Planta (2005) 222: 141–150
DOI 10.1007/s00425-005-1506-8

ORIGINAL ARTICLE

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The composition and timing of flower odour emission by wild *Petunia axillaris* coincide with the antennal perception and nocturnal activity of the pollinator *Manduca sexta*

Received: 6 November 2004 / Accepted: 14 January 2005 / Published online: 13 May 2005

Abstract In the genus *Petunia*, distinct pollination syndromes may have evolved in association with bee-visitation (*P. integrifolia* spp.) or hawk moth-visitation (*P. axillaris* spp). We investigated the extent of congruence between floral fragrance and olfactory perception of the hawk moth *Manduca sexta*. Hawk moth pollinated *P. axillaris* releases high levels of several compounds compared to the bee-pollinated *P. integrifolia* that releases benzaldehyde almost exclusively. The three dominating compounds in *P. axillaris* were benzaldehyde, benzyl alcohol and methyl benzoate. In *P. axillaris*, benzenoids showed a circadian rhythm with an emission peak at night, which was absent from *P. integrifolia*. These characters were highly conserved among different *P. axillaris* subspecies and *P. axillaris* accessions, with some differences in fragrance composition. Electroantennogram (EAG) recordings using flower-blends of different wild *Petunia* species on female *M. sexta* antennae showed that *P. axillaris* odours elicited stronger responses than *P. integrifolia* odours. EAG responses were highest to the three dominating compounds in the *P. axillaris* flower odours. Further, EAG responses to odour-samples collected from *P. axillaris* flowers confirmed that odours collected at night evoked stronger responses from *M. sexta* than odours collected during the day. These results show that timing of odour emissions by *P. axillaris* is in tune with nocturnal hawk moth activity and that flower-volatile composition is adapted to the antennal perception of these pollinators.

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Keywords Electroantennogram studies · Flower volatile collection · Gas chromatography · *Manduca sexta* · *Petunia axillaris* · *Petunia integrifolia*

Abbreviation EAG: Electroantennogram · EAD: Electroantennogram detection · QTL: Quantitative trait loci · GC: Gas chromatogram · MS: Mass spectrometer

Introduction

The species *Petunia axillaris* and *Petunia integrifolia* differ in most of their pollination-related flower-characters (Wijsman 1982, 1983; Ando et al. 1995a, b, 2001; Tsukamoto et al. 1998; Stuurman et al. 2004). The two species can generally be crossed in the laboratory and produce viable hybrids (Wijsman 1983; Watanabe et al. 1996; Tsukamoto et al. 1998; Ando et al. 2001). Although *P. axillaris* and *P. integrifolia* share the same habitats in nature (sympatric populations), hybrids have never been found. It is believed that one of the major reasons for the lack of hybrids in nature is that two different insect orders pollinate the two *Petunia* species (Ando et al. 2001). In Brazil, the bee *Leioproctus* subgen. *Hexantheda* was observed feeding on *P. integrifolia* flowers, whereas the hawk moth *Manduca diffissa* and *Manduca contracta* were observed feeding from *Petunia axillaris* flowers (Ando et al. 2001). In Uruguay, we observed the same bee feeding on *P. integrifolia* flowers during the day and we observed the hawk moths *M. diffissa*, *Manduca sexta* and *Eumorpha fasciata* feeding on *P. axillaris* flowers mostly soon after sunset but also later during the night (M.E. Hoballah, personal observation, February 2004). The specific flower characters of *P. integrifolia* and *P. axillaris* appear to be adapted to their pollinators.

As has been pointed out previously (Bradshaw et al. 1998; Stuurman et al. 2004), one of the major issues in the

evolution of pollination syndromes is the number of genes involved in the transitions of one syndrome to another. We have previously performed a quantitative trait loci (QTL) analysis for pollination related traits (Stuurman et al. 2004). For the traits colour, shape, nectar and fragrance the QTL analysis revealed a few loci with relatively large effects (Stuurman et al. 2004). For flower fragrance we found a major QTL on chromosome VII of both *P. axillaris* and *P. integrifolia* that appears to control the level of general volatiles output (Stuurman et al. 2004). We expect that more detailed chemical analysis of the fragrance will enable us to detect genetic differences at the level of single odour components. Hawk moth pollinated flowers tend to emit complex blends of volatiles that may differ between plant species. For our genetic analysis, it is essential to first determine the composition of the odours emitted by *P. axillaris* accessions and second to determine how single compounds contribute to the reaction of the hawk moth pollinator antennae.

The first goal of the present study was to determine qualitative and quantitative differences in odour blends among wild *Petunia* spp. and accessions. Flower-odours of cultivated *Petunia hybrida* (cv Mitchell) have been collected in previous studies (Kolosova et al. 2001; Verdonk et al. 2003; Boatright et al. 2004), but no information is available for the flower-odours of wild *Petunia* species, apart from our previous work on the differences between the odour blends of *P. integrifolia inflata* vs. *P. axillaris parodii* wild species (Stuurman et al. 2004). We collected the odours of different accessions for both wild species to find out if they release similar flower-odour blends. In fact, adaptation to different pollinators or florivores of the different accessions could have as consequence a difference in the quality of the flower-odour blends among accessions grown in different habitats. We also measured the rhythm in odour emission for one accession per wild species.

