1 and H-3 resonances indicative of (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (1) (Fig. 3), and no signal from its enantiomer (1*S*,4a*R*,7*R*,7a*S*)-nepetalactol (2) was detected.

The mean ratio of the two identified components, based on entrainment samples taken from four different batches of *D. plantaginea* from two different apple cultivars over two years, was calculated as 3.7:1 (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol: (4a*S*,7*S*,7a*R*)-nepetalactone (Table 2).

Diel patterns in pheromone release

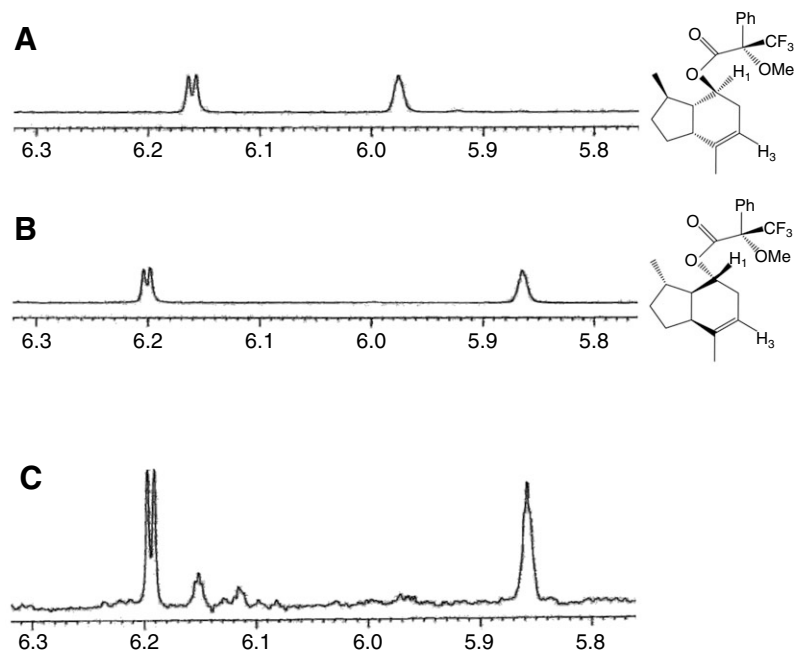The data show a distinct cycling in the levels of pheromone collected (as measured by summed

Fig. 3. ^{1}H NMR spectrum showing H-1 and H-3 resonances: (A) Mosher's ester of (1*S*,4a*R*,7*R*,7a*S*)-nepetalactol, (B) Mosher's ester of (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol and (C) derivatised air entrainment sample from *Dysaphis plantaginea*. The oviparae-produced compound is identical to the Mosher's ester of (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (B).

Table 2. *Ratios of (1R,4aS,7S,7aR)-nepetalactol (1) and (4aS,7S,7aR)-nepetalactone (5) collected from different batches of Dysaphis plantaginea oviparae*

Life-stage collected	Apple cultivar	Year	Analytical replicates Ratio ol:one	Batch mean Ratio ol:one
Oviparae	Infested branch of Braeburn	2003	3.66:1 3.67:1 3.68:1	} 3.67:1 (78.6%)
Oviparae	Infested excised leaves of Braeburn	2003	3.58:1 3.62:1 3.64:1	} 3.61:1 (78.3%)
Gynoparae	Collected from Braeburn. Oviparae reared on Cox	2003	3.56:1 3.99:1 3.94:1	} 3.83:1 (79.3%)
Gynoparae	Collected from Cox and oviparae reared on Cox	2004	Mean from 95 oviparae. Adult stadium aged 2–14 days	} 3.69:1 (78.7%)
Overall mean	Braeburn and Cox	2003/2004		3.70:1 (78.7%)

quantities of nepetalactol and nepetalactone), with significant differences between the photophase and scotophase (Fig. 4) (both analyses $P<0.001$, $N=20$). With the onset of photophase, pheromone production increased gradually for the first three hours of photophase and thereafter remained at high levels until the end of the photophase sampling ($P<0.001$, $N=20$; 0–3 h lower than 3–6, 6–9 or 9–12 h). With the onset of scotophase, pheromone production decreased rapidly, although low levels were detected throughout the scotophase ($P<0.001$, $N=20$; 0–3 h higher than 3–6, 6–9 or 9–12 h).

