Silene otites (Caryophyllaceae): Attraction of nectar-seeking mosquitoes to inflorescence odours, and temporal variation of flower scent and flower visitors

Dissertation

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General Introduction

Mosquitoes, with more than 3400 species (Backer 1989), represent a significant threat to human health because of their ability to transmit pathogens that afflict millions of people world-wide (WHO 1992, Pinheiro and Corber 1997, WHO/CTD 1998). Although they are only a few millimetres long, they could be considered as the most dangerous animals in the world. The World Health Organization (WHO) estimates that mosquitoes infect up to 700 million people a year and are responsible for the deaths of more than 2 million (Day 2001). Therefore, mosquitoes are the focal point of entomological studies world-wide, especially in connection with their human-medical significance as vectors of dangerous diseases like malaria, yellow fever, dengue fever, and filariasis.

Mosquitoes are extremely successful organisms due to their ability to adapt to a wide range of habitats. They for example feed/drink on a broad spectrum of environmental elements: plants (Sandholm and Price 1962; Grimstad and DeFoliart 1974; Smith and Gadawski 1994, Burkett et al. 1999; Manda et al. 2007), animals (Gad et al. 1999; Konate et al. 1999; van den Hurk et al. 2003; Rodrigues and Maruniak 2006), water (Weber and Tipping 1990, 1993; Weber et al. 1991) and even solid sugar (Eliason 1963). The factors involved in attracting mosquitoes to these sources are complex and not fully understood (Bowen 1991; Davis and Bowen 1994; Keystone 1996). However, it is at least known that mosquitoes use visual, thermal, and olfactory stimuli to locate a blood host of which the olfactory cue is probably the most important (Mark and Fradin 1998). Another cue, humidity, which is related with the thermal cue, also has been shown as an important cue in some studies (Smart and Brown 1957; Gilbert et al. 1966, Mukabana 2002).

For both autogenous mosquitoes (females can lay eggs without taking any blood-meal) and anautogenous mosquitoes (females must take blood-meal to develop their eggs) carbohydrates are an important determinant of survivorship (Nayar and Sauerman 1971a,b), and it has been proven that sugar ingestion plays a critical role in longevity, fecundity, flight capacity, and host-seeking behaviour (Harada et al.1971; Harada et al. 1976; Nayar and Sauerman 1975; Magnarelli 1978; Klowden 1986). At least for females it is known that sugars serve as precursors for the synthesis of fats (Van Handel 1984). Most mosquitoes obtain carbohydrates primarily from floral nectars (Haeger 1955; Sandholm and Price 1962; Grimstad and DeFoliart 1974) but also from honeydew, plant phloem, or damaged and rotting fruits (Yuval 1992). They prefer some plants over others as nectar source (Grimstad and DeFoliart 1974; Magnarelli 1978; Gadawaski and Smith 1992). Plant fluids are also referred to as important source of water for mosquitoes in arid climates (Reisen et al. 1986). Generally, sugar sources are more attractive than blood-hosts (e.g. humans) during first foraging periods of females (Foster and Takken 2004) and autogenous as well as anautogenous teneral females prefer sugar over a meal of blood and are preferentially attracted to plant related volatiles (Bowen et al. 1995). In some cases sugar feeding appears to be pre determined in the sense that host-seeking and blood-feeding cannot take place unless the female first takes a sugar meal (Nayar and Pierce 1980; Hancock and Foster 1993). In contrast to females which are strongly attracted to carbohydrates only in specific stages of their life (Foster and Takken 2004), males are strongly attracted to this food source throughout their whole life; they only ingest carbohydrates (Reisen et al. 1986).

Mosquitoes are known to use floral scent for finding nectar sources (Jepson and Healy 1988; Bowen 1991; Foster and Hancock 1994; Mauer and Rowley 1999). Consequently floral scent is an important factor in the attraction of mosquitoes to flowers (Vargo and Foster 1982). Nevertheless, little is known about the specific cues (compounds) used by mosquitoes for finding nectar plants, and only few studies addressed this topic (Bowen 1992; Mauer and Rowley 1999; Howse 2003). For finding effective nectar-related attractants, it is important to determine which plant species produces the most attractive floral compounds for mosquitoes and to identify those compounds.

It can be hypothesized that plant species, which are adapted to mosquitoes for their pollination, emit more specific mosquito attracting compounds than plant species pollinated primarily by other pollen agents. However, effective pollination by mosquitoes has been described only in two cases world-wide. 1) in the orchid *Habenaria obtusata* (Banks ex Pursh) Richardson (Stoutamire 1968), and 2) in the Caryophyllaceae *Silene otites* (L.) Wibel (Brantjes and Leemans 1976). Accordingly these two mosquito pollinated species are supposed to emit effective mosquito attracting floral compounds. *H. obtusata* is distributed in nearctic regions, occurring from Labrador and the New England states westward to the Aleutian Islands and British Columbia (Stoutamire 1968). *Silene otites* on the other hand is widely distributed in Middle, East and South Europe as well as in Central Asia. The present work focuses on the chemical attractiveness of *S. otites* to flower visiting mosquitoes. *Silene otites* is usually perennial and dioecious (Wringley 1986). Flowers are small and white-greenish, arranged in terminal cymes. It is a nocturnal plant, the floral scent emission of which is strongest at night (Jürgens et al. 2002). The male flowers remain functional for two nights whereas the female flowers emit scent over several days until they get pollinated (Brantjes and Leemans 1976).

Jürgens et al. (2002) analysed the scent of this species, and they found the monoterpenoid lilac aldehyde as main compound. However, these authors only studied the scent of one plant individual, and nothing is known about the variability in the scent of this plant among populations, and between males and females. Compounds with low variability may be more important for attraction of pollinators compared to compounds with high variability (Ayasse et al. 2000). Further, although mosquitoes are known to be pollinators of *S. otites*, nothing is known about the importance of floral scent emitted by *S. otites* for attraction of its flower-visiting mosquitoes (e.g. *Culex pipiens* L. and *Culiseta annulata* Schrank), though already Brantjes and Leemans (1976) assumed that olfactory cues are responsible for attraction of mosquitoes to *S. otites*. Therefore, if the flower odour of *S. otites* is proved as the reason for mosquitoe’s attraction, the identification of electrophysiologically and behaviourally active compounds might lead to identify potential mosquito attractants. The most attractive floral odour compounds might be helpful for developing new pest control strategies, which would be applicable in different life stages of both male and female mosquitoes, preferably as bait in traps.

Furthermore, in other pollination systems it is known that previous experience strongly influences the attractiveness of floral scent. In the sexually deceptive orchid *Ophrys sphegodes* Miller, pollinated by males of the solitary bee *Andrena nigroaenea* (Kirby 1802), it has been shown that male bees learned the odour bouquets of that orchid during mating attempts and recognized them in later encounters (Ayasse et al. 2000). Nevertheless, nothing is known about the learning ability of adult mosquitoes with regard to floral scent compounds.

Though *S. otites* is described as being pollinated during night (Brantjes and Leemans 1976), preliminary observations revealed that this plant species is also visited by several insects during day-time. Therefore, it would also be interesting to see the pattern of floral emission in *S. otites* as well as the visitor spectrum of this species over a day. During the day, *S. otites* might emit compounds attractive to day-active visitors, and during night, compounds attractive to the night-active visitors and pollinators, i.e. mosquitoes and moths (Brantjes and Leemans 1976).

The research in present study is directed towards understanding the cues responsible for mosquitoe's visitation of the flowers of *S. otites* with three broad goals in mind: 1) The determination of the variability in flower scent composition in *S. otites* and the evaluation of the attraction of mosquitoes to inflorescence odours of different *S. otites* populations. 2) The identification of *S. otites* compounds electrophysiologically active in mosquitoes to get knowledge on the compounds of *S. otites* detected by the mosquitoes. 3. The identification of the behaviourally active compounds finally responsible for attraction of mosquitoes to *S. otites*.

Goals and Experimental Design

The main aim of this study was to know how mosquitoes find the flowers of *Silene otites* for nectaring. As mosquitoes (e.g. *Culex pipiens*) described as pollinators of this plant species are night-active, it was hypothesized that mosquitoes find *S. otites* flowers due to their scent. Most of the experiments in the present study were done using *Culex pipiens pipiens* biotype *molestus* Forskal 1775 (European strain). The following series of experiments were conducted to test the hypothesis and finally to find out the key compounds responsible for attraction of mosquitoes to *S. otites*:

1. Assessment of variability in floral odour of *Silene otites.* To estimate the variability in inflorescence scent composition among populations and between sexes of *S. otites*, 63 plant individuals of nine geographic locations were used to collect odours by dynamic headspace methods. For collection of floral scent emitted from *S. otites*, 1-4 flowering stems were enclosed within a polyester oven bag (Toppits®) in order to accumulate the emitted scent. The emitted volatiles were trapped for 2 min in an adsorbent tube through the use of a membrane pump (G12/01 EB, ASF Thomas, Inc.). A power supply and a flow meter were used to standardise and to adjust the flow rate to 0.2 l/min. The adsorbent tube was built from a quartz vial by cutting the closed end, and filling the tube with 3 mg of adsorbent (Tenax® TA:CarbotrapTM = 1:1). The adsorbents were fixed in the tubes using glass wool.

After collecting the scent, the adsorbent tubes were stored in a freezer until analysis in a GC-MS (gas chromatography coupled to mass spectrometry) system. Therefore, the adsorbent tubes were introduced via ChromatoProbe in the 1079 PTV injector of a Varian 3800 GC for thermal desorption. The same system, temperatures, and settings were used for the analyses as described in Dötterl et al. (2005a). The data were analysed by multivariate statistical methods (nonmetric multidimensional scaling, analysis of similarity) and the hypothesis that floral scent is differing among populations and between sexes was tested.

2. Examination of mosquitoes attraction to the odour emitted by *Silene otites* inflorescences. To know whether or not the mosquitoes are attracted to the odour of *S. otites* in absence of visual stimuli, bioassays using *S. otites* inflorescences were conducted in a wind tunnel (Fig. 1). Additionally it was tested whether or not there is any difference in the attractivity of odours emitted from plants of different populations.

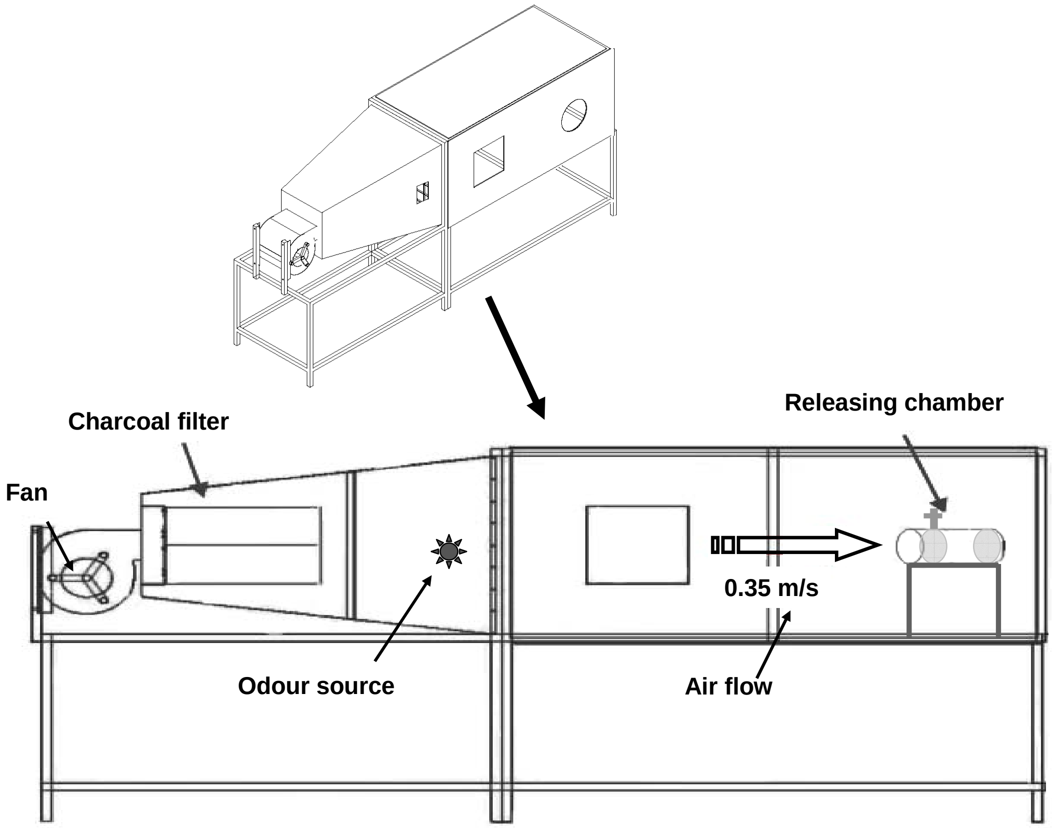

Figure 1. Schematic representation of wind tunnel used for bioassays.