The second goal of this study was to assess the perception of the flower-odour by the antennal olfactory receptor cells of the nocturnal hawk moth *M. sexta*, a pollinator that visits *P. axillaris* in its natural habitat. Work on the neurobiology of *M. sexta* olfaction dates back 30 years, comprising studies on interneurons in the brain (Itagaki and Hildebrand 1990), detection of sex pheromone components by olfactory receptor neurons (Kalinová et al. 2001), antennal olfactory receptor cells responses (Shields and Hildebrand 2001), glomerular organization of antennal lobes (Hildebrand 1996) and their importance for odour-modulated flight (Willis et al. 1995). Some information also exists on the learning capability of this hawk moth species (Daly and Smith 2000; Daly et al. 2001). Electroantennogram (EAG) studies with *M. sexta* have been conducted with leaf odours of *Lycopersicon esculentum* (tomato), *Capsicum annuum* (bell pepper), *Datura wrightii* and *Proboscidea parviflora* and with single compounds that are known to be released by leaves of host plants (Fraser et al. 2003). Here, we measured antennal responses to flower-odours of different wild *Petunia* spp. and accessions, to single compounds that were detected in *Petunia* flower-odours, and to night-versus day-released odours.

Materials and methods

Plants and insects

Petunia species and lines used for the experiments are listed in Table 1 with the sources from which they were obtained. Plants were grown in a greenhouse (13:11 L:D cycle, 18–25°C) in pots (11 cm height, 12.5 cm diameter) with commercial soil (70% Klasman substrate, 15% Seramis clay granules, 15% quartz sand). Fertilization occurred each week (NPK and Fe). *Manduca sexta* female-pupae were obtained from NCSU-Entomology Insectary (Raleigh, NC, USA) and reared under laboratory climatic conditions. Light was provided by six fluorescent tubes (Philips TDL, 36 W) with a 16:8 L:D cycle. Relative humidity in the laboratory was enhanced by the use of a humidifier (Defensor 3001, 60% RH). Prior to emergence pupae were placed in rearing cages (BugDorm-2, http://www.megaview.com.tw) containing a PVC-box filled with tap water. Adults used for the experiments were 4–5 days old and unmated.

Odour collection of flowers

We collected the flower-odours and analysed them by capillary gas chromatography, the method of choice for analysing complex blends of volatile organic molecules (Dobson 1994; Schiestl and Marion-Poll 2002). *Petunia* plants were placed in a climate chamber (Conviron, E95 Mode, and IG, Switzerland) held at 20°C, 70% RH and

Table 1 List of *Petunia* spp. used in this study and their origin

No.	Species	Received from (origin in nature)
1	*P. integrifolia integrifolia* (A)	Bot. Garden Dresden
2	*P. integrifolia inflata* (S6)	University Amsterdam
3	*P. integrifolia integrifolia* (B)	Bot. Garden Rostock, received as *P. integrifolia violacea*
4	*P. axillaris axillaris* (N)	Bot. Garden Rostock
5	*P. axillaris axillaris* (O)	Bot. Garden Dresden (Argentina, Prov. Buenos Aires)
6	*P. axillaris axillaris* (Q)	Bot. Garden Leipzig (Northern Argentina)
7	*P. axillaris axillaris* (P)	Bot. Garden Bern
8	*P. axillaris parodii* (S7)	University Amsterdam

with a light intensity of 14,500 lm/m^2 at plant height (16x Sylvania 150 W VHO Cool White and 12x Sylvania, 100 W Satin, 12:12 L:D cycle). Six glass cylinders (9.5 cm diameter, 54 cm high) were placed over a flower (no vegetation was present in the cylinder) and rested on a Teflon disk divided in two with a central hole for the stem of the flower. For details on this method see Turlings et al. (1998). Air was pumped (1.1 l/min) through a charcoal filter and introduced from the top of each cylinder over the flower. Super-Q traps (25 mg, 80/100 mesh, Alltech, Deerfield) were attached laterally at the base of each cylinder. Traps were connected to flow meters (Automated Volatile Collection System-ASU, ARS, Gainesville, USA) through which the air was sucked out at 0.8 l/min for 4 h. The collection period was programmed with the use of software (TESS,Version 1.0, ARS, Gainesville, USA). After each collection, traps were rinsed with 150 µl dichloromethane and 200 ng of *n*-octane and nonyl acetate were added as internal standards. Three microlitre aliquots of the samples were injected splitless with an automated injection system into a Hewlett Packard model HP 6890 gas chromatograph (GC) equipped with a flame ionisation detector. The apolar HP-1 capillary column (30 m, 0.25 mm internal diameter, 0.25 µm film thickness, Agilent) was held at 50°C for 3 min and then programmed at 8°C/min to 230°C and held for 9.5 min. Helium (24 cm/s) was used as carrier gas.

We collected and analysed the odour of four *P. axillaris axillaris* accessions (N: $n=4$, Q: $n=3$, O: $n=3$, P: $n=3$), *P. axillaris parodii* ($n=3$), two accessions of *P. integrifolia integrifolia* (each $n=3$) and *P. integrifolia inflata* ($n=3$) over 24 h for 4 h intervals. Each replicate was carried out with a new plant. The plants were placed inside the climate chamber 3 days prior to an odour collection. Agilent ChemStation software was used to quantify all major components. Identification of the compounds benzaldehyde, benzyl alcohol, benzeneacetaldehyde, methyl benzoate, phenylethyl alcohol, methyl salicylate, eugenol, vanillin, isoeugenol and benzyl benzoate was based on comparison of retention times of unknowns with that of synthetic samples (Aldrich, Fluka, Sigma, purity of synthetic standards: 95–99%). A few compounds were identified only with the use of the Wiley library after GC-mass spectrometry analysis (Agilent 5973 mass selective detector, transfer line 230°C, source 230°C, quadrupole 150°C, ionization potential 70 eV, scan range 0–400 amu). These compounds are hexadecanoic acid methyl ester, *Z*-9,17-octadecadienal, benzyl salicylate and benzyl acetate. An ANOVA was used to compare the quantity of single volatiles emitted between lines and over different periods. Rhythmicity in odour emissions was determined for *P. axillaris axillaris* N and *P. integrifolia integrifolia* by collecting over 4 days (for 4 h intervals) under continuous light (all other conditions same as previous collections). We collected the odours from two flowers simultaneously during this experiment and found that eucalyptol, in addition to benzaldehyde, was also produced by *P. integrifolia*. This is the reason why we included eucalyptol in the EAG experiments (see below). ANOVA was used to analyse for differences in quantity of flower-volatiles collected among *Petunia* spp. and among collection periods.