Total quantity of pheromone collected over consecutive scotophases and photophases

The quantity of pheromone collected during scotophase increased over days 2–6 of the adult stadium (Fig. 5). No pheromone was detected on the second day, and the highest scotophase levels were recorded on the eighth day. Total quantities then decreased slightly (days 9 and 10) and remained low until the end of the experiment (days 10–21). The quantity of pheromone collected during photophase increased rapidly over days 2–6 of the adult stadium (Fig. 5), with greatest quantities recorded between days 5 and 12, before declining from day 13 onward. During days 3–21 of the adult stadium, there was on average 3.2 times more pheromone collected during the photophase than in the preceding scotophase.

Greatest total quantity of pheromone (24 h periods: scotophase + photophase) was recorded between days 5–12 of the adult stadium, with the highest level recorded on the eighth day. On the eighth day of the adult stadium, each ovipara released on average 99.6 ng of sex pheromone. During the photoperiod of the eighth day of the adult stadium, we also recorded the maximum mean release rate in our experiment: 8.4 ng per ovipara per hour (8–9 h of photophase).

Changes in the ratio of nepetalactol:nepetalactone

Over adult stadium ages 2–21 days, the cohort of 95 oviparae produced a total of 76.6 μg of (1R,4aS,7S,7aR)-nepetalactol (1) and 22.9 μg of (4aS,7S,7aR)-nepetalactone (5), thus giving a blend of 3.34:1 (77.0% nepetalactol). However, analysis found a significant age effect ($P<0.001$) (Fig. 6A), with ratios remaining steady until the fourteenth day of the adult stadium, and thereafter the relative amount of nepetalactol in the mixture declined. Total quantity of pheromone based on ages 2–14 days of the adult stadium were 58.4 μg and 15.8 μg for 1 and 5, respectively, which gives a ratio of 3.69:1 (78.7% nepetalactol).

There was also a significant effect of scoto/photophase on ratios recorded ($P=0.009$) (Fig. 6B). Higher levels of nepetalactol (mean 3.4%) were recorded in the mixture during scotophase compared with photophase.

Observations of calling behaviour

On all three days that behaviour was recorded, a similar pattern of characteristic behaviours attributed to sex-pheromone release was observed (Fig. 7). In the laboratory, no calling behaviour was exhibited at the onset of photophase (12 h of scotophase, also 0 h of
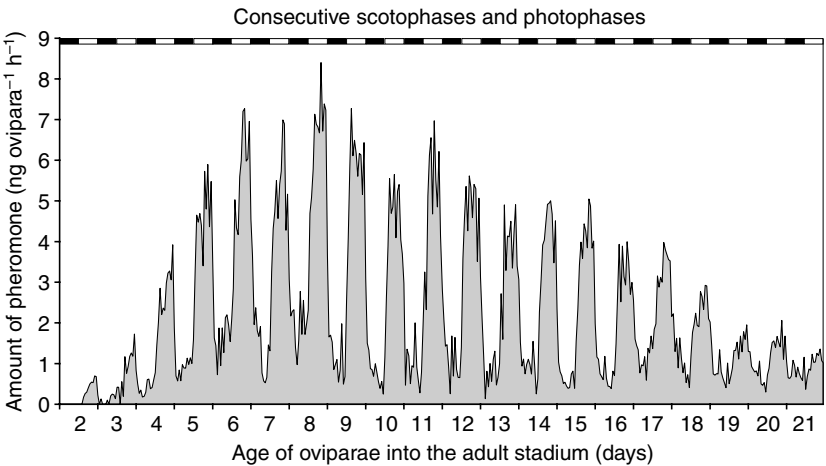