A Fischbach speed controller fan (D340/E1, FDR32, Neunkirchen, Germany) continuously circulated air through the tunnel with an air speed of 0.35 m/sec. The incoming air was cleaned through four charcoal filters (145 mm × 457 mm, carbon thickness 16 mm, Camfil Farr). Bioassays were conducted in the second and third hour after onset of darkness, when *S. otites* emits most of its volatiles (Chapter 4). The inflorescences were placed at the upwind end of the tunnel behind gauze and different aluminium screens, so that they were invisible to the mosquitoes. As the behaviours of mosquitoes were not found to be influenced by the opposite sex (see Chapter 3), therefore, a group of 10-15 randomly chosen male and/or female mosquitoes were released from a releasing chamber (16 cm × 8 cm) at the downwind end of the tunnel and their behaviour was observed for 1 hr. The behaviour of single mosquitoes in term of their attraction to the floral odour was assessed based on their landing on the gauze in front of the odour source (20 cm × 10 cm). To avoid counting any responding mosquito twice, landing mosquitoes were removed from the wind tunnel 15 sec. after their landing with the aid of an aspirator. To evaluate the attractiveness of mosquitoes to different *S.otites* populations, 25 bioassays using *S. otites* of six populations were conducted.

3. Estimation of antennal sensitivity by Electroantennography. Electroantennographic recordings (EAG) of mosquito antennae were done using 13 compounds common in *S. otites* of all populations to test whether mosquitoes can detect (smell) the common odour compounds of *S. otites*, to compare the antennal sensitivity among different compounds, and to record the way of changes in sensitivity with changes of compounds doses. The compounds used in this study contributed 97% of the total floral scent of *S. otites* in average. Four- to five-day-old mosquitoes were used for measurements. An excised antenna was mounted between glass micropipette electrodes filled with insect ringer. The electrodes were connected to silver wires. Signals were interfaced with a two-channel USB acquisition controller (provided by Syntech, Hilversum, Netherlands) to a PC as described in Dötterl et al. (2005b). 20 µl of test compounds were placed onto a piece of filter paper (2.5 × 1.5 cm^2) inside a 5 ml plastic syringe (Omnifix, B/Braun, Melsungen). Separate syringes were used for each stimulus. Stimuli were released into a continuous flow of humidified air passing over the antenna with a pulse duration of 0.5 sec, and a flow of 10 ml/sec regulated by a CS-01 Stimulus Controller (Syntech). Each compound and each dilution was tested on 4-6 mosquito antennae.

4. Evaluation of antennal responses with a GC-EAD system. As not only the main and most common compounds of *S. otites* tested in the EAG studies, but also the minor compounds of *S. otites* may be important attractants, GC-EAD (gas chromatography coupled to electroantennographic detection) measurements were conducted to identify the compounds of *S. otites* scent samples detected by mosquitoes (Fig. 2). For these measurements 1 µl of dynamic-headspace samples, eluted with acetone, of different populations of *S. otites* were used. The same GC-EAD system and settings as described by Dötterl et al. (2005b) was used. Only compounds eliciting signals in at least two runs were treated as electrophysiologically active. The lifespan of cut mosquito antennae is very short (ca. 10 min) due to which it was not possible to use the cut antennae for GC-EAD measurements as used for EAG recordings, because one run for GC-EAD recording lasts 20 min. Therefore, it was necessary to develop another preparation method. It was found that the lifespan of antennae was increased when the antennae were kept with the head of mosquito cut from the thorax and the postoccipital region was subsequently placed in the glass capillary electrode containing insect ringer. The tip of one antenna was cut off, as done during EAG recordings, and placed in another glass capillary electrode containing also insect ringer. Antennae of 9 males and 24 females of *C. pipiens* were measured on the inflorescence samples, while 10 male and 8 female antennae were additionally tested on authentic standard compounds.

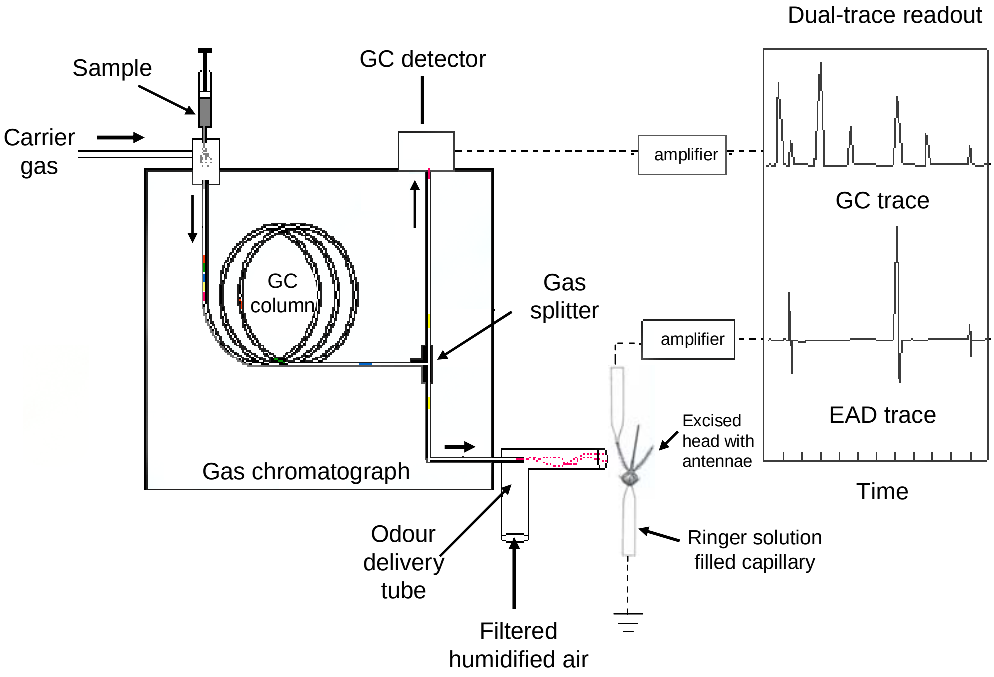

Figure 2. Simultaneous recordings of signals from a FID detector and an insect antenna (EAD). Modified from: http://www.srs.fs.usda.gov/4501/what_is_gc.htm.

GC-EADs were not only conducted with *C. pipiens* to identify the compounds of *S. otites* detected by this pollinator, but also with day-active *Aedes aegypti* to test the hypothesis that night-active mosquitoes respond more sensitively to floral scent compounds than day-active mosquitoes. Night-active visitors have to use floral scent as cues for nectar-plant finding because visual cues are insufficient, while day-active visitors do not need to rely solely on floral scents for host-plant finding, as additionally they may use visual advertisements. Therefore, night-active mosquitoes may have specific adaptations in order to sensitively detect floral scents. Here, a total of 20 males and

6 females of *A. aegypti* were tested on floral scent samples of *S. otites,* and 7 males and 19 females were tested on authentic standard compounds.

5. Attractiveness of antennal sensitive compounds. Bioassay experiments with *C. pipiens* were done using the antennal sensitive compounds as mixtures in different dilutions and singly to reveal the effect of compound concentrations for attraction of mosquitoes and to identify the most attractive compounds, respectively. All the assays were conducted in the wind tunnel, as described above with few exceptions. Here, five mosquitoes per group were released to evaluate their attractiveness, and paraffin oil was used as a negative control.

6. Two-stimuli choice experiments. In wind tunnel experiments the four most attractive compounds were tested against a mixture of all behaviourally attractive compounds, and also against phenyl acetaldehyde, a compound known to be attractive for mosquitoes (Howse 2003) and dominant in most of the *S. otites* samples. The aim was to test whether a scent mixture comparable to the scent emitted by *S otites* is more attractive than a subset of these compounds, and whether a mixture of compounds is more attractive than the main compound of *S. otites*.

7. Learning experiments. Wind tunnel bioassays with flower scent naïve and flower scent experienced (conditioned) mosquitoes were done to explore whether or not a behavioural modification exists in mosquitoes due to their experience with flower odour. It was hypothesised that conditioned mosquitoes are more strongly attracted to flower scent compounds compared to naïve mosquitoes. Learning may help mosquitoes in repeated finding of the most suitable host-plant species. For conditioning, unfed mosquitoes were kept together in small cylindrical boxes and got access for 1 hour to a filter paper sucked with a sugar solution, in combination with a scent stimulus. The control group of naïve mosquitoes was offered a sugar solution without a scent stimulus. Only individuals that had consumed sugar solutions were used for subsequent wind tunnel bioassays after a specific starvation period. During bioassay experiments the same scent stimuli as used for conditioning were offered to naïve as well as conditioned mosquitoes and the percentage number of mosquitoes attracted was compared between these two groups.

8. Scoring of day-rhythmic variation. Though *S. otites* is described as pollinated during night, preliminary observations revealed that this plant species is also visited by several insects during day-time. Therefore, the day-rhythmic variation in floral scent and flower visitors of *S. otites* were investigated in detail. It was tested whether there is pollinator-mediated selection on floral scent composition or not. *Silene otites* might emit specific compounds during day-time for attraction of day-active pollinators, and different compounds during night for attraction of night-active visitors.

Results and Discussion

Many mosquito species visit plants for nectaring, but little is known about these mosquito-plant interactions. Most of the investigations available focused on the description of habitats, where mosquitoes were found visiting plants for nectaring (Sandholm and Price 1962; Grimstad and DeFoliart 1974), and only few studies working on the signals used by mosquitoes for nectar-plant finding are available (Jepson and Healy 1988; Foster and Hancock 1994; Mauer and Rowley 1999). This dissertation provides data on the importance of olfactory cues for mosquitoes in finding flowering plants. It shows that in absence of visual stimuli mosquitoes are effectively attracted to the inflorescences of *Silene otites*, a plant pollinated, besides other insects, by mosquitoes.

In this study the geographic variability of inflorescence scent in *S. otites* was determined by GC-MS, and the attractivity of inflorescence odour of plants of different populations was tested in wind tunnel bioassays with *Culex pipiens molestus* (Chapter 1). Further, electroantennographic measurements (EAG, GC-EAD) were conducted to identify the scent compounds of *S. otites* detected by mosquitoes, and to compare the antennal responses to different compounds (Chapter 1, Chapter 2). Bioassays with the electrophysiologically active compounds demonstrated that these compounds are indeed responsible for attraction of mosquitoes to this plant species (Chapter 2). Learning experiments revealed that mosquitoes can learn flower scent compounds, which might help them in finding and visiting the nectar-rich plants repeatedly (Chapter 3). Finally, an investigation on the day- and night-active flower visitors revealed that *S. otites*, described as nocturnal, is not visited only during night, but also during day-time by several insect species. The day-active visitors might also be attracted to flowers of *S. otites* by their scent because this plant emits scent during day-time as well. However, much higher amount of scent was found at night, and qualitative differences in scent between day and night are pointing towards pollinator mediated selection of floral scent in *S. otites* (Chapter 4).

This is the first study incorporating flower scent analyses, electrophysiological recordings, and behavioural assays in order to identify flower-related attractants for mosquitoes. The understanding of the floral odour components responsible for olfactorial and behavioural responses in mosquitoes will assign us to use synthetic floral odours for monitoring and controlling of mosquito species.

Flower odour variation in *Silene otites* populations

The analyses of floral odour of *S. otites* revealed the presence of compounds of different classes, mainly benzenoids, monoterpenoids, and fatty acid derivatives (Chapter 1). The benzenoid compound phenyl acetaldehyde was the most dominant one among all odorant compounds followed by lilac aldehyde, (Z)-3-hexenyl acetate, linalool oxide (pyranoid), (Z)-3-hexen-1-ol, benzaldehyde, phenylethyl alcohol, linalool, linalool oxide (furanoid), lilac alcohol, acetophenone, methyl salicylate and hexanol. Most of the compounds found in this study have been reported earlier as part of the floral odour bouquet in other angiosperms (Knudsen et al. 2006), but only nine of the compounds identified in this study were also found in the *S. otites* sample analysed by Jürgens et al. (2002). On the other hand, Jürgens et al. (2002) identified nine compounds that were not detectable in the samples analysed in the present study. Furthermore, only small amounts of phenyl acetaldehyde were found in the study of Jürgens et al. (2002), though this compound was found as the dominant compound in most samples of present study.

The differences between the findings of these two studies might be ascribed to different scent collection methods, but probably such differences could also be due to the sampling of plants of different geographical origin. The populations of *S. otites* studied here emitted population-specific scent profiles (Fig. 2 in Chapter 1) and out of 38 inflorescence volatiles, only 19 were common to all plants. The differences in scent chemistry found among plants of different populations could not be explained by the geographic origin of the plants. There was no correlation between the scent and the distance matrix of the populations indicating that populations close to each other were not more similar in their scents than distant populations. In *S. otites* the reason for the observed variability is unknown, but it may be that different populations depend on different pollinators. Plants dominated by lilac aldehyde are possibly more frequently visited by moths compared to plants dominated by phenyl acetaldehyde and visited especially by mosquitoes. Lilac aldehyde is the most attractive compound for nocturnal moths, such as *Autographa gamma* (Plepys et al. 2002 a,b), a known visitor of *S. otites*, and it is known that lilac aldehyde is more attractive for noctuids than phenyl acetaldehyde (Dötterl et al. 2006). In this study the compounds, such as hexanol or (Z)-3-hexenyl acetate, which are not exclusively emitted by flowers but also by vegetative plant parts (Pare and Tumlinson 1999), were also included. Insects attracted to the plants may detect green leaf volatiles as well as floral odours (Chapter 1, Chapter 2), and may use these two groups of odours for host-plant finding.