EAG experiments

A previous study carried out with *M. sexta* showed that male and female antennae react similarly to plant odours (Fraser et al. 2003). This permitted us to use females exclusively. An antenna of a female *M. sexta* (4–7 days old) that was not exposed to flower-odours was excised at the base and the distal tip was cut off. It was then mounted between two glass capillary electrodes filled with 0.1 M KCl and connected via an Ag–AgCl wire to the amplifier. The antenna was held in a humidified air stream (90–100% RH, 23 ± 2°C) delivered at 1 m/s via a water-jacketed glass tube (6 mm internal diameter) whose outlet was about 1 cm from the preparation. The EAG signal was fed into an AC/DC amplifier (×100) via a high impedance preamplifier (×10), recorded on the hard disk of a PC via a 16-bit analogue-digital IDAC card (Syntech, The Netherlands), and monitored simultaneously with an oscilloscope (Tektronix 5103, USA). Antennae were stimulated as described in Guerenstein and Guerin (2001) by passing 1 ml of charcoal-filtered air through a 5 ml polypropylene syringe containing the stimulus. The latter consisted of either a *Petunia*-flower (cut at the pedicel and inserted in the syringe), a synthetic *Petunia*-flower volatile (sources were Aldrich, Sigma and Fluka, Switzerland, purity ranged from 95% to 99% and was unknown for eugenol and phenylethyl alcohol) at 100 µg or a flower-volatiles extract collected from *P. axillaris axillaris* O at different periods during the day (4×4 h periods, 5 µl). Benzyl alcohol and benzeneacetaldehyde had very similar retention times thus, they were identified relatively late in our GC-MS analyses. It is for this reason that benzeneacetaldehyde was not included in our EAG studies. An aliquot (10 µl of 10 µg/µl solution) of a stimulus chemical dissolved in dichloromethane (Merck, analytical grade) was deposited on a filter paper strip that was placed in the syringe after evaporation of the solvent. Dichloromethane alone was used as a control. The EAG amplitudes presented are the percentages of the absolute amplitudes in millivolts generated by the stimuli expressed as a fraction of the standard (*Z*-3-hexenol, 100 µg, mean of the values recorded at the beginning and end of each test series). An ANOVA was used to compare differences in antennal responses to the treatments.

Gas chromatography coupled electroantennogram detection (GC-EAD)

The methodology is described in Steullet and Guerin (1994). *M. sexta* female antennae were employed as

detectors to locate biologically active volatiles in the *Petunia* flower-odours. These odours were separated on a polar capillary column (30 m BGB FFAP, 0.25 mm internal diameter, 0.25 µm film thickness) in a gas chromatograph (Carlo Erba Instruments HRGC 5300, Italy) with an on-column injector and a flame ionization detector (FID, at 260°C). Hydrogen (30 cm/s) was used as carrier gas. Injections were made at 40°C, and the column temperature was programmed at 10°C/min to 220°C (held for 10 min). The column was equipped with a deactivated precolumn (1 m OV 1701-OM, 0.25 mm internal diameter, 0.25 µm film thickness). The column effluent was split (50:50) between the FID and the EAD and simultaneously monitored by the FID and a *M. sexta* antenna, and both signals were recorded simultaneously on a PC using a GC-EAD software program (Syntech, The Netherlands). To identify biologically active *Petunia*-flower volatiles, the retention times of peaks were compared with that of synthetic chemicals (see Odour collection of flowers section) injected under the same GC conditions.

Results

Composition and rhythm of *Petunia* flower odour release

Petunia species, subspecies and accessions differed in the quality of floral volatiles emitted (Fig. 1). The flower odour-blends of the hawk moth-pollinated *P. axillaris* accessions contained multiple compounds, the major ones being phenylpropanoids. In contrast, benzaldehyde (compound 1, Fig. 1) was the only compound that we collected from the bee-pollinated *P. integrifolia* flowers. Traces of other compounds collected from single flowers were present, but at levels too low to identify them consistently across experiments. *P. axillaris parodii* emitted significantly more volatiles than the other petunias (ANOVA, $P = 0.0001$, $F = 7.802$, Fig. 1) and we observed lower total floral odour emission for *P. integrifolia* compared to *P. axillaris* (note the log scale in Fig. 1). *P. axillaris parodii* produced methyl salicylate (Fig. 1, compound 6), which the other subspecies *P. axillaris axillaris* did not produce, whereas it did not produce isoeugenol (Fig. 1, compound 9) that was produced by the other subspecies. We found major qualitative differences also among accessions of *P. axillaris axillaris* (Fig. 1). Accessions N and Q emitted benzeneacetaldehyde and benzyl benzoate (Fig.1, compounds 2 and 10), whereas the accessions P and O released hexadecanoic acid methyl ester and *Z*-9,17-octadecadienal (Fig. 1, compounds 12 and 13). Benzyl acetate was produced only by *P. axillaris parodii* and accession O (Fig. 1, compound 5). Phenyl ethyl alcohol was produced only by the accession Q (Fig. 1, compound 4). The major floral odour constituent's methyl benzoate and benzaldehyde were produced by all *axillaris* subspp. and accessions (Fig. 1). The third major constituent, benzyl alcohol, was produced by all *axillaris* subspp. with exception of accession N that produced benzeneacetaldehyde (Fig. 1). The present fragrance analysis shows an increased qualitative complexity and intensity in *P. axillaris* species as compared to *P. integrifolia* species that results from addition of several volatile phenyl propanoids and other compounds to a basic *P. integrifolia* scent that has benzaldehyde as the major component.