Fig. 4. Mean quantity of pheromone [sum of (4aS,7S,7aR)-nepetalactone and (1R,4aS,7S,7aR)-nepetalactol] released per ovipara over 20 consecutive scoto- and photophases. Samples were taken hourly and were started at the onset of scotophase on the second day of the adult stadium.
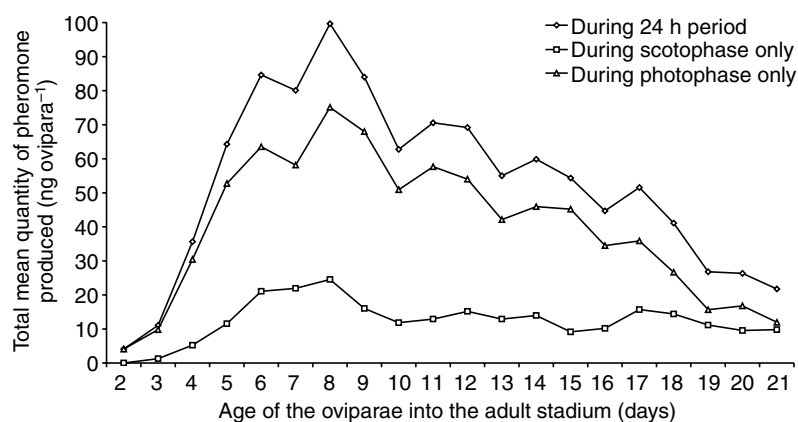
Fig. 5. Mean total quantity of pheromone released per ovipara in either the whole 24 h period, the scotophase or the photophase. Sampling from adult ages 2–21 days.

photophase). During the first 3 h of photophase (0–3 h), an increasing number of oviparae were observed to display calling behaviours; for the remainder of the photophase (4–11 h), >90% of the oviparae were exhibiting calling behaviour. Whilst >90% of oviparae were calling, oviparae were never observed all calling at the same time. The last observation of the day was made 5–10 min into scotophase, and no oviparae were observed to be calling at this time. It was assumed that throughout the rest of the scotophase there was no calling behaviour; this is what others have reported in *Aphis spiraecola* (e.g. Jeon et al., 2003).

Discussion

Identification of components and determination of ratios

Like many other species of aphid in the subfamily Aphidinae (Table 1), *D. plantaginea* oviparae release two iridoid components in their sex pheromone. We have determined conclusively that only one enantiomer of nepetalactol, (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (1), is released from *D. plantaginea*. Exact biosynthesis of these compounds is unknown but it has been suggested that 1 is produced from a glycoside precursor and a portion of 1 is selectively oxidised to

the nepetalactone (Dawson et al., 1990; Pickett et al., 1992). Since 1 and the nepetalactone are believed to be on the same biosynthetic pathway, and given that *D. plantaginea* produces 1, the enantiomer of nepetalactone is most likely to be (4a*S*,7*S*,7a*R*)-nepetalactone (5) because inversion of three chiral centres is unlikely to occur easily. A typical ratio of components for *D. plantaginea* would be 3.7:1 (1:5) and, in an olfactometer, male *D. plantaginea* are attracted to a synthetic mixture of these components (J.D.F., unpublished data).