Variable floral odour and equal attractivity

In presence of only olfactory stimuli, male and female mosquitoes were equally attracted to different chemotypes of *S. otites* irrespective of the total amount of scent emitted and the sex of the inflorescences used in bioassays (Chapter 1). It raises the question, why different chemotypes attracted equal percentage numbers of mosquitoes?

The wind tunnel bioassays with single electrophysiologically active compounds revealed that mosquitoes are attracted to a wide range of odorant compounds (Fig. 2 in Chapter 2). These data explain why mosquitoes were similarly attracted to different odours consisting of different mixtures/ratios of these compounds. Furthermore, mosquitoes were not only effectively attracted to different compounds, but the attraction was similar to different concentrations of compounds (Fig. 3 in Chapter1, Fig. 1 in Chapter 2). This finding is inconsistent with the results of Bowen (1992) showing that the behavioural response is increasing with stimulus concentration. However, these two studies are not comparable, because Bowen (1992) used a much higher and unnatural dosage of the compounds compared to this study. In conclusion, mosquitoes are very generalistic in their attractiveness to the floral odours of *S. otites*. It can be assumed that mosquitoes are not only attracted to the odours tested in this study, but to several further odour compounds being emitted by other plants visited by mosquitoes. The attraction of mosquitoes to many flower scent compounds might explain why mosquitoes are found on quite different plant species for nectaring.

Antennal sensitivity of day- and night-active mosquitoes to floral scent volatiles of *Silene otites*

Both male and female *C. pipiens molestus* were equally attracted to inflorescence odours of different *Silene otites* populations, and therefore, it was assumed that the floral odour compounds, which were common to all populations, might have been responsible for attracting mosquitoes. However, a presupposition would be that mosquitoes have antennal receptors for these compounds. Indeed, mosquitoes responded to all of the 13 compounds common in all populations in the electroantennographic (EAG) measurements (Fig. 4 in Chapter 1). Some compounds were more sensitively detected than others. Further, mosquitoes additionally responded to another compound, benzyl alcohol, in GC-EAD studies using dynamic headspace samples of *S. otites* inflorescences (Chapter 2). All these compounds may be used by *C. pipiens* for host-plant finding.

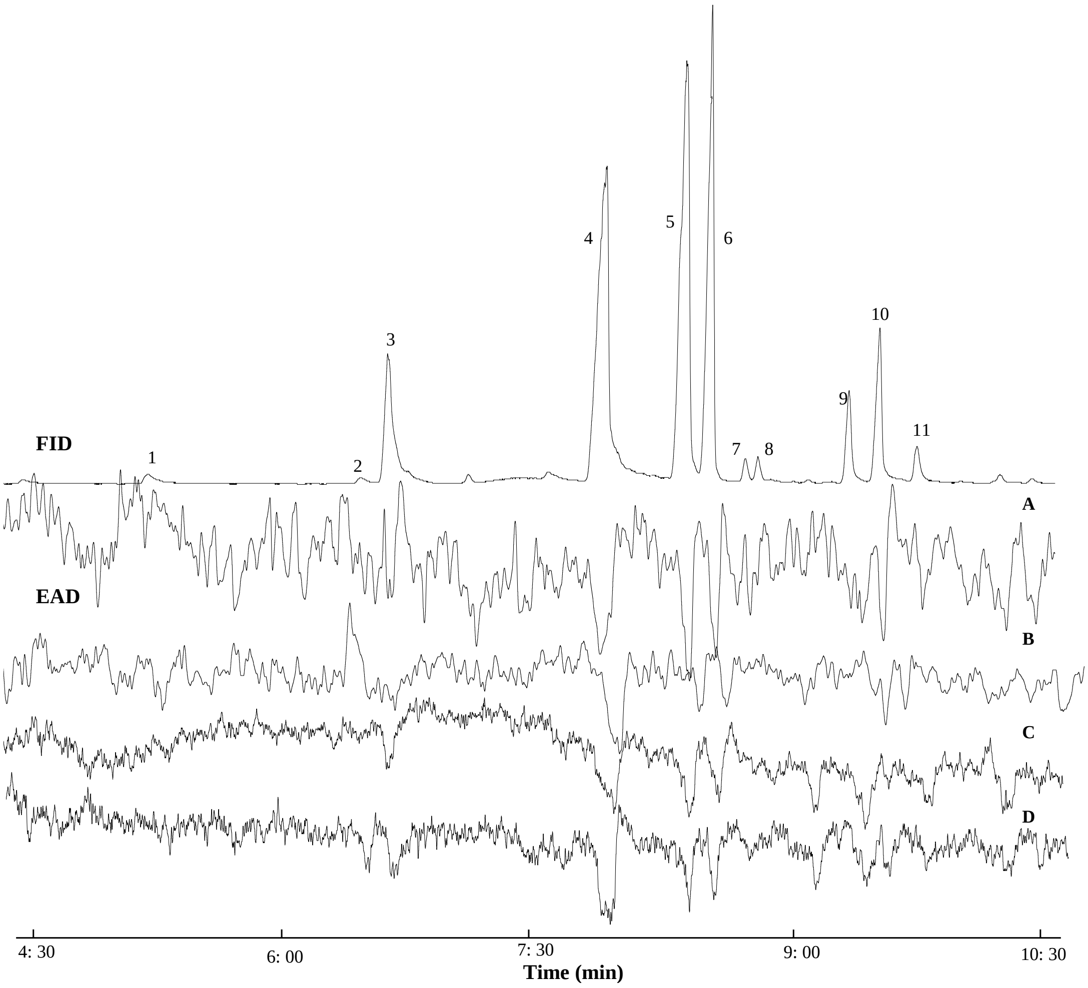

Figure 3. Coupled gas chromatographic and electroantennographic detection (GC-EAD) of a *Silene otites* inflorescence scent samples using antennae of A-B: *Culex pipiens molestus* (male and female, respectively), and C-D: *Aedes aegypti* (male and female, respectively). 1. Benzaldehyde; 2. Benzyl alcohol; 3. Phenylacet aldehyde; 4. Phenylethyl alcohol; 5. Lilac aldehyde A; 6. Lilac aldehyde B+C; 7. Lilac aldehyde D; 8. (Z)-Linalool oxide (pyranoid); 9. Lilac alcohol A; 10. Lilac alcohol B+C; 11. Lilac alcohol D.

The comparison of antennal sensitivity between night- and crepuscular-active *Culex pipiens molestus* and day-active *Aedes aegypti* revealed in general no differences between both sexes, at least in their qualitative detection of compounds (Fig. 3; Table 2 in Chapter 2), though some differences seem to exist at least in some analyses. Therefore, not only night-active mosquitoes, which have to depend on floral scent for finding suitable nectar host-plants, but also day-active mosquitoes, which could efficiently use the optical flower signals for finding nectar plants, seem to have similar receptors on their antennae for detecting floral scents, and both may rely on chemical signals for finding suitable flowers for nectaring. Although the behavioural attractiveness of *A. aegypti* to the antennal active compounds was not evaluated in present study, *A. aegypti* is known to be attracted to *S. otites* odours (K. Jahreiß, personal communication) as well as odours of other plants (Jepson and Healy, 1988). Therefore, day- as well as night-active mosquitoes rely on floral scents for finding nectar host-plants.

The compounds identified as antennal stimulants in mosquitoes could be neutral, attractive as well as repellent to mosquitoes. Few studies showed that the antennal sensitivity was not always directly correlated with behavioural attractiveness. For example linalool oxide furanoides elicited strong EAG response in *Pieris rapae crucivora* Boisd., but acted as weak deterrent in proboscis extension reflex tests, and weak repellents in flower visiting tests (Ômura et al. 2000). On the other hand, dipropyl disulphide elicited weak EAG response in the female of *Acrolepiopsis assectella* Zell., but was highly attractive to this moth (Lecomte et al.1998). As mosquitoes are attracted to the inflorescence odours of *S. otites* (Table 2 in Chapter 1), it is likely that at least some of the compounds eliciting signals in the antennae are attractive to mosquitoes as a mixture thereof or singly.

Behavioural attraction of mosquitoes to floral volatiles of *Silene otites*

With regard to floral volatiles only four compounds are known to be detected by mosquitoes (phenylethyl alcohol, benzyl alcohol, Mauer and Rowley 1999), or to be behaviourally active (phenyl acetaldehyde, Howse 2003; linalool, Kline et al. 2003). In this study in total 14 *S. otites* volatiles, including those four compounds, were found to be electrophysiologically (Chapter 1, Chapter 2) as well as behaviourally (Chapter 2) active in mosquitoes. All these compounds are also known to elicit antennal responses and/or to be potent behavioural attractants in other insects, e.g. Coleoptera (Lampman et al. 1987; Bartlet et al. 1997; Dickens 1999), Hemiptera (James 2005), Homoptera (Han and Chan 2002), Hymenoptera (Du et al. 1998; Wei and Kang 2006), Lepidoptera (Honda et al.1998; Landolt et al. 2001; Dötterl et al. 2006), and Neuroptera (Han and Chan 2002). Interestingly, some of these compounds were found to repel other insects, for example phenyl acetaldehyde repels the ants *Solenopsis geminate* Fabricus and *Pheidole morrisi* Forel (Blum et al. 1982), linalool oxide (pyranoid and furanoid) the butterfly *Pieris rapae crucivora* Boisd. (Ômura et al. 2000), methyl salicylate the aphids *Rhopalosiphum padi* (Pettersson et al. 1994) and *Aphis fabae* Scop. (Hardie et al. 1994), acetophenone the beetles *Dendroctonus pseudotsugae* (Pureswaran and Borden 2004) and *Dendroctonus brevicomis* LeConte (Erbilgin et al. 2007), and linalool *Lygaeus kalmii* Stal and *Tetraopes tetrophthalmus* Forster (Theis 2006).

In present study weak antennal sensitive compounds were responsible for strong behavioural attraction and vice versa. For example, acetophenone, phenyl acetaldehyde and phenylethyl alcohol were found to elicit only weak antennal responses, whereas in the behavioural assays mosquitoes were strongly attracted by these compounds. In case of linalool, the opposite was true. Therefore, antennal sensitivity to flower volatiles cannot be correlated with behavioural attractivity of mosquitoes to these compounds.

The preference of mosquitoes to floral over vegetative odour

The more effective attraction of mosquitoes to the mixture of the four most attractive floral odour compounds than to the mixture of floral and vegetative compounds indicate the importance of floral odours for mosquitoes in finding flowering plants. The equal attraction of mosquitoes to the mixture of the four most attractive odour compounds and phenyl acetaldehyde show that there was no synergistic effect of the four compound mixture, and phenyl acetaldehyde alone was as attractive as the mixture of the most attractive compounds which also contained phenyl acetaldehyde. In natural environment, green leaf compounds are widespread and would not necessarily help insects to find their accurate location of flowers (Honda et al. 1998). However, in this study mosquitoes were attracted by several vegetative odours without offering additionally typical flower scent compounds (Fig. 2 in Chapter 2,). Therefore, it seems that mosquitoes in search for food are attracted by vegetative scents when no typical flower signals are available, but mosquitoes prefer and rely on typical flower scents over a mixture of flower and vegetative scents for finding the flowers effectively.

Learning of flower odour compounds by mosquitoes

The learning capability of mosquitoes is known with regard to oviposition kairomones (McCall and Eaton 2001; Kaur et al. 2003), repeated finding of suitable breeding sites (Charlwood et al. 1988) as well as swarming sites (Service 1994), and repeated finding of a specific blood meal host species (Mwandawiro et al. 2000). In this study, the significantly higher attraction of experienced than naïve mosquitoes to three odour compounds, tested as mixture and singly, exhibits the evidence of learning capability of mosquitoes in relation to flower odour compounds. Furthermore, the conditioned mosquitoes were not only more effectively attracted to floral scent compounds, they also showed a different behaviour after landing on the gauze compared to naïve mosquitoes. While in naïve mosquitoes the ‘sitting’ response dominated, the most conditioned mosquitoes actively searched for food and penetrated the gauze with their proboscis. This finding strongly indicates that, based on their previous experience, the conditioned mosquitoes have learned the odour as a signal for food. The learning of compounds or compound mixtures may help mosquitoes in repeated finding of the most suitable host-plant species. The fact that learning influences the behaviour of mosquitoes also needs to be considered when using floral scent as bait in traps. To successfully use floral scent for trapping mosquitoes it might be necessary analysing the scent of flowers visited by mosquitoes in the area of the trap to be able to mimic the naturally occurring odour compounds leading possibly to an increased effectivity of the trap.