Emission of total floral odour and individual phenylpropanoids and benzenoids (here we present only the data for benzaldehyde as single compounds) were higher during the night than during the day for *P. axillaris* flowers (Fig. 2). In contrast, *P. integrifolia* species did not show a difference between day and night odour emissions (data not shown). Interestingly, emissions of *Z*-9,17-octadecadienal and hexadecanoic acid methyl ester released by the *P. axillaris axillaris* accessions P and O did not differ between night and day (Fig. 2).

We determined whether the day–night rhythm was environmentally induced or caused by an endogenous clock. For this we transferred plants from a 12:12 L:D cycle to continuous light and measured volatile output. A clear circadian rhythm for odour production was found for benzaldehyde (Fig. 3) and the other phenylpropanoids (data not shown) for *P. axillaris*, but not for *P. integrifolia* (Fig. 3). Hence, an endogenous circadian rhythm in flower odour emission is present in the night-active hawk moth pollinated *P. axillaris*, but absent in the bee pollinated *P. integrifolia*.

Perception of floral odours by *M. sexta*

Antennal responses of *M. sexta* to different flower volatiles were measured using the EAG method. This method permits the recording of shifts in potential (in millivolts) induced in response to antennal stimulation with adequate chemostimuli (Schneider 1957). During stimulation with the volatile component, this potential drops by some millivolts. At first, the odours of freshly excised flowers of different wild petunias were presented to the antennae. Among odours of flowers from different *Petunia* spp. the hawk moth-pollinated *P. axillaris* spp. elicited significantly higher responses from the antenna of *M. sexta* than flowers of *integrifolia* spp. (Fig. 4a).

Next we tested individually the components present in the flower scents at a standard amount of 100 µg. Of all the compounds present in *Petunia* flower-odours, benzyl alcohol elicited the highest EAG responses followed by methyl benzoate and benzaldehyde (Fig. 4b). These three compounds were the predominant components of hawk moth-pollinated *Petunia* spp. (Fig. 1). Benzyl benzoate and isoeugenol, which are major odour components of the flowers of some *P. axillaris* subspp. and accessions, elicited marginal EAG responses from the antennae of *M. sexta* females (Fig. 4b).

Comparing day (10 a.m.–2 p.m. and 2 p.m.–6 p.m. collection periods) and night (6 p.m.–10 p.m. and 10