Diel patterns in pheromone release

The sequential sampling device has enabled us to gather the most in-depth dataset on sex pheromone release for any species of aphid. The data presented here substantiates, in part, previous conclusions made by other workers who recorded calling behaviour only during the photophase (Marsh, 1972; Eisenbach and Mittler, 1987; Guldemond and Dixon, 1994; Thieme and Dixon, 1996; Jeon et al., 2003). We observed no calling behaviour at the onset of photophase or shortly after the onset of scotophase but did record intense calling behaviour during most of the photophase. Following hourly analysis of pheromone release, it can be seen that there is a clear cycling in the release of sex pheromone from *D. plantaginea* oviparae. High levels of pheromone were always associated with the photoperiods, there was then a rapid drop, and low levels were detected throughout the scotophase. Other workers have reported similar findings; Dawson et al. (Dawson et al., 1990) and Jeon et al. (Jeon et al., 2003) both collected headspace samples from oviparae (*Schizaphis graminum* and *A. spiraecola*, respectively) during the scotophase when calling behaviour was not exhibited, and both detected pheromone in low quantity relative to photophase measurements. These scotophase measurements could have been contamination left over from the previous photophase; for example, on the glass parts of the apparatus. However, in our experiment, adsorption onto the glass with continual slow release would be unlikely over such extended periods with continuous aeration (Stewart-Jones and Poppy, 2006). Another possibility is that pheromone was absorbed into the waxy cuticles of the apple leaves or the aphids themselves. Again, this explanation is unlikely because pheromone levels would be expected to tail and gradually decrease with time into the scotophase. Rather than decreasing during scotophase, in the first five days of the experiment pheromone levels appeared to be increasing during scotophase (Fig. 4) and this might reflect a gradual development of biosynthetic pathway capacity for pheromone production as the aphids matured. An important factor to
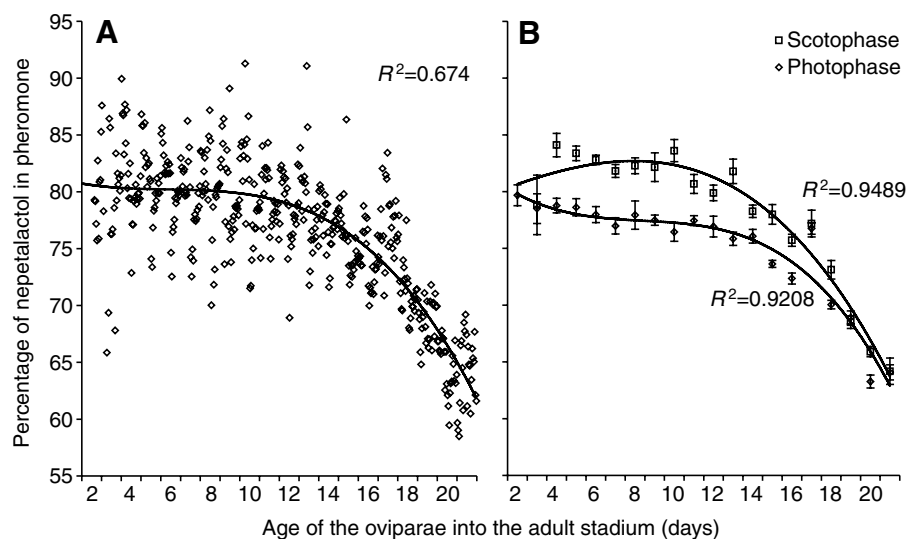

Fig. 6. (A) Component ratios sampled at hourly intervals. (B) Mean daily ratios for scoto- and photophases. Oviparae sampled were 2–21 days into adult stadium.
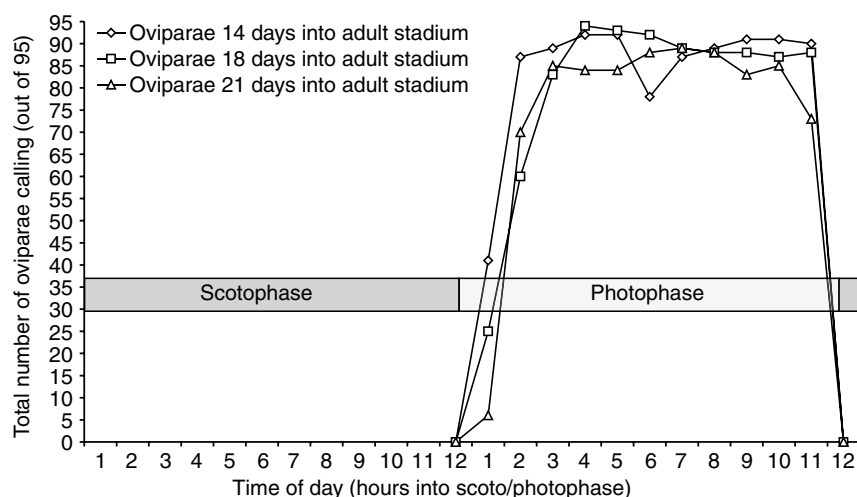
Fig. 7. Behavioural observations of the oviparae under air entrainment. Hourly counts of the number of individuals that were displaying calling behaviours on three different days (adult ages 14, 18 and 21 days).