Temporal variation of flower visitor and flower scent of *Silene otites*

Flower visitor observations showed that *Silene otites*, although regarded as adapted to microlepidoptera and mosquitoes as pollinators, is visited by a wide range of insects during the night and day with almost 60 species of 5 insect orders (Table 1 in Chapter 4). During day beetles, brachyceran flies and hymenopteran species were found, at night flowers were visited by nocturnal Lepidoptera, nematoceran flies, and Neuroptera individuals. Most flower visitors were observed visiting many flowers consecutively, and visiting male and female flowers, making them to potential pollen vectors. Therefore, *S. otites* might not be pollinated only during night, but also during day-time.

The analyses of flower scent collected at night and during the day revealed that *S. otites* emitted the scent abundantly at night and scarcely during day-time. Emitted amount of volatiles along with their chemical constituents were also highly variable.

These differences in scent in a course of the day might be the results of pollinator mediated selection. *S. otites* emitted most of its scent immediately after the darkness, the time when most of the nocturnal visitors, i.e. moths and mosquitoes, were observed visiting the flowers. During that time the scent was characterized by high percentage amounts of phenyl acetaldehyde, 2-phenylethanol, and lilac aldehyde (Table 2 in Chapter 4). All these compounds are known to be attractants for moths (Haynes et al. 1991; Heath et al. 1992; Meagher 2001; Meagher 2002; Plepys et al. 2002; Dötterl et al. 2006) and/or mosquitoes (Chapter 2), the main flower visitors of *S. otites* at night. In the afternoon, when especially bees and syrphids were frequently found, the flowers emitted several compounds known to be attractive for these insects. As an example, linalool, the most abundant compound emitted in the afternoon is known as attractant for bees (Henning et al. 1992), and methyl salicylate, also emitted mainly in the afternoon, is known as attractant for syrphid flies (James 2005). In conclusion, day and night visiting insects of *Silene otites* may act as important pollen agents for the reproductive success of this species. The ‘fine tuned’ odour emission of this plant seems to be an adaptation to the olfactory abilities and preferences of the day- and night-active pollinators, respectively.

Future outlook

This thesis provides the evidence that mosquitoes are attracted to the emitted odour of *Silene otites*, that mosquitoes can detect 14 of the odour compounds emitted, and that these compounds are responsible for attraction of mosquitoes to this species. In a next step field experiments need to be conducted to test, whether these compounds, especially the four most attractive ones, are useful as bait in traps for the manipulation and monitoring of population dynamics of mosquitoes.

Summary

In this investigation the role of flower odours in attracting mosquitoes to *Silene otites* were evaluated. Although mosquitoes are known to visit diverse flowers for nectaring, *S. otites* is one of only two plant species world-wide in which mosquitoes have been described as pollinators. In several investigations flower odours were described as effective attractants for mosquitoes, however, in none of the studies the compounds responsible for attraction were identified. Therefore, in the present study the aim was to investigate the chemical components in *Silene otites* that play a role in the attraction of mosquitoes. The information obtained can be useful in developing odour-baited traps for monitoring and controlling mosquito populations.

Inflorescence odour composition of 63 *Silene otites* samples of nine populations was determined. There was variability in scent composition among populations and between sexes, and few chemotypes dominated by different compounds were identified (Chapter 1). Most populations and samples were dominated by phenyl acetaldehyde, but in some samples high relative amounts of lilac aldehyde or (Z)-3-hexen-1-ol and (Z)-3-hexenyl acetate were present. One sample was characterized by a high percentage amount of linalool. Populations geographically close to each other were not more similar in their scent than distant populations. Nevertheless, in wind tunnel bioassay experiments with inflorescence odours of *S. otites* of six populations and *Culex pipiens molestus*, no differences in attractivity of different populations were observed. Therefore, different scent spectra seem to have the same attractiveness for mosquitoes.

In electroantennographic studies with the 13 most common and abundant odour compounds of *S. otites*, *Culex pipiens molestus* males and females responded similarly

to dilution series but the strength of the responses differed among compounds. Linalool oxide (furanoid) and linalool evoked the strongest responses in male and female mosquitoes, and (Z)-3-hexenyl acetate was strongly active in females. Medium responses were evoked in males by (Z)-3-hexenyl acetate, in females by benzaldehyde and methyl salicylate, and in both sexes by lilac aldehyde, lilac alcohol, and linalool oxide (pyranoid). Further, the differences in antennal responses between night- and crepuscular-active *Culex pipiens molestus* and day-active *Aedes aegypti* to headspace flower odour samples of *S. otites* were investigated using coupled gas chromatographic–electroantennographic detection (GC-EAD). Night-active visitors have to use floral scent as cue for nectar plant finding because visual cues are insufficient, while day-active visitors need not solely to rely on floral scents for host-plant finding; additionally they may use visual advertisements. Therefore, it was hypothesised that night-active *C. pipiens* more strongly respond to floral scents than day-active *A. aegypti*. However, no striking differences in antennal responses to the odour compounds have been found between the two mosquito species (Chapter 2).

To identify the compounds responsible for attraction of mosquitoes to *S. otites*, wind tunnel bioassays with antennal sensitive compounds and *C. p. molestus* were conducted. Mosquitoes responded to 14 compounds in different magnitudes (65-20%) and acetophenone, linalool oxide (pyranoid), phenyl acetaldehyde and phenylethyl alcohol were found as more attractive in comparison to the least attractive compound, hexanol (Chapter 2). Further, in two-stimulus choice tests, mosquitoes were significantly more attracted to the mixture of the four most attractive compounds (all typical flower scent compounds) than the mixture of all 14 compounds (including flower and vegetative scents of *S. otites*). Therefore, it seems that mosquitoes prefer flower scent over a combination of flower and vegetative scents when searching for flowers.

Wind tunnel bioassays were not only conducted with naïve mosquitoes but also with conditioned mosquitoes to test the hypothesis that mosquitoes can learn flower scent compounds (Chapter 3). Learning may help the mosquitoes to find the most suitable host-plant that they visit more frequently thereafter. Indeed, the significantly higher attraction of conditioned mosquitoes compared to unconditioned ones to different floral odours, as mixture and singly, proved the learning capacity of mosquitoes to floral volatiles.

Though *Silene otites* is described as night-pollinated, preliminary observations revealed that this plant species is also visited by several insects during day-time. Therefore, the temporal variations of flower scent and flower visitors were investigated in more detail (Chapter 4). Although *S. otites* emits highest amounts of floral volatiles at night, just after sunset, it also emits a smaller amount of volatiles during day-time. Interestingly, the temporal variations differed among scent compounds where five different emission patterns of compounds could be categorized: (1) mainly few hours after sunset (e.g. phenyl acetaldehyde, lilac aldehyde), (2) during whole night (e.g. α-pinene), (3) mainly in the afternoon (e.g. linalool), (4) during the forenoon as well as the afternoon (e.g. (E)-β-Ocimene), and (5) during the afternoon, and the first half of the night (e.g. benzaldehyde). During day and night, a total of 60 flower-visiting insect species were recorded. During day beetles, brachyceran flies and hymenopteran species were found, whereas, at night the nocturnal Lepidoptera, nematoceran flies, and neuropteran individuals were found to visit the flowers. Insect species visiting the flowers during daytime may be attracted by the scent compounds emitted during day, while nocturnal insects may be attracted by compounds emitted during night. These differences in scent in the course of the day might be the results of pollinator mediated selection, and the ‘fine tuned’ odour emission of this plant seems to be an adaptation to the olfactory abilities and preferences of the day- and night-active pollinators, respectively. Pollination experiments are now needed to test, whether day-active visitors are indeed pollinators of this plant, and to determine the contribution of day- as well as night-active visitors to reproductive success.

Zusammenfassung

In der vorliegenden Dissertation wurde die Funktion von Blütendüften des zweihäusigen Ohrlöffelleimkrauts *Silene otites* bei der Anlockung von Stechmücken untersucht. Mücken besuchen eine Vielzahl von Pflanzenarten, um an deren Nektar zu trinken, wobei *Silene otites* neben der Orchidee *Habenaria obtusata* die einzige Pflanzenart ist, bei der Mücken als Bestäuber beschrieben sind. Seit einiger Zeit ist bekannt, dass Blütendüfte beim Auffinden der Nektarpflanzen eine große Rolle spielen, wobei nicht bekannt war, welche Stoffe für die Lockwirkung verantwortlich sind. Es kann angenommen werden, dass Pflanzen, die auf Mücken als Bestäuber angepasst sind, effektivere Lockstoffe für Mücken abgeben als Pflanzen, die primär von anderen Tieren bestäubt werden. Ziel dieser Arbeit war es daher, die Substanzen von *S. otites* zu identifizieren, die für die Anlockung von Stechmücken verantwortlich sind. Effektive Lockstoffe könnten dann beim Monitoring von Mückenpopulationen in Fallensystemen eingesetzt werden.

Die Untersuchung des Blütenduftes von 63 Individuen von *Silene otites* unterschiedlicher Herkunft ergab, dass sich Pflanzen von verschiedenen Populationen in ihrer Duftzusammensetzung unterscheiden und dass es auch geringe Unterschiede im Duft zwischen männlichen und weiblichen Pflanzen gibt (Kapitel 1). Der Duft der meisten Populationen bzw. Proben war von Phenylacetaldehyd dominiert, andere von Lilakaldehyd, (Z)-3-Hexen-1-ol oder (Z)-3-Hexenylacetat. Eine Duftprobe war von Linalool dominiert. Pflanzen, die aus geographisch nah zusammen liegenden Populationen kamen, dufteten nicht ähnlicher als Pflanzen aus weit entfernten.

Trotz dieser Unterschiede im Duft unterschiedlicher Populationen war der Infloreszenz-Duft von *S. otites* Pflanzen verschiedener Populationen im Windkanaltest gleich attraktiv für Stechmücken.

Elektroantennographische Messungen mit *Culex pipiens* haben gezeigt, dass Mücken viele Stoffe riechen können, die von *S. otites* Blütenständen abgegeben werden. Besonders große Signale bei den Messungen wurden durch Linalooloxid (furanoid) und Linalool in den Antennen von männlichen wie weiblichen Mücken und durch (Z)-3-Hexenylacetat nur in weiblichen Antennen hervorgerufen. Mittelgroße Ausschläge wurden in männlichen Antenne durch (Z)-3-Hexenylacetat, in weiblichen Antennen durch Benzaldehyd and Methylsalicylat, und in den Antennen beider Geschlechter durch Lilakaldehyd, Lilakalkohol und Linalooloxid (pyranoid) ausgelöst (Kapitel 1).

Um zu prüfen, ob nachtaktive Mücken Blütendüfte empfindlicher riechen als tagaktive, wurden Blütendüfte von *S. otites* nicht nur an Antennen der nachtaktiven Art *C. pipiens* getestet, sondern auch an der tagaktiven Gelbfiebermücke *Aedes aegypti*. Blütendüfte sind besonders nachts wichtig, wenn Optik kaum eine Rolle bei der Wirtspflanzenfindung spielt, während tagsüber optische Blütensignale eine größere Bedeutung haben könnten. Entgegen der Annahme konnten jedoch keine Unterschiede bezüglich der Detektion von Blütendüften bei tag- und nachtaktiven Stechmücken festgestellt werden (Kapitel 2).

Um die Stoffe zu identifizieren die letztendlich für die Anlockung von Stechmücken eine Rolle spielen, wurden alle 14 elektrophysiologisch aktiven Substanzen an *C. pipiens* im Windkanal getestet. Die Duftstoffe lockten im Mittel zwischen 20% und 65% der Mücken an, wobei Acetophenon, Linalooloxid (pyranoid), Phenylacetaldehyd und Phenylethylalkohol am attraktivsten waren und Hexanol die wenigsten Mücken anlockte (Kapitel 2).

In einem Zwei-Wahl-Test wurden signifikant mehr Mücken von den vier attraktivsten Stoffen angelockt als von einem Gemisch, das alle 14 Stoffe enthielt. Im Gegensatz zu der 4-Komponenten Mischung beinhaltete die 14-Komponenten Mischung auch Stoffe, die nicht nur von Blüten, sondern auch von vegetativen Pflanzenteilen abgegeben werden. Auf der Suche nach Nektar scheinen die Mücken daher Blütendüfte gegenüber einer Kombination mit Blattdüften zu bevorzugen (Kapitel 2).

Um zu testen, ob Stechmücken Blütendüfte lernen können, wurden Lernexperimente durchgeführt (Kapitel 3). Die Ergebnisse haben gezeigt, dass Mücken in der Tat Blütendüfte erlernen können und signifikant mehr konditionierte als naive Mücken von Einzelsubstanzen sowie von einem Duftstoffgemisch angelockt werden können. Das Lernen solcher Blütensignale könnte Stechmücken helfen, eine geeignete Nektarpflanzenart wieder zu finden.