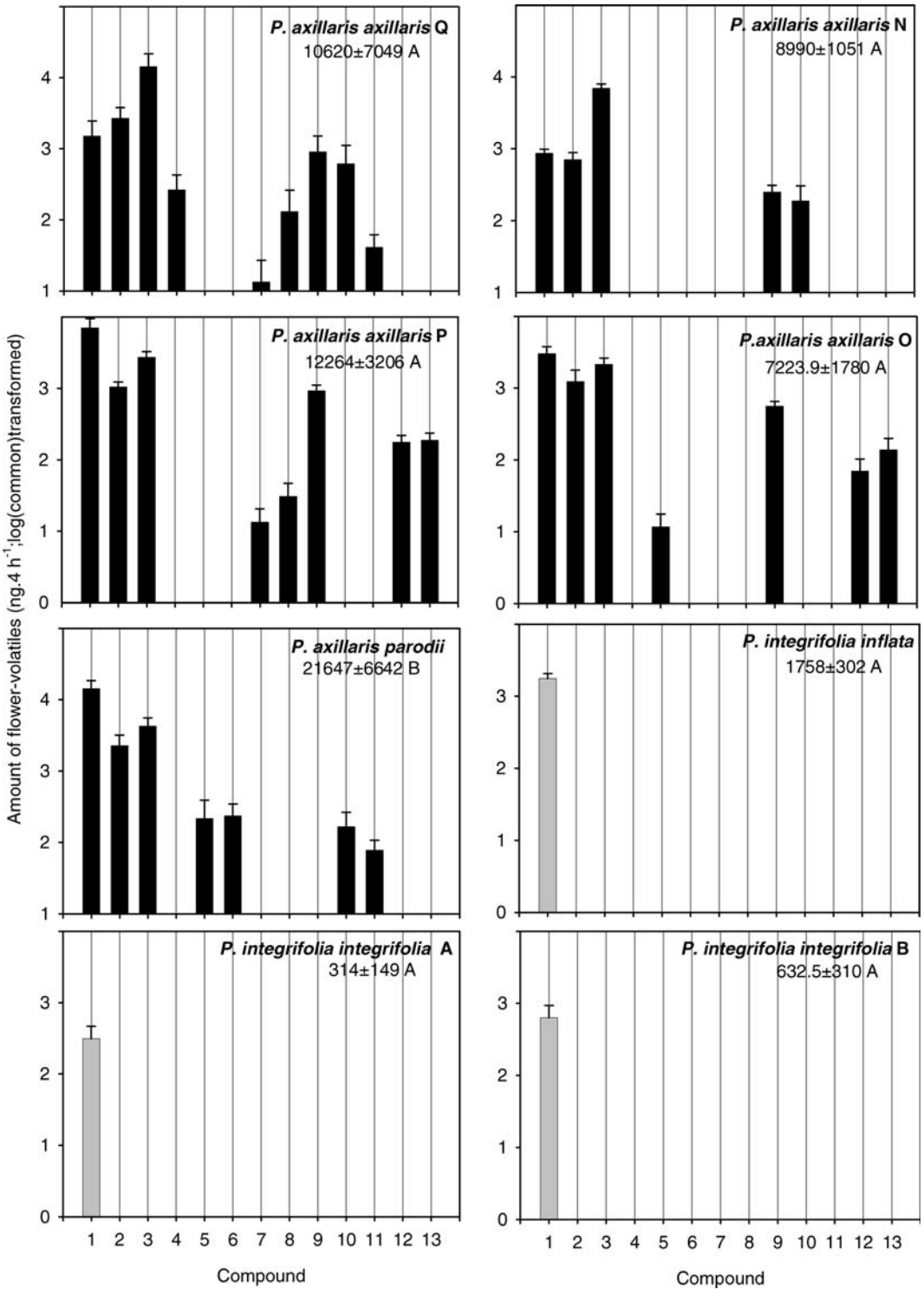

Fig. 1 Mean (± SE) levels of 13 volatiles (*bars*) emitted from single flowers of different *Petunia* spp. (*axillaris* spp. in *black* and *integrifolia* spp. in *grey*). *Different letters* after means (± SE) indicate a significant difference in total flower-volatiles produced among *Petunia* species (after ANOVA followed by SNK post hoc test). Abscissa is: *1* benzaldehyde, *2* benzyl alcohol or benzenacetaldehyde for accession N or benzyl alcohol/benzeneacetaldehyde mixture for accession Q, *3* methyl benzoate, *4* phenylethyl alcohol, *5* benzyl acetate, *6* methyl salicylate, *7* eugenol, *8* vanillin, *9* isoeugenol, *10* benzyl benzoate, *11* benzyl salicylate, *12* hexadecanoic acid methyl ester, *13* *Z*-9,17-octadecadienal

p.m.–2 a.m.) collected odours from *P. axillaris axillaris* N (Fig. 5a), the night-collected odours stimulated the antenna significantly more (Fig. 5b). The higher response of the antenna to the night-collected odours could be explained simply by the higher quantity of flower-volatiles collected during this period.

Gas chromatography coupled electroantennogram detection

To detect if trace components of the *P. axillaris* flower-odour blends are important for the antenna-responses of *M. sexta* we utilized a system that couples EAD (Arn et al. 1975). In this set-up the responses of the antenna is measured to the individual components of the floral scents at the relative quantities at which they are emitted by the flowers. Three constituents of the trapped volatiles from *P. axillaris axillaris* accession P elicited very high responses from the antenna of *M. sexta* i.e. benzaldehyde, benzyl alcohol and methyl benzoate (Fig. 6). The strongest antennal response was elicited by methyl benzoate (Fig. 6), which is produced in high amounts by all *P. axillaris* subspp. and accessions (Fig. 1). Other compounds present in the floral extract did not elicit a response from the *M. sexta* antenna.

Discussion

Petunia species differ in flower-odour in accordance with the biology of their pollinators

The differences in flower-odour blends between the *Petunia* species *axillaris* and *integrifolia* were found to be both qualitative and quantitative: *P. axillaris* spp. released higher amounts and more compounds than *P. integrifolia* spp.. A similar situation has been found for *Clarkia* (Onagraceae), for three genera of Nyctaginaceae and for *Nicotiana* (Solanaceae): moth-pollinated species produce a large quantity of volatiles, whereas bee or hummingbird pollinated species produce less or almost no scent (Raguso and Pichersky 1995; Levin et al. 2001; Raguso et al. 2003). Odour is very important for the attraction of hawk moths as it activates their feeding behaviour and is used to locate flowers over distance (Brantjes 1978). Hawk moths use odour cues over longer distances than bees do (Dobson 1994) and therefore odour quantity has probably to be higher to be detected. A study conducted with *M. sexta* has shown that there is synergy between odour and visual cues (Raguso and Willis 2002). In fact, colours can be distinguished by hawk moths even at night (Kelber et al. 2002). Odours attract the moths from a distance, but at short distance from a flower they will feed on both scented and non-scented flowers (Raguso and Willis 2002). For long distance attraction the quantity of the few major compounds may be more important than the quality of the odour blend. The compounds detected in our study are released by different plant families that are pollinated by nocturnal hawk moths (Knudsen et al. 1993).

There were considerable differences in the quality of the flower-odour blend among *axillaris* subspp. and among *axillaris axillaris* accessions, which are thought to be pollinated by the same or similar nocturnal moths. Here again the quantity of a few major compounds may be of more selective importance for hawk moths attraction than the overall quality of the blend. However, we cannot exclude that differences in the flower-odour blends do not reflect odour adaptation to different pollinators or adaptation to florivores. In fact, in *Nicotiana attenuata*, it was shown that dynamics of