remember relates to the morphology of oviparae, who produce the pheromone in their hind tibia from glandular cells just below a highly thinned area of cuticle that is traversed by many large secretory canals (Petterson, 1970b; Zeng et al., 1992). In contrast to most female Lepidoptera, who physically expose the sex pheromone gland from within their bodies, aphid oviparae have no such behavioural control over release. Essentially what is biosynthesised by the glandular cells of oviparae probably diffuses out with little other control. Thus, whilst calling behaviour in Lepidoptera can regulate release at a secondary level to biosynthesis, for example in *Trichoplusia ni* (Noctuidae) (Tang et al., 1989), calling behaviour in aphid oviparae probably serves simply to aid dispersal. Although unknown for aphids, changes in biosynthesis are likely to be mediated by neurohormones in the PBAN (pheromone biosynthesis activating peptide) family, as has been found to be the case in five other insect orders (Rafaeli and Jurenka, 2003). Therefore, the changes we recorded are most likely the result of pathway upregulation and downregulation. While we know that pheromone is biosynthesised *de novo* (Pickett et al., 1992), our results and those of others (Dawson et al., 1990; Jeon et al., 2003) suggest that, although downregulated during scotophase, biosynthesis does not cease completely. Future study into pathway regulation might focus on gene expression analysis of pheromone biosynthesis genes using genomic techniques such as aphid microarrays or real time-polymerase chain reaction (rt-PCR).

From the current study, what is not known is whether pheromone production is truly under circadian control, i.e. is it driven by an endogenous clock or is it driven by external stimuli only? Others using behavioural measures have concluded that pheromone release is driven by an endogenous circadian rhythm (Marsh, 1972; Eisenbach and Mittler, 1980). By analysing pheromone collected in headspace from three different-aged aphids under constant darkness, Jeon et al. also concluded that pheromone release was under circadian control (Jeon et al., 2003).

Diel peaks in pheromone release

Other than a photophase–scotophase pattern in pheromone release, there is no laboratory evidence to suggest that *D. plantaginea* oviparae have narrow time periods within the photophase during which particularly high levels of sex pheromone are released. In the autumns of 2003, 2004 and 2005, mating in the field was observed throughout the photoperiod and such observations would suggest that distinct periods of high pheromone release do not occur in the field either. *D. plantaginea* were observed mating in the field at 09.30, 10.45, 11.15, 11.20, 13.00, 13.20, 14.50 and 16.30 h BST (A.S.-J., unpublished data).

During photophase, laboratory-recorded patterns of pheromone release were in agreement with laboratory observations of calling behaviour. Nearly all oviparae were calling 3 h into the photophase and continued to do so until the onset of scotophase. Similarly, Marsh concluded that maximum pheromone release is reached 2 h into the photophase (Marsh, 1972). Although Marsh found that calling in *M. viciae* waned before the onset of scotophase in old and young oviparae (Marsh, 1972), in the current study this was not found to be the case. In the laboratory under constant conditions, calling might indicate release of pheromone; however, there are significant age influences on quantity (see below). In the field, where rain or wind can curtail calling behaviour (Goldansaz and McNeil, 2003), we stress that, due to the morphology of aphid oviparae (discussed above), an observed absence of calling behaviour may not necessarily indicate no release of pheromone. In rain or high wind conditions, which might occur regularly in the field, release of pheromone without calling could still serve to attract males in the immediate vicinity. Aphids are relatively weak fliers compared with Lepidoptera and it has been suggested that when trying to locate oviparae at close range, upwind walking by the males is particularly important and this is only punctuated by brief flights when weather conditions are favourable (Goldansaz and McNeil, 2006). If aphids such as *D. plantaginea* were to restrict sex pheromone release to a narrow time period within the photophase, the probability of a successful mating would be dramatically reduced given weather variability. We therefore think that, in contrast to Lepidoptera, most aphids are unlikely to have temporally narrow periods of very high sex pheromone release, and for *D. plantaginea* this is supported by our data. In a diverging species complex or races that coexist sympatrically on the same plant, there are reports of temporally narrow periods of calling behaviour (e.g. Guldemond and Dixon, 1994; Thieme and Dixon, 1996). In these particular situations, temporal partitioning might exist, but this would need to be verified by actual measurements of pheromone release.

Effect of age on pheromone quantity and calling behaviour

Oviparae of potato aphids (*Macrosiphum euphorbiae*) do not display calling behaviour until 2–3 days into the adult stadium (Goldansaz and McNeil, 2003), and the pheromone components

were not detected until the second day into the adult stadium for the vetch aphid (*M. viciae*) (Hardie et al., 1990). Our first day of sampling was on the second day of the adult stadium, and low levels of pheromone were detected. Data show that sex-pheromone release in *D. plantaginea* was highest on the eighth day of the adult stadium before gradually decreasing again. This is consistent with other behavioural studies that found male *M. viciae* responsiveness to calling oviparae to be strongest when oviparae were 6 days into the adult stadium (Marsh, 1972). Also, *Aphis fabae* calling behaviour was observed to intensify until day 8 of the adult stadium (Thieme and Dixon, 1996). Similarly, chemical studies that sampled for 24 h periods from *M. viciae* found pheromone release to be highest on day 6 (Hardie et al., 1990). These slight variations in age-related maximal pheromone output/attractiveness might be species characteristics or may be due to different temperatures used in rearing.