Obwohl *Silene otites* als nachtbestäubt gilt, konnten bei Voruntersuchungen auch tagsüber Bestäuber an den Blüten beobachtet werden. Basierend auf diese Erkenntnisse wurden der Blütenduft sowie die Blütenbesucher im Tagesverlauf detailliert untersucht (Kapitel 4). Insgesamt konnten 60 verschiedene Insektenarten an den Blüten beobachtet werden, tagsüber vor allem Käfer, Fliegen und Hautflügler und nachts Stechmücken, Nachtfalter sowie Netzflügler. All diese Insekten sind potentielle Bestäuber von *S. otites*.

Die Blütenduftuntersuchungen ergaben, dass *Silene otites* den meisten Duft nachts, kurz nach Sonnenuntergang, abgibt. Eine kleinere Duftstoffmenge wurde aber auch tagsüber abgegeben. Interessanterweise unterschieden sich einzelne Duftstoffe deutlich in ihrer Tagesrhythmik und die Stoffe konnten in fünf Kategorien eingeteilt werden: Stoffe, die (1) wenige Stunden nach Sonnenuntergang (z.B. Phenylacetaldehyd, Lilakaldehyd), (2) die ganze Nacht (z.B. α-Pinene), (3) vornehmlich am Nachmittag (z.B. Linalool), (4) sowohl vormittags wie nachmittags (z.B. (E)-β-Ocimen) und (5) nachmittags und in der ersten Nachthälfte (z.B. Benzaldehyd) emittiert werden.

Die unterschiedliche Duftabgaberhythmik könnte eine Anpassung an die olfaktorische Ausstattung und Vorlieben der tag- wie nachtaktiven Blütenbesucher darstellen. Stoffe wie Linalool, die tagsüber abgegeben werden, dürften bei der Anlockung von tagaktiven Bestäubern (z.B. Bienen) eine Rolle spielen, während Stoffe wie Phenylacetaldehyd und Lilakaldehyd effektive Lockmittel für Mücken und Nachtfalter sind. Bestäubungsexperimete müssen nun zeigen, ob tagaktive Blütenbesucher in der Tat auch Bestäuber sind, und welchen Anteil sie am Reproduktionserfolg von *S. otites* haben.

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Darstellung des Eigenanteils

Kapitel 1-3: Sämtliche Daten wurden von mir erhoben und ausgewertet. Die Manuskripte wurden von mir (80%), Andreas Jürgens (10%) und Stefan Dötterl (10%) verfasst.

Kapitel 4: Die Daten über die Blütenbesucher wurden von Katerin Jahreiß erhoben. Die Blütenduftproben wurden von mir (3 von 4) sowie von Katrin Jahreiß (1 von 4) gesammelt und analysiert. Das Manuskript hat Stefan Dötterl und Andreas Jürgens geschrieben.

FLORAL ODORS OF *Silene otites* (CARYOPHYLLACEAE): THEIR VARIABILITY AND ATTRACTIVENESS TO MOSQUITOES

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Abstract Inflorescence scent samples from nine populations of dioecious *Silene otites*, a plant pollinated by moths and mosquitoes, were collected by dynamic headspace. Sixty-three scent samples were analyzed by gas chromatography - mass spectrometry. Out of 38 compounds found, 35 compounds were identified, most of which were monoterpenoids, fatty acid derivatives, and benzenoids. Phenyl acetaldehyde was the most dominant compound in most of the samples. The variability in scent composition was high, and population as well as sex differences were found. Nevertheless, wind tunnel experiments proved similar attraction of *Culex pipiens pipiens* biotype *molestus* mosquitoes to the inflorescence odor of *S. otites* of different populations, indicating that different blends are similarly attractive to mosquitoes. The electrophysiological responses of mosquitoes to the 12 most common and abundant odor compounds of *S. otites* differed. Linalool oxide (furanoid) and linalool evoked the strongest responses in male and female mosquitoes, and *(Z)*-3-hexenyl acetate was strongly active in females. Medium responses were evoked in males by *(Z)*-3-hexenyl acetate, in females by benzaldehyde and methyl salicylate, and in both sexes by lilac aldehyde, lilac alcohol, and linalool oxide (pyranoid).

Key Words *Silene otites*; flower odor variability; wind tunnel bioassays; *Culex pipiens pipiens* biotype *molestus*; electroantennography; attraction; nectar host plant

INTRODUCTION

Carbohydrates are vital resources for adult male and female mosquitoes. Up-take of sugar plays a critical role in longevity, fecundity, flight capacity, and host-seeking behavior (Harada et al., 1971; Nayar and Saurman, 1971, 1975; Magnarelli, 1978; Klowden, 1986). The primary sugar source for mosquitoes is nectar (Haeger, 1955; Sandholm and Price, 1962; Grimstad and DeFoliart, 1974), and mosquitoes prefer some plants to others as nectar sources (Grimstad and DeFoliart, 1974; Magnarelli, 1978; Gadawaski and Smith, 1992). However, the specific cues that mosquitoes use to find and to select nectar sources are not well understood. Many flower visitors, mosquitoes included, are known to be attracted to floral scents (Vargo and Foster, 1982; Dudareva and Pichersky, 2000).

For finding effective nectar-related attractants for biological control of mosquitoes, it is important to determine which plant species produces the most attractive floral compounds and to identify these compounds. Plant species adapted to mosquitoes as pollinators are expected to emit more mosquito-attracting compounds than plants pollinated primarily by other pollen vectors.

Worldwide, effective pollination by mosquitoes has been described only in the orchid *Habenaria* (*Platanthera*) *obtusata* (Banks ex Pursh) Richardson (Stoutamire, 1968) and in *Silene otites* L. Wibel (Caryophyllaceae) (Brantjes and Leemans, 1976), which is a usually perennial and dioecious species widely distributed in Middle, East and South Europe as well as in Central Asia. The small and white-greenish flowers are arranged in terminal cymes. Jürgens et al. (2002) described the floral scent composition of *S. otites*. The scent of a few plants of a single *S. otites* population was analyzed. Therefore, nothing is known about the variability in the scent of this plant among populations, or between males and females.

Compounds with low variability may be more important for attraction of pollinators than compounds with high variability (Ayasse at al., 2000), as pollinators may exert selective pressure on the scent composition in order to emit attractive compounds, resulting in regular emission of the attractive compounds, while the non-attractive compounds may be more variable. So far, only a single major volatile component of *S. otites,* phenyl acetaldehyde, has been shown to attract mosquitoes (Jhumur et al., 2006) while the importance of the total floral scent emitted by *S. otites* is unknown for attraction of its flower-visiting mosquitoes (e.g., *Culex pipiens* L. and *Culiseta annulata* Schrank; Brantjes and Leemans, 1976).

The aim of this study was therefore to analyze the geographic variability of the floral scent composition of *Silene otites* (L.) Wibel (Caryophyllaceae), and to assess the attractiveness of floral bouquets of different *S. otites* populations to *Culex pipiens pipiens* biotype *molestus* Forskal 1775. Further, the antennal electrophysiological responses of *C. p. molestus* to the most common and abundant odor compounds in *S. otites* were measured.

METHODS AND MATERIALS

Plant material Inflorescence scent samples were collected from 63 individuals of nine different populations. The geographic origin of eight populations as well as the number of females and males sampled are shown in Fig. 1. For one population (h), from which three males and four female individuals were sampled, the geographic origin is unknown. Seed of the different populations were provided by several botanical gardens. To reduce environmental variation between different populations, plants were grown under the same conditions (e.g., soil, temperature) in pots in the greenhouse until they built up a rosette, and thereafter the pots were placed in flower beds in the field.

Odor collection S. otites is a nocturnal plant. Its floral scent emission is strongest in the early night hours (Jürgens et al., 2002). The male flowers remain functional for two nights, whereas the female flowers emit scent over several days until they are pollinated (Brantjes and Leemans, 1976). For studying variability of floral scents, floral odors of *S. otites* were collected from 1-4 inflorescences of each individual plant 2-3 d after onset of floral bloom when most of the flowers in an inflorescence had opened for the first time. Thus, the inflorescences used were of the same age, however, the flowers of these inflorescences were in different developmental stages. It is unclear whether there is variation in scent of *S. otites* between flowers of different ages in the same plant, and whether this possible variation contributed to the observed variability among populations. However, as scent was collected from inflorescences of the same age, the possible variation in scent among flowers of different ages is not expected to have influenced our measurements. Further, in a closely related species, *S. latifolia*, no differences in scent composition of flowers of different stages were found (Dötterl et al., 2005b).

To collect odors, potted plants were placed under the extractor hood in the laboratory. Volatiles were collected using the dynamic headspace method described by Dötterl et al. (2005b). The inflorescences were enclosed in a polyester oven bag (20 cm×8 cm; Toppits®, Germany) 1-1½ hr after sunset, and volatiles were trapped in an adsorbent tube for 2 min using a membrane pump (ASF Thomas, Inc.) with a flow rate of 200 ml/min. The adsorbent tubes were filled with a mixture (1:1) of 3 mg Tenax-TA (mesh 60–80) and Carbotrap (mesh 20–40). To distinguish between plant volatiles and ambient contaminants, surrounding air was collected for comparison. Furthermore, to discriminate odor emitted by flowers from odor deriving from vegetative parts, scent was also collected from non-flowering shoots. However, as insects attracted to the plants may detect green leaf volatiles as well as floral odors, we also included vegetative odors in subsequent analyses (see below).

Preparation of plant material for bioassays To facilitate the work with the night-active plant-flower visitor system, plants were shifted from the flower beds to a climatic chamber with an inverted day and night rhythm shortly before onset of flowering. The maintenance of the climatic chamber was dark (9 hr: from 9 a.m. – 6 p.m.) and light (15 hr: from 6 p.m. – 9 a.m.) with 20.5°C and 24.5°C, respectively. One or two days after moving, when flower opening had adjusted to the changed day and night rhythm, inflorescences were used for bioassays. Flower odors were collected before and after each bioassay and expressed as the mean total amount of emitted odors during bioassays. Flowering inflorescences (3-5) of males or females of a population were cut and placed together in small glass bottles filled with water. Within 5 min, the inflorescences were bagged, and thereafter emitted volatiles were collected for 2 min as described above. With the exception of higher amounts of green leaf odors in cut plants, the scent compositions of clock-shifted plants were the same as those of *in situ* plants (Jhumur, unpublished data).

Preparation of insects for bioassays We used flower-naïve individuals of the autogenous *Culex pipiens pipiens* biotype *molestus* Forskal 1775 (European strain) for experiments. Mosquitoes were reared according to Jhumur et al. (2006) with an inverted day and night rhythm in accordance with the designed bioassays. For bioassays, the sugar supply was removed 61-63 hr prior to the experiment. For electrophysiological measurements, regularly fed mosquitoes were used.

Chemical scent analysis Scent samples were analyzed on a Varian Saturn 2000 mass spectrometer coupled to a Varian 3800 gas chromatograph equipped with a 1079 injector that had been fitted with the ChromatoProbe kit. The adsorbent tube containing sample was placed in the Chromatoprobe and then inserted into the modified GC injector. The injector split vent was opened (1/20), and the injector was heated to 40°C to flush any air from the system. The split vent was closed after 2 min, and the injector was heated to 200°C (200°C/min); this temperature was held for 4.2 min. Then, the split vent was opened again (1/10) while the injector was cooled down. For analyses, a ZB-5 column (5% phenyl polysiloxane; 60 m long, i.d. 0.25 mm, film thickness 0.25 µm, Phenomenex) was used. A constant flow of carrier gas (helium: 1.8 ml/ min) was maintained by electronic flow control. The GC oven temperature was held for 7 min at 40°C, then increased by 6°C per min to 250°C, and held for 1 min. The MS interface was 260°C, and the ion trap worked at 175°C. The mass spectra were taken at 70 eV (in EI mode) with a scanning speed of 1 scan sec $^{-1}$ from m/z 30 to 350. The GC-MS data were processed using the Saturn Software package 5.2.1. Component identification was carried out using the NIST 02 mass spectral data base or MassFinder 2.3, and confirmed by comparison of retention times with published data (Adams, 1995). Identification of individual components was confirmed by comparison of both mass spectra and GC retention data with those of authentic standards.

For quantification of odors emitted from inflorescences, known amounts of lilac aldehydes, trans-β-ocimene, *(Z)*-3-hexenyl acetate, benzaldehyde, phenyl acetaldehyde, and veratrole were injected in the GC for calibration.

Bioassays A 160×75×75 cm wind tunnel (Dötterl et al., 2006; Jhumur et al., 2006) was used for bioassays. A Fischbach speed controller fan (D340/E1, FDR32, Neunkirchen, Germany) continuously circulated the air through the tunnel with an air speed of 0.35 m/sec. The incoming air was cleaned through four charcoal filters (145×457 mm, carbon thickness 16 mm, Camfil Farr). To allow mosquitoes to adapt to the wind tunnel environment, they were kept in the wind tunnel room for about 12 hr before the experiment started. In order to avoid contamination, all equipment was cleaned with ethanol, burned in flame, and then sterilized at 200°C, and surgical gloves were worn during mosquito handling and bioassays.