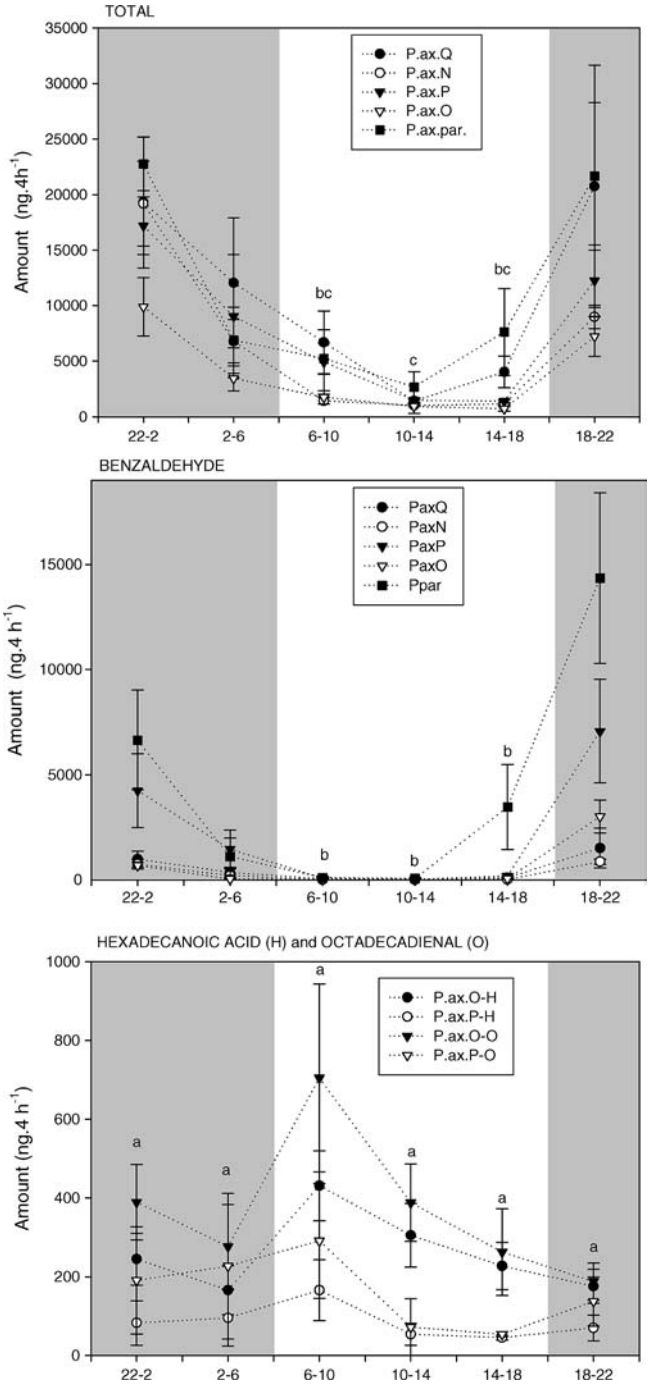

Fig. 2 Mean (±SE) of total and single compounds flower volatile emissions from *P. axillaris* subspp. and accessions for each of six collection periods (4 h collection periods, three dark periods in *grey* and three light periods in *white*). *Different letters on bars of each graph* indicate significant differences among periods (after ANOVA followed by SNK post hoc test)

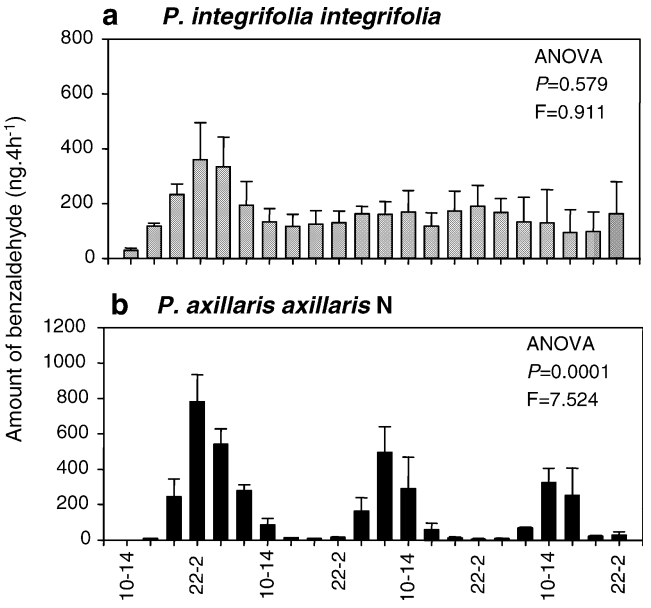

Fig. 3 Mean (±SE) emission rates of benzaldehyde from *P. integrifolia integrifolia* (*grey*) and *P. axillaris axillaris* (*black*) over 4 h collection periods (*bars*) carried out for 4 days by placing plants in continuous light. See ANOVA data in the right corner of each graph

the corolla pools of benzyl acetetone and nicotine throughout the day are consistent with their roles in advertisement and defense (Euler and Baldwin 1996).

P. axillaris flower emission is nocturnal and controlled by an endogenous clock

We detected nocturnal rhythmic emission of several compounds for *P. axillaris* subspp. and accessions. Rhythmic emissions of methyl benzoate, benzaldehyde, isoeugenol, benzeneacetaldehyde and benzyl benzoate have also been detected in *Petunia hybrida* (Kolosova et al. 2001; Verdonk et al. 2003; Negre et al. 2003). The non-rhythmic emission of hexadecadienal methyl ester and *Z*-9,17-octadecadienol (Fig. 2) suggests that some floral volatiles are produced for purposes other than pollinator attraction, such as herbivore and nectar robber repellence or pathogen resistance.