The maximum pheromone output for the vetch (*M. viciae*) and spiraea (*A. spiraecola*) aphids has been reported to be over 200 ng per ovipara per day. However, for both species, more typical levels were in the region of 100–150 ng per ovipara per day (Hardie et al., 1990; Jeon et al., 2003). For *D. plantaginea*, the maximum we calculated was 99.6 ng per ovipara per day, with typical levels in the region of 60–90 ng per aphid per day. Even after 3 weeks, *D. plantaginea* were still calling and able to release pheromone. However, the quantity of pheromone being released was in decline and there may have been only limited release beyond the 3-week period. This long period over which adult *D. plantaginea* oviparae can release sex pheromone might be particularly relevant to mating success in the field because, in autumn, extended periods of poor weather, which delays mate location by the males, may be quite common.

Comparison of our recorded calling behaviour and the quantified levels of pheromone released showed a poor relationship. Whereas observations of aphids aged 14, 18 and 21 days into the adult stadium showed no significant difference in the number of oviparae calling (Fig. 7), direct measurement of pheromone during these photophases found significant differences (means of 45.9, 26.7, 12.0 ng per aphid during respective photophases). Similarly, Jeon et al. noted intense calling behaviour in 3-week-old oviparae but detected no pheromone (Jeon et al., 2003). Again, these examples reinforce the idea that calling behaviour is an unreliable indicator of quantity of pheromone being released.

Effect of age on (1R,4aS,7S,7aR) nepetalactol:(4aS,7S,7aR)-nepetalactone ratios

Unlike another study, which reported a significant fluctuation in ratios with age of *M. viciae* (Hardie et al., 1990), we did not find significant changes in iridoid ratios for *D. plantaginea*, and only a slight decrease in the relative amount of nepetalactol was recorded when the aphids were more than 14 days into the adult stadium. By sampling 24 times per day, we have certainty in our measurements, and the low variability in our dataset does support the idea that species-specific ratios are an important component of intraspecies recognition. Interestingly, the two other studies in which ratios have been quantified over age also report a slight decrease in the relative amount of nepetalactol

with age (Jeon et al., 2003; Goldansaz et al., 2004). There may be a biological function for this change – for example, to optimise attractant or aphrodisiac properties of the pheromone – as suggested by Hardie et al. (Hardie et al., 1990). Alternatively, this decrease might suggest that feedback pathways between biosynthesis of nepetalactol and selective oxidation to nepetalactone may be less synchronous in older aphids, or it may be a symptom of senescence in the glandular pheromone-producing cells.

Conclusions

Dysaphis plantaginea oviparae predominantly release sex pheromone during the photophase; however, low levels are released throughout the scotophase. Pheromone release is greatest on the eighth day of the adult stadium and this species releases a typical blend of 3.7:1 (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol:(4a*S*,7*S*,7a*R*)-nepetalactone. Calling behaviour is poorly correlated with quantities of pheromone released across different ages; conversely, due to aphid morphology, lack of calling may not necessarily indicate no release of pheromone. We therefore suggest caution when extrapolating calling behaviour to make inferences on pheromone release. Lack of a temporally narrow and distinct period of high sex-pheromone release during photophase suggests that alternative mechanisms or factors for species recognition and isolation exist. Further study should aim to understand how aphids in the field avoid cross-communicating, given that many share the same iridoid components in their sex pheromone. Questions relating to the influence of graded environmental variables, such as wind, light or temperature, could be explored with regard to measured release of pheromone and the ratio of components. Of particular interest would be a better understanding of the mechanisms regulating biosynthesis of sex pheromone in aphids. Aphids represent a fascinating superfamily of insects with unusual life cycles. Many species are of great agricultural importance, and continued progress in our understanding is vital if we are to successfully exploit our biological and chemical knowledge of these insects for human benefit.

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