At the conditions described above, *S. otites* emitted the highest amounts of floral odors in the 2nd and 3rd h after onset of darkness (Jhumur, unpublished data). Therefore, bioassays were conducted within this time frame. The inflorescences, the cut ends of which were already inserted in water, were placed at the upwind end of the tunnel behind gauze and different aluminium screens. They were invisible to the mosquitoes.

A group of 10-15 randomly chosen male and/or female mosquitoes (the behaviors of mosquitoes were not influenced by the opposite sex, see also Jhumur et al. 2006) were released from a chamber (16×8 cm) at the downwind end of the tunnel. The mosquitoes were observed for 1 hr. Landing of mosquitoes on the gauze (20×10 cm) in front of the odor source was considered as attraction to the source. Additionally, the latency time before landing was measured. Further, after landing, the behavior was classified into two types: “sitting” and “searching”. “Sitting” was characterized simply as sitting without moving or doing anything on the gauze during 15 sec after landing, and “searching” was characterized by excited movement of mosquitoes on the gauze and repeated penetration of gauze with their proboscis, presumably in search for a food source. To avoid recording the behavior of any responding mosquito twice, landing mosquitoes were removed from the wind tunnel after 15 sec with an aspirator.

From other tests with mosquitoes in the same wind tunnel, we know that almost no mosquitoes landed just by chance in front of the odor source (Jhumur et al., 2006). Therefore, we did not test the mosquitoes´ response to clean air or room air. Further, given that a small number of mosquitoes would land just by chance on the gauze in front of the odor source, this number should be similar for odor from all *S. otites* populations, and thus not affect the comparison of attractiveness of *S. otites* odor from different populations.

Dependent upon availability of flowers, 25 bioassays were conducted with *S. otites* plants of six populations. Male and female inflorescences were tested separately. However, female inflorescences were not available for the ‘a’, and ‘c’ populations (Table 2). Nine bioassays were conducted with population ‘i’, six with ‘f’, four with ‘g’, and two each with ‘c’, ‘b’, and ‘a’. Most of the inflorescences of one plant (one bioassay) were tested with two groups of mosquitoes, and the behavioral responses (percentage of individuals landing) of these 20-30 mosquitoes were used for subsequent statistical analyses (see below). However, for population ‘a’, only one group of mosquitoes was used for each of the two inflorescence samples. In total, 113 male and 531 female mosquitoes were tested. Male mosquitoes were not available during the bioassays with male inflorescences of populations ‘a’, ‘c’, and ‘g’, and female inflorescences of population ‘b’.

Electrophysiology

Authentic standard compounds The most frequently found 12 floral scent compounds of *S. otites* were used for electrophysiological measurements. Among these compounds, lilac aldehyde was synthesized as described by Dötterl et al. (2006); lilac alcohol and linalool were provided by Karlheinz Seifert (purity >99%), and the other compounds were purchased from Sigma-Aldrich (hexanol, *(Z)*-3-hexen-1-ol and *(Z)*-3-hexenyl acetate >98%; benzaldehyde 99%; phenylethyl alcohol 99%, acetophenone 98%; linalool oxide [furanoid] 97%; phenyl acetaldehyde 90%; methyl salicylate 98%) or Wako (linalool oxide [pyranoid] 98%). To obtain dose-response curves for the tested compounds, and to compare the sensitivity of mosquitoes to different compounds, electroantennographic (EAG) recordings were performed with a dilution series of standard compounds (Schütz et al., 1999). Dilutions were prepared in paraffin oil (Uvasol, MERCK, Darmstadt, Germany).

Preparation Four- to five-d-old *C. p. molestus* were used for EAG. For measurements, an excised antenna was mounted between glass micropipette electrodes filled with insect ringer (8.0 g/L NaCl, 0.4 g/L KCl, 0.4 g/L $CaCl_2$). The electrodes were connected to silver wires. Signals were interfaced with a two-channel USB acquisition controller (provided by Syntech, Hilversum, Netherlands) to a PC as described by Dötterl et al. (2005a). Twenty µl of a test compound were placed onto a piece of filter paper (2.5×1.5 cm^2) inside a 5 ml plastic syringe (Omnifix, B/Braun, Melsungen). Separate syringes were used for each stimulus. Stimuli were released into a continuous flow of humidified air passing over the antenna with a pulse duration of 0.5 sec, and a flow of 10 ml/sec regulated by a CS-01 Stimulus Controller (Syntech). Each compound and each dilution was tested on 4-6 mosquitoes. In all EAG tests, antennae were stimulated at 30-40 sec intervals. To discriminate between the antennal response elicited by the air flow or by paraffin, and by the tested scent compound, a filter paper containing only paraffin was tested as the first and last measurement on each antenna. To counterbalance for the loss of antennal sensitivity during the measurements, the antennal response to a syringe containing *(Z)*-3-hexen-1-ol (10^{-1} in paraffin) was recorded as the second measurement from the beginning and the end. *(Z)*-3-hexen-1-ol is a compound frequently found in sampling of *S. otites*. As this compound was used as the standard for EAG recordings, it was not used to obtain dose response curves.

Statistical analysis We used the Primer 6 programme (Clarke and Warwick, 2001; Clarke and Gorley, 2006) to assess the variability in scent of *S. otites* individuals of different populations. Semiquantitative data of compounds (percentages = relative amounts with respect to total peak areas) were used, because the total amount of emitted volatiles varied greatly among different individuals (see also Dötterl et al., 2005b). We used multidimensional scaling (MDS) based on Bray-Curtis similarities to detect similarities among samples. To evaluate how well or poorly the particular configuration produces the observed distance matrix, the stress value is given. The smaller the stress value, the better is the fit of the reproduced ordination to the observed distance matrix (Clarke, 1993). We used *ANOSIM* (two-way crossed design, factors: sex, population) in Primer to test for differences in scent between male and female flowers as well as among populations. *SIMPER* (two-way crossed design, factors: sex, population) was used in Primer to identify the compounds responsible for dissimilarities between sexes and among populations. *RELATE* was used in Primer to correlate the scent matrix with the distance matrix (in km) of the populations. To obtain the scent matrix, mean relative amounts of compounds were calculated for the different populations, and these values were used to calculate the Bray-Curtis similarities finally used for the analysis.

Chi-square tests were used to assess the differences in attractiveness between male and female mosquitoes (number of males responding-males not responding vs. number of females responding-females not responding) to male and female inflorescences of different populations of *S. otites,* and no differences in responses between male and female were found (Jhumur, unpublished data). Therefore, the responses of males and females were pooled for further analyses.

In individual bioassays with specific inflorescences, the number of landing (attractive) mosquitoes (%) was determined at first, and among the landed mosquitoes thereafter the proportion of searching mosquitoes (%) was calculated. *Kruskal-Wallis-ANOVA* followed by the *Tukey-Kramer* post hoc test for nonparametric data in STATISTICA (StatSoft, Inc. 2004) was used to compare these behavioral responses to the flower odors of different populations. *ANOVA* was used to compare the latency time of individual mosquitoes to different populations. Normality was tested using the *Kolmogorov-Smirnov test*; homogeneity of variances was tested using the *Hartley test*.

For analyzing the EAG recordings, at first the responses from the blank syringes were measured and subtracted from the recordings in between. Then, the response to *(Z)*-3-hexen-1-ol as the second measurement from the beginning of each measurement was set to 100%. As the sensitivity of antennae decreased during measurements, the response to *(Z)*-3-hexen-1-ol was also measured as the second measurement from the end, to determine the loss of sensitivity and to compensate for this. The responses to different compounds and dilutions are given as proportions of the responses to *(Z)*-3-hexen-1-ol. These data were directly used without transformation for further analyses. A General Linear Model (GLM) in STATISTICA was used to compare the differences in responses of males and females to different dilutions and different compounds. The alpha level for all statistical analyses was 0.05.

RESULTS

Variability in floral scents of Silene otites Thirty-eight compounds were detected in the inflorescence odor samples of *S. otites* of nine geographical locations, 35 of which have been tentatively identified by comparing mass spectra and retention index with literature data (Adams, 1995). Additionally, the identity of 27 of these compounds was confirmed by authentic standards (see Table 1). Among these, six compounds were also emitted from leaves. The identified compounds belong to five classes: fatty acid derivatives (8), benzenoids (6), nitrogen bearing compounds (1), monoterpenoids (18), and sesquiterpenoids (2). The benzenoid phenyl acetaldehyde (PAA) was the most dominant odor compound in most of the individuals. However, one specimen emitted no PAA but instead high relative amounts of lilac aldehyde. Nineteen out of 38 compounds were common to the scent samples of all populations.

Semiquantitative differences in the odor samples based on Bray-Curtis similarities are shown in Fig. 2. The variation among the samples was high with significant differences among the samples from different populations (within sexes; two-way *ANOSIM*: *R*: 0.454; $P < 0.001$). *SIMPER* analyses revealed the compounds responsible for differences among populations. Most populations and samples were strongly dominated by phenyl acetaldehyde, but in some samples high relative amounts of lilac aldehyde (e.g., samples of population 'g') or *(Z)*-3-hexen-1-ol and *(Z)*-3-hexenyl acetate (e.g., samples of population 'h') were present. One sample of population 'i' was characterized by a high percentage (33%) of linalool. There was no correlation between the scent and the distance matrix of the populations (*RELATE: Rho* = -0.02, *P* = 0.52) indicating that populations close to each other were not more similar in their scents than distant populations.

Within populations, we found significant differences in scent between male and female plants (two-way *ANOSIM*: *R*: 0.129; *P* = 0.038). However, the differences between males and females were less pronounced than the observed differences among populations. Within populations, both males and females emitted the same compounds, but the proportions of some compounds differed between males and females. According to *SIMPER* analysis, phenyl acetaldehyde and *(Z)*-3-hexenyl acetate were the main compounds responsible for differences between males and females (PAA: 38% in males, 31% in females; *(Z)*-3-hexenyl acetate: 8% in females, 6% in males).

Behavioral responses of mosquitoes to odors of Silene otites inflorescences In the wind tunnel bioassays about 50% of tested mosquitoes were attracted to the scents emitted from the inflorescences of *S. otites* of different populations. Male and female inflorescences were equally attractive to mosquitoes (‘b’: *Chi-square test*: $X^2_{df=1} = 0.25$, $P = 0.62$; ‘f’: $X^2_{df=1} = 0.03$, $P = 0.87$; ‘g’: $X^2_{df=1} = 1.26$, $P = 0.26$; ‘i’: $X^2_{df=1} = 1.74$, $P = 0.19$). Therefore, the responses to female and male inflorescences were pooled for further analyses. No differences in attractiveness among populations were found (*Kruskal–Wallis-ANOVA*: H (5, 25) = 4.3; $P = 0.5$). There was high variability in attraction within populations, which could not be explained by the different total amounts of scent emitted (Table 2, Fig. 3). As an example, most inflorescences of populations “i” emitted similar total amounts of floral scent, but their attractiveness differed strongly (34%-73%).

The latency time of mosquitoes did not differ among populations (*ANOVA*: F (5, 314) = 0.33; $P= 0.89$), and was on average 30 min. However, overall significant differences were found in the post-choice behavior (*Kruskal–Wallis-ANOVA*: H (5, 25) = 11.139; $P = 0.0487$). The ‘searching’ behavior was recorded most often when inflorescences of population ‘a’ were offered to the mosquitoes, and less often when they were offered inflorescences of population ‘c’. Nevertheless, there were no significant differences in post-hoc tests.

Electrophysiological recordings Electroantennographic responses of male and female *C. p. molestus* to several odor components of *S. otites* are shown in Fig. 4. All tested compounds elicited EAG responses, and the effect of dilution was evident for each compound. The EAG responses generally increased with increasing dose of the tested compounds. However, the mosquitoes responded differently to the compounds tested, and we also recorded differences in the responses of males and females to different compounds (Table 3). The strongest responses (110-151%) were elicited by linalool oxide (furanoid) and linalool. Furthermore, females responded strongly to *(Z)*-3-hexenyl acetate. Weak responses (< 80%) were obtained from both sexes to phenyl acetaldehyde, phenylethyl alcohol, acetophenone, and hexanol.

DISCUSSION

Most compounds found in this study have earlier been reported as part of the floral odor bouquet in other angiosperms (Knudsen et al., 2006), but only nine of the compounds identified in our study were also found in the *S. otites* samples analyzed by Jürgens et al. (2002). In total, we found 22 new compounds in the floral scent of *S. otites*, which have not been reported previously in that species. On the other hand, Jürgens et al. (2002) identified nine compounds that were not detectable in our samples. Furthermore, only small amounts of phenyl acetaldehyde were found in that study, but we have found that this was the dominant compound in nine populations. Some of these differences might be ascribed to different scent collection methods used by Jürgens et al (2002) and in our study, but probably such differences are also due to the sampling of plants of different geographical origin. Different populations of *S. otites* emit population-specific scent profiles, with only 19 out of 38 inflorescence volatiles being common to plants of the nine populations studied here.