We found that nocturnal floral odour production in *P. axillaris* is under the control of an endogenous circadian clock. The same is known for other flower species and for various biochemical classes of volatiles (Overland 1960; Altenburger and Matile 1988; Loughrin et al. 1991). A shortened period of volatile production probably serves to save energy and resources. Soon after pollination, the production of methyl benzoate, benzaldehyde and benzeneacetaldehyde decreases in *Petunia* (Negre et al. 2003), another indication of a strategy to reduce costs of volatile production. The production of flower volatiles restricted to the night could also be an adaptation of the plant to avoid seed predators, as was

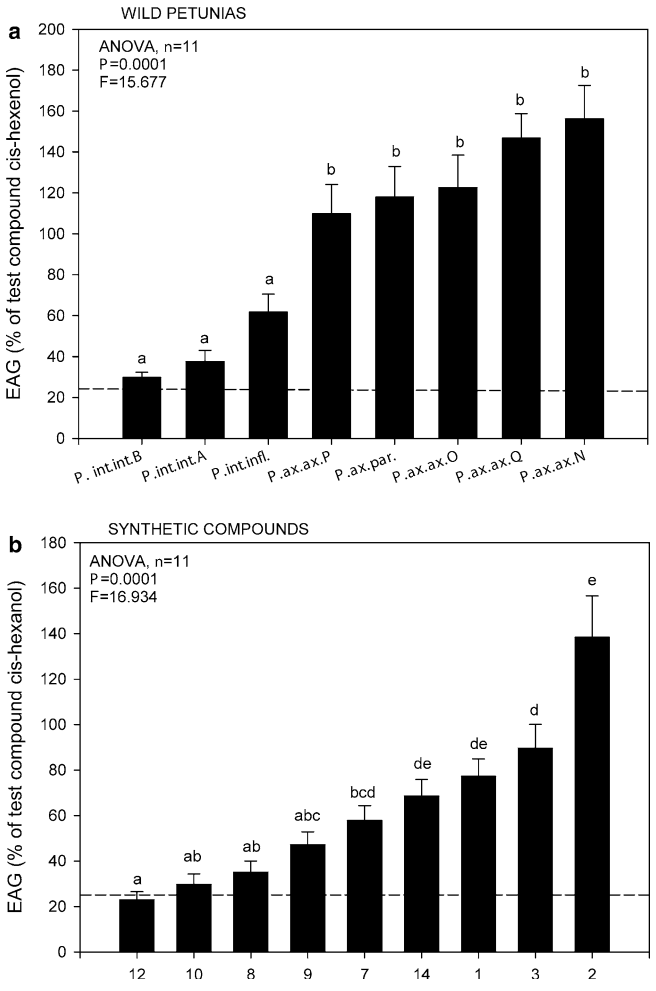

Fig. 4 **a** Mean ± SE EAG responses (absolute amplitudes expressed as a fraction of the standard *Z*-3-hexenol recorded at the beginning and end of each test) of unmated female *Manduca sexta* to single flowers of different *Petunia* spp. **b** Mean ± SE EAG responses of unmated female *M. sexta* to 100 µg source doses of nine *Petunia* odour constituents represented on the abscissa: *12*, hexadecanoic acid; *10*, benzyl benzoate; *8*, vanillin; *9*, isoeugenol; *7*, eugenol; *14*, eucalyptol; *1*, benzaldehyde; *3*, methyl benzoate; *2*, benzyl alcohol. The *dotted line* represents the response to the solvent dichloromethane. *Different letters above bars* indicate significant differences between treatments (Student Newman–Keuls post hoc test after ANOVA; see ANOVA data at the left corner of each graph)

shown by Baldwin et al. (1997) for *N. attenuata*. *Antirrhinum* flowers (Kolosova et al. 2001), *Artemisia annua* (Lu et al. 2002) and *Rosa hybrida* (Helsper et al. 1998) also posses an internal clock controlling floral odour production, but they reach maximum levels during the day. We did not detect an endogenous circadian rhythm in the volatiles collected from *P. integrifolia*.

Manduca sexta antennal response is tuned to *P. axillaris* flower fragrance

Benzaldehyde, benzyl alcohol and methyl benzoate elicited the strongest EAG and EAD responses from *M. sexta* and these volatiles are major constituents of *P. axillaris* flower scent. A recent study on GC-EAD