Although intraspecific variation in floral scent has been observed for many angiosperms, comprehensive screening for population/geographic variation in floral scent composition has been investigated only in few species, e.g., *Yucca filamentosa* L (Agavaceae; Svensson et al., 2005), *Magnolia kobus* DC (Magnoliaceae; Azuma et al., 2001), *Geonoma macrostachys* Mart. (Arecaceae; Knudsen, 2002), *Silene latifolia* L. (Caryophyllaceae; Dötterl et al., 2005b), and *Ophrys* species (Orchidaceae; Mant et al., 2005). The intraspecific variability found in our dataset was comparable to the variability found in other taxa studied so far. Such variability in scent may be the result of genetic drift or the result of natural selection (Tollsten and Bergström, 1993). Further, different chemotypes may be adapted to different pollinators (Whitten and Williams 1992; Tollsten and Bergström, 1993).

So far, we do not know the evolutionary factors triggering the observed odor variability among *S. otites* population. Different pollinators associated with the different populations might exert different selective pressures on the odor. Only a few species of nocturnal Lepidoptera and mosquitoes have been recorded as pollinators in this species (Brantjes and Leemans, 1976), among them *Autographa gamma* L. and *Culex pipiens*. While *A. gamma* is known to be strongly attracted by lilac aldehyde (Plepys et al., 2002a, b), *C. pipiens* is known to respond strongly to phenyl acetaldehyde (Jhumur et al., 2006).

In this study, phenyl acetaldehyde was the most dominant and abundant odor compound, followed by lilac aldehyde, *(Z)*-3-hexenyl acetate, linalool oxide (pyranoid), *(Z)*-3-hexen-1-ol, benzaldehyde, phenylethyl alcohol, linalool, linalool oxide (furanoid), lilac alcohol, acetophenone, methyl salicylate, and hexanol. Most of these compounds are known to elicit strong antennal responses and/or to be attractive to moths such as *Hadena bicruris* Hufn. (Lepidoptera: Noctuidae, Dötterl et al., 2006), *Sphinx perelegans* Edwards (Lepidoptera: Sphingidae, Raguso and Light, 1998), *Hyles lineata* L. (Lepidoptera: Sphingidae, Raguso et al., 1996), *Argyresthia conjugella* Zeller (Lepidoptera: Argyresthiidae, Bengtsson et al., 2007), *Cydia pomenella* L. (Lepidoptera: Tortricidae, Bengtsson et al., 2007), and *Mamestra brassicae* L. (Lepidoptera: Noctuidae, Rojas, 1999), while so far, only phenyl acetaldehyde has been reported as being attractive to mosquitoes (Howse, 2003; Jhumur et al., 2006). Interestingly, 19 out of 35 identified compounds in *S. otites* were also found in other closely related *Silene* species, which have been described as moth-pollinated flowers

(Jürgens et al., 2002). Thus it is not surprising that besides mosquitoes also moths have been reported as pollinators of *S. otites* (Brantjes and Leemans, 1976).

Our study showed that in the absence of visual stimuli, mosquitoes were attracted to male and female inflorescences of *S. otites* by scent only. The attractiveness of both sexes of this dioecious plant was similarly strong in bioassays, even though female and male inflorescences differed with respect to relative amounts of scent compounds. We found no significant differences in intensity or latency time of response to the inflorescence scents of six different populations. Therefore, different compound mixtures seem to have the same attractiveness.

Even within *S. otites* populations that showed low qualitative and semiquantitative scent, no positive relation between the total amount of scent emitted and the number of mosquitoes attracted was found. This finding is in contrast to the results of Bowen (1992), who found that behavioral response increased with stimulus concentration. Microclimatic conditions in the wind tunnel, such as temperature (which ranged from 20 to 25°C), humidity, and atmospheric pressure, might have influenced the results obtained in the present study (Grimstad and DeFoliart, 1975). Further, the inflorescences might have emitted not only attractive compounds, but also compounds repellent to mosquitoes (Kessler and Baldwin, 2007). The effect of repellency could increase with increasing concentration of these repellent compounds. Jhumur et al. (2006) found that the most dominant odor compound of *S. otites*, phenyl acetaldehyde, attracted about 65% of *C. p. molestus* , whereas only about 50% of the mosquitoes were attracted to the entire *S. otites* inflorescence odor in the present study. This finding supports the hypothesis that *S. otites* does not emit only attractive, but also repellent compounds.

EAG studies were conducted to examine whether mosquitoes are able to detect components of the *S. otites* odor profile other than phenyl acetaldehyde. Mosquitoes responded to all compounds tested, and all these compounds may be involved in host-plant finding by mosquitoes. Bioassays are now needed to test these compounds in behavioral assays on *C. pipiens*. The compounds employed in the EAG studies were representative of the floral scent composition of *S. otites,* accounting for 97% on average of all samples of this species. Both male and female mosquitoes detected all 13 floral scent compounds tested (including *(Z)*-3-hexen-1-ol) till 10^{-5} dilutions. Therefore, if some of these compounds also proved to be attractive, then they might be used as reliable cues for finding *S. otites*, and be used as long-range attractants by *C. pipiens*.

From this and previous studies, mosquitoes have been proven so far to detect or be attracted to 15 floral volatiles (Mauer and Rowley, 1999; Howse, 2003; Kline et al., 2003; Jhumur et al., 2006). Interestingly, the ranking of the EAG responses did not correlate with the dominance of the volatiles in floral scent profiles. For example, phenyl acetaldehyde elicited only weak responses in EAGs although it is the main compound (35% mean percentage amount) in the scent of *S. otites*. Further, this compound was shown to be very attractive to mosquitoes (Jhumur et al., 2006). On the other hand, the mean percentage amounts of linalool and linalool oxide (furanoid) were only 3% and 2%, respectively, but elicited the strongest EAG responses. Several studies provide evidence that release of linalool oxide (furanoid) and linalool may reflect adaptations by plants to attract lepidopteron pollinators (Raguso et al., 1996; Raguso and Light, 1998; Andersson et al., 2002; Andersson and Dobson, 2003). Linalool also occurs in plants pollinated by bats, bees, flies, beetles, and wasps (Borg-Karlsson et al., 1996; Raguso and Pichersky, 1999). These monoterpenoids may also be important for attraction of mosquitoes, and could explain the mixed pollinator guild found in *S. otites,* mainly moths and mosquitoes. Indeed, the attractiveness of linalool for mosquitoes was confirmed by Kline et al. (2003). In a dual-port olfactometer, more *Aedes aegypti* (L.) individuals were attracted by linalool than by a control. Though these two oxygenated monoterpenes are generally assumed to be pollinator attractants, Ômura et al. (2000) reported that linalool oxide (furanoid) acted as a weak deterrent in proboscis extension responses and a weak repellent in flower alighting tests with the cabbage butterfly *Pieris rapae* L., indicating that this compound can be repellent as well as attractive to insects. Bioassays are needed to determine the behavioral response to linalool oxide in mosquitoes.

Similarly to phenyl acetaldehyde, phenylethyl alcohol (3% mean percentage) elicited only weak EAG responses, although this compound may also be attractive to mosquitoes. Mauer and Rowley (1999) found that *C. pipiens* was attracted to the scent of the common milkweed *Asclepias syriaca* L., which is dominated by phenylethyl alcohol and benzyl alcohol. The authors assumed that these two benzenoides were responsible for attraction of mosquitoes to *A. syriaca*, but failed to attract mosquitoes in a dual-port olfactometer to a synthetic mixture of these two compounds.

Interestingly, in our study, *C. pipiens* also responded to the typical green leaf odors of *S. otites*, such as *(Z)*-3-hexenyl acetate. These compounds are not only released from several plant species in response to herbivory, but also serve as attractants for a variety of predatory and parasitic insects (see Röse et al., 1998; James, 2005). In the natural environment, green leaf compounds are widespread and would not necessarily guide insects directly to flowers (Honda et al., 1998), though being directed to vegetation would certainly increase the probability of finding flowers.

In summary, floral scent compositions of *Silene otites* populations from different geographical origin are highly variable, but nevertheless similarly attractive to *Culex* *pipiens molestus* mosquitoes. Mosquitoes can detect the most common and abundant scent compounds of *S. otites* inflorescences, but we still lack knowledge about the biological significance (e.g., attractant or repellent) of most of the compounds. By means of bioassay, we are presently evaluating the role of these compounds in the plant-pollinator interactions of *S. otites* and mosquitoes, which might lead to the development of new means of pest control and mosquito attractants as well as repellents.

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Figure legends

Figure 1

Geographic origin of eight out of nine *Silene otites* populations analyzed (a-f, i; the geographic origin of population 'h' is unknown). The number of sampled male and female individuals of each population is given in brackets.

Figure 2

Multidimensional scaling (MDS) based on Bray-Curtis similarities of the odor composition of 63 inflorescences from nine populations of *Silene otites*. Most of the samples were dominated by high relative amounts of phenyl acetaldehyde, however, in some samples high relative amounts of other compounds, *such as* lilac aldehyde, were found.

Figure 3

Total amount of scent emission from *Silene otites* inflorescences and % mosquitoes attracted in the wind tunnel (N= 100 %= 170, 98, 260 for 'f', 'g' and 'i' population, respectively).

Figure 4

Electroantennographic responses (EAG) of male (rectangular) and female (triangular) *Culex pipiens pipiens molestus* to different dilutions (in paraffin) of common floral scent compounds of *Silene otites* of different populations. Twenty µl of each dilution of 12 scent compounds were tested on 4-6 mosquitoes. The antennal responses are given in relation to a standard stimulus (*Z*-3-Hexen-1-ol) Odor compounds have been sorted according to their mean percentage amounts in *S. otites*.

TABLE 1. RELATIVE AMOUNTS OF COMPOUNDS (MEAN ± SE) IN INFLORESCENCE ODORS OF 63 *SILENE OTITES* PLANTS FROM DIFFERENT POPULATIONS. THE NUMBER OF INDIVIDUALS SAMPLED IN EACH POPULATION (A I) IS GIVEN IN BRACKETS

Compounds	**a (7)**	**b (7)**	**c (9)**	**d (5)**	**e (7)**	**f (6)**	**g (8)**	**h (7)**	**i (7)**
Fatty acid derivatives									
Hexanol*	0.36 ± 0.16	0.14 ± 0.03	0.13 ± 0.05	0.15 ± 0.06	0.12 ± 0.04	0.19 ± 0.06	0.26 ± 0.22	0.04 ± 0.02	0.28 ± 0.08
(Z)-3-Hexen-1-ol*,[a]	3.64 ± 1.92	6.32 ± 1.75	2.33 ± 1.25	1.83 ± 0.47	3.39 ± 2.22	7.21 ± 2.09	3.2 ± 1.69	15.01 ± 6.01	8.74 ± 1.71
(E)-3-Hexen-1-ol	0.53 ± 0.16	0.14 ± 0.06	0.4 ± 0.19	0.41 ± 0.22	0.36 ± 0.35	0.19 ± 0.08	0.01 ± 0.01	0.23 ± 0.08	0.45 ± 0.14
(Z)-3-Hexenyl acetate*,[a]	4.27 ± 1.84	10.33 ± 2.73	1.85 ± 0.81	5.36 ± 1.89	8.12 ± 4.1	6.95 ± 2.65	5.31 ± 2.68	15.18 ± 5.39	7.26 ± 1.64
(E)-2-Hexenyl acetate*	0.11 ± 0.05	0.04 ± 0.04	0.08 ± 0.06	0.06 ± 0.04	0.73 ± 0.55	0.02 ± 0.02	-	-	0.03 ± 0.02
Hexyl acetate*	-	0.09 ± 0.08	0.03 ± 0.02	0.01 ± 0.01	2.61 ± 1.71	0.17 ± 0.1	-	-	0.14 ± 0.08
(Z)-3-Hexenyl butyrate[a]	-	0.23 ± 0.08	0.01 ± 0.01	0.08 ± 0.07	-	0.35 ± 0.17	0.01 ± 0.01	0.05 ± 0.03	0.33 ± 0.14
(E)-4,8 Dimethyl 1,3,7 nonatriene[a]	-	0.01 ± 0.01	0.15 ± 0.08	-	-	-	-	-	-
Benzenoids									
Benzaldehyde *,[a]	4.62 ± 0.67	7.26 ± 1.37	2.08 ± 0.37	7.1 ± 1.02	4.05 ± 1.18	8.26 ± 1.74	6.67 ± 1.26	2.59 ± 0.73	5.12 ± 1.08
Benzyl alcohol[a]	0.02 ± 0.02	0.17 ± 0.06	0.01 ± 0.01	0.04 ± 0.03	0.01 ± 0.01	0.84 ± 0.42	0.14 ± 0.06	0.48 ± 0.26	4.68 ± 1.43
Phenyl acetaldehyde[a]	47.71 ± 4.43	38.97 ± 4.78	35.94 ± 1.54	42.27 ± 7.64	40.89 ± 11.69	41.02 ± 4.42	31.66 ± 3.29	14.85 ± 4.64	26.05 ± 4.29
Acetophenone[a]	0.36 ± 0.22	0.31 ± 0.17	0.48 ± 0.32	0.58 ± 0.36	0.32 ± 0.29	1.38 ± 0.56	3.01 ± 2.01	0.07 ± 0.07	1.08 ± 0.5
Phenylethyl alcohol[a]	0.82 ± 0.25	5.18 ± 0.74	1.82 ± 0.47	2.02 ± 0.72	1.87 ± 0.83	5.31 ± 0.68	1.25 ± 0.38	1.44 ± 0.65	6.98 ± 0.96
Methyl salicylate[a]	0.07 ± 0.03	3.27 ± 1.16	0.16 ± 0.06	0.03 ± 0.03	-	0.41 ± 0.17	0.03 ± 0.03	2.36 ± 1.32	0.07 ± 0.04
N- bearing compounds									
3-Methyl-butyl-aldoxime(syn/anti) [a]	-	-	0.01 ± 0.01	-	-	-	0.01 ± 0	0.01 ± 0.01	-
Monoterpenoids									
α-Pinene[a]	-	0.02 ± 0.02	-	0.15 ± 0.09	0.08 ± 0.04	0.13 ± 0.06	0.12 ± 0.05	0.35 ± 0.12	0.14 ± 0.1
β-Pinene[a]	0.05 ± 0.04	0.01 ± 0.01	-	0.04 ± 0.02	0.05 ± 0.03	0.12 ± 0.05	0.01 ± 0.01	0.23 ± 0.13	0.25 ± 0.08
D-Limonene[a]	-	-	-	-	0.04 ± 0.02	-	-	-	-
(E)-ß-Ocimene[a]	-	-	tr	-	-	-	0.06 ± 0.06	0.31 ± 0.2	0.54 ± 0.54