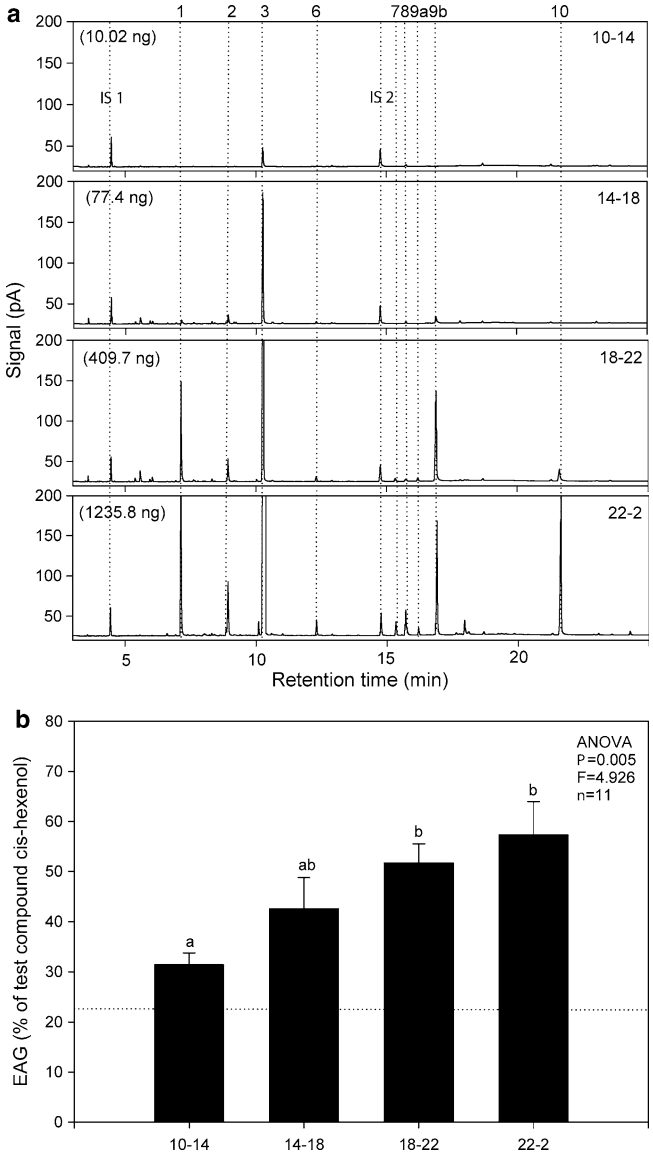

Fig. 5 **a** The four chromatograms represent the four extracts used for the EAG assays (IS1 internal standard *n*-octane, IS2 internal standard nonyl-acetate, *1* benzaldehyde, *2* benzeneacetaldehyde, *3* methyl benzoate, *6* methyl salicylate, *7* eugenol, *8* vanillin, *9a* isoeugenol 1, *9b* isoeugenol 2, *10* benzyl benzoate). **b** Mean ± SE EAG responses of unmated *M. sexta* females to 5 µl of samples of *Petunia axillaris axillaris* N flower-volatile extracts (4 h collections, photophase collections lasted from 10 a.m.–6 p.m. and scotophase collections from 6 p.m.–8 a.m.). The *dotted lines* represent EAG response of *M. sexta* to the solvent dichloromethane. *Different letters above bars* indicate significant differences among treatments (Student Newman–Keuls post hoc test after ANOVA)

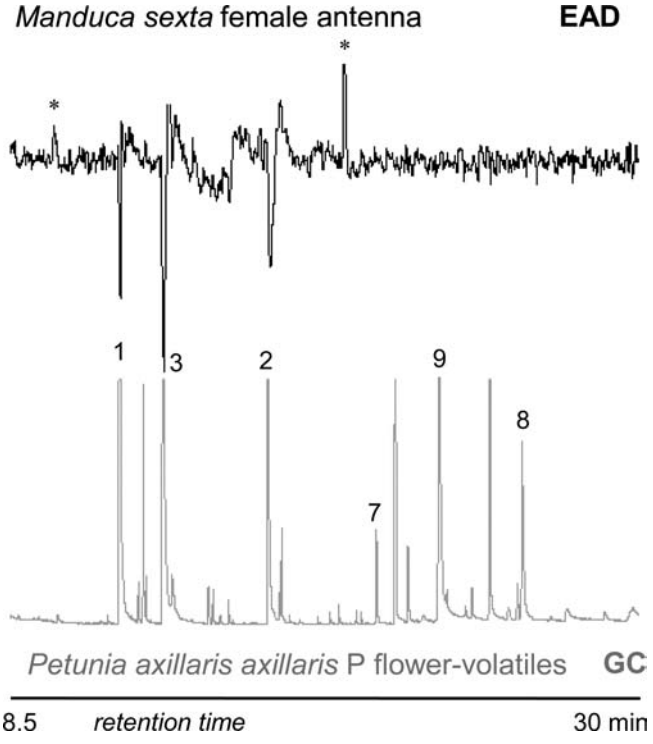

Fig. 6 Analysis of injected flower-volatiles collected from *Petunia axillaris* (subsp. *axillaris* accession P) by GC-EAD with a *M. sexta* antenna. The grey trace is the flame ionization detector (FID) response and the black trace is the electroantennographic detector EAD response generated during elution of the biologically active constituents of the headspace odour from the gas chromatographic column (*1* benzaldehyde, *3* methyl benzoate, *2* benzyl alcohol, *7* eugenol, *9* isoeugenol, *8* vanillin). The upward deflections marked with *asterisks* are due to occasional lack of contact between the recording electrode and the cut tip of the antenna

from *M. sexta* female antennae showed that several host-plant compounds elicit strong EAD-responses, including benzyl alcohol and methyl salicylate (Fraser et al. 2003).

Specific olfactory receptor cells of *M. sexta* females have been shown to respond to benzyl alcohol, benzyl benzoate, methyl benzoate and methyl salicylate but no receptor cells were found for eugenol and isoeugenol (Shields and Hildebrand 2001). EAG-recordings from the nocturnal hawk moth *Sphinx perelegans* (Raguso and Light 1998) yielded preferentially strong responses to the same three compounds, but benzaldehyde elicited a stronger response from the *S. perelegans* antennae than benzyl alcohol and methyl benzoate, the opposite of what was found here for *M. sexta*. Although rank orders of sensitivity may differ, many moths are expected to use the same compounds to locate nectar sources.

In conclusion, *P. axillaris* flowers produce three major volatile compounds, benzaldehyde, benzyl alcohol and methyl benzoate, which may be readily detected by hawk moths, their principal pollinators. These compounds are released only during the night when these pollinators are active, implying that the scent of *P. axillaris* flowers is produced to ensure pollination by the attraction of *M. sexta* and other hawk moths.

In future we will create near isogenic lines that differ in single loci or single genes (involved in total flower output, circadian rhythm or benzaldehyde, benzyl alcohol and methyl benzoate final biosynthesis step) to test if the difference in single gene expression in *Petunia* can influence pollinator behaviour, as was shown for the trait colour in *Mimulus* by Bradshaw and Schemske (2003). This will also enable us to determine the species-specific

differences in the structure of the genetic loci underlying fragrance production.

Acknowledgements Special thanks to C. Ball, R. Alder (University of Bern, Institute of Plant Sciences) and C. Faria (University of Neuchâtel, Institute of Zoology) for plant care, and to L. Broger, Dr. J. Moore, C. Galliot, R. Schreyer and A. Dell'Olivo (University of Bern, Institute of Plant Sciences) for insightful comments on an earlier version of the manuscript. This work was part of the National Centre for Competence in Research "Plant Survival" with financial support by the Swiss National Science Foundation and the Canton Bern.

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