Compounds	**a (7)**	**b (7)**	**c (9)**	**d (5)**	**e (7)**	**f (6)**	**g (8)**	**h (7)**	**i (7)**
(Z)-Linalool oxide furanoid[a]	0.28 ± 0.15	tr	0.87 ± 0.21	-	0.78 ± 0.52	0.02 ± 0.02	0.01 ± 0.01	-	0.16 ± 0.04
(E)-Linalool oxide furanoid[a]	-	0.6 ± 0.11	0.01 ± 0.01	4.45 ± 2.35	0.12 ± 0.07	1.46 ± 0.39	0.03 ± 0.03	7.17 ± 1.16	3.04 ± 0.39
Linalool[a]	-	3.13 ± 1.09	0.84 ± 0.22	0.49 ± 0.19	0.06 ± 0.04	4.26 ± 1.63	0.52 ± 0.12	5.64 ± 1.72	8.11 ± 4.24
Hotrienol	-	2.16 ± 0.48	0.25 ± 0.2	1.52 ± 0.88	0.1 ± 0.04	1.63 ± 0.5	0.66 ± 0.16	0.6 ± 0.39	2.57 ± 0.49
2,2,6-Trimethyl-2-vinyl-5-ketotetrahydropyran	0.4 ± 0.13	0.05 ± 0.01	0.71 ± 0.16	0.53 ± 0.21	0.42 ± 0.16	0.16 ± 0.05	0.06 ± 0.04	0.73 ± 0.33	0.73 ± 0.11
Lilac aldehyde A[a]	12.37 ± 2.01	6.94 ± 0.86	14.42 ± 1.43	10.46 ± 1.43	8.14 ± 1.91	6.27 ± 0.65	20.16 ± 2.23	12.03 ± 2.45	5.96 ± 1.58
Lilac aldehyde B + C[a]	13.72 ± 1.9	8.32 ± 1.13	16.53 ± 1.11	11.31 ± 1.36	10.43 ± 2.35	7.48 ± 0.59	19.42 ± 1.26	10.19 ± 1.78	6.47 ± 1.52
Lilac aldehyde D[a]	2.9 ± 0.6	2.29 ± 0.52	4.22 ± 0.47	3.02 ± 0.41	2.73 ± 0.5	1.94 ± 0.29	4.67 ± 0.61	2.29 ± 0.49	1.12 ± 0.31
(Z)-Linalool oxide pyranoid[a]	5.99 ± 2.97	0.33 ± 0.13	10.11 ± 4.99	0.23 ± 0.22	8.71 ± 4.95	0.76 ± 0.23	1.47 ± 0.95	0.43 ± 0.17	0.84 ± 0.19
(E)-Linalool oxide pyranoid[a]	0.66 ± 0.3	0.59 ± 0.21	4.36 ± 1.21	6.74 ± 2.08	3.83 ± 1.58	2.45 ± 0.66	0.1 ± 0.05	5.28 ± 2.3	5.76 ± 1.02
Lilac alcohol A[a]	0.32 ± 0.07	0.39 ± 0.18	0.96 ± 0.19	0.4 ± 0.12	0.71 ± 0.49	0.19 ± 0.07	0.37 ± 0.11	1.3 ± 0.41	0.54 ± 0.12
Lilac alcohol B+C[a]	0.36 ± 0.08	0.49 ± 0.13	1.03 ± 0.18	0.51 ± 0.15	0.94 ± 0.68	0.36 ± 0.1	0.59 ± 0.15	0.81 ± 0.27	0.7 ± 0.16
Lilac alcohol D[a]	0.03 ± 0.02	0.13 ± 0.04	0.2 ± 0.04	0.04 ± 0.02	0.22 ± 0.17	0.05 ± 0.03	0.11 ± 0.03	0.05 ± 0.03	0.07 ± 0.02
1-Hydroxy linalool	-	1.55 ± 0.49	-	0.04 ± 0.02	-	0.29 ± 0.28	0.05 ± 0.05	-	1.79 ± 0.88
Monoterpenoid 43, 67, 79, 91, 105, 121	-	0.01 ± 0.01	-	0.08 ± 0.06	0.09 ± 0.06	0.05 ± 0.03	0.01 ± 0.01	0.2 ± 0.06	0.01 ± 0.01
Monoterpene oxide 39, 65, 79,91,105, 121, 135, 150	-	0.01 ± 0.01	-	0.03 ± 0.03	0.02 ± 0.02	0.06 ± 0.06	-	-	0.01 ± 0.01
Sesquiterpenoids									
(E)-ß-Caryophyllene[a]	-	0.1 ± 0.1	-	0.02 ± 0.01	0.04 ± 0.02	0.03 ± 0.02	-	0.06 ± 0.06	-
Geranyl Isovalerate	0.38 ± 0.25	0.38 ± 0.38	0.02 ± 0.02	-	-	-	-	-	-
Unknown									
43, 67, 93, 109, 123, 151	0.04 ± 0.02	0.04 ± 0.02	-	-	-	-	-	-	-

*: *compounds were also found in samples collected from leaves*

[a]: *compounds were identified by comparing mass spectra and retention times with authentic standards*

TABLE 2. ATTRACTION, POST-CHOICE BEHAVIOR, AND LATENCY TIME OF MOSQUITOES WITH RESPECT TO THE EMITTED SCENT FROM *Silene otites* INFLORESCENCES (3-5) OF DIFFERENT POPULATIONS (a-i).

Population (numbers of female (f) and male (m) inflorescence samples tested in bioassays)	Number of mosquitoes tested	Odor emission of inflorescence samples (ng/2 min) Median (Min-Max)	Number of landed (attracted) mosquitoes (%) Median (Min-Max)[1]	Number of landed mosquitoes showing searching behavior (%) Median (Min-Max)[2]	Latency time of mosquitoes until landing (min) Median (Min-Max)[3]
a (2 m)	30	357 (337-378)	49 (36-63)	75 (50-100)	25 (5-53)
b (1 m, 1 f)	46	240 (223-378)	50 (44-55)	55 (50-60)	20 (1-59)
c (2 m)	40	219.3 (217-221)	60 (47-64)	18 (18-18)	31 (1-59)
f (2 m, 4 f)	170	146 (9-370)	51 (35-72)	40 (12-80)	25 (1-60)
g (1 m, 3 f)	98	463 (329-1387)	38 (30-52)	32 (0-33)	23 (3-59)
i (5 m, 4 f)	260	115 (82-234)	53 (34-73)	20 (0-35)	25 (1-59)

[1] *Kruskal–Wallis-ANOVA: H (5, 25) = 4.3; P = 0.5*

[2] *Kruskal–Wallis-ANOVA: H (5, 25) = 11.13; P = 0.05*

[3] *ANOVA: F (5; 314) = 0.33; P = 0.89*

TABLE 3. MULTIPLE COMPARISONS BASED ON A GENERAL LINEAR MODEL (GLM) OF ANTENNAL RESPONSES OF MALE AND FEMALE MOSQUITOES TO DIFFERENT COMPOUNDS AND DILUTIONS

Effect	*Df*	MS	*F*	*P*
Intercept	1	1181681	11128.31	< 0.001
Sex	1	569	5.36	0.02
Dilution	4	92933	875.18	< 0.001
Compound	12	2603	24.51	< 0.001
Sex*Dilution	4	87	0.82	0.511
Sex*Compound	12	841	7.92	< 0.001
Dilution*Compound	48	960	9.05	< 0.001
Sex*Dilution*Compound	48	135	1.27	0.116
Error	455	106		

Fig. 1

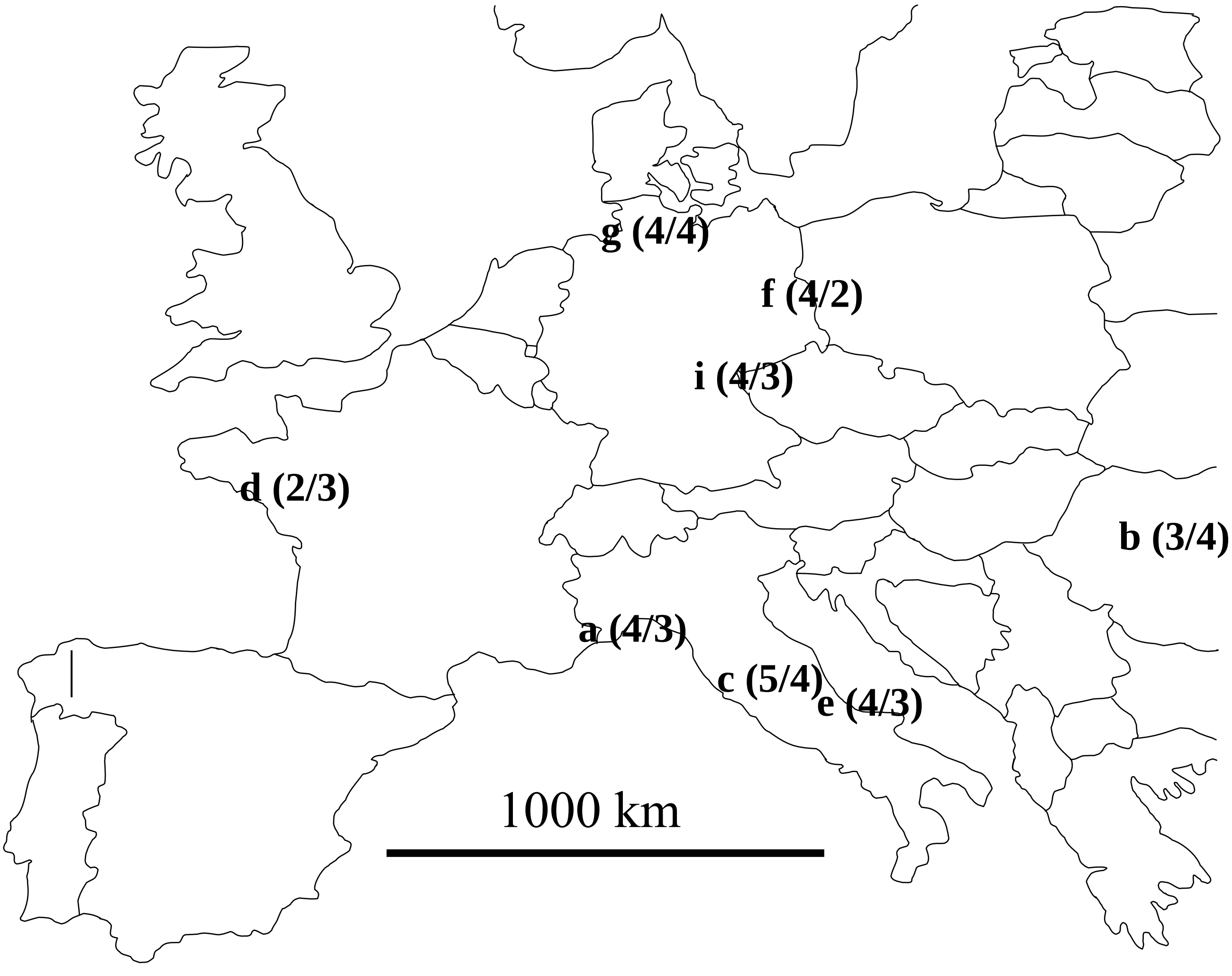


Fig. 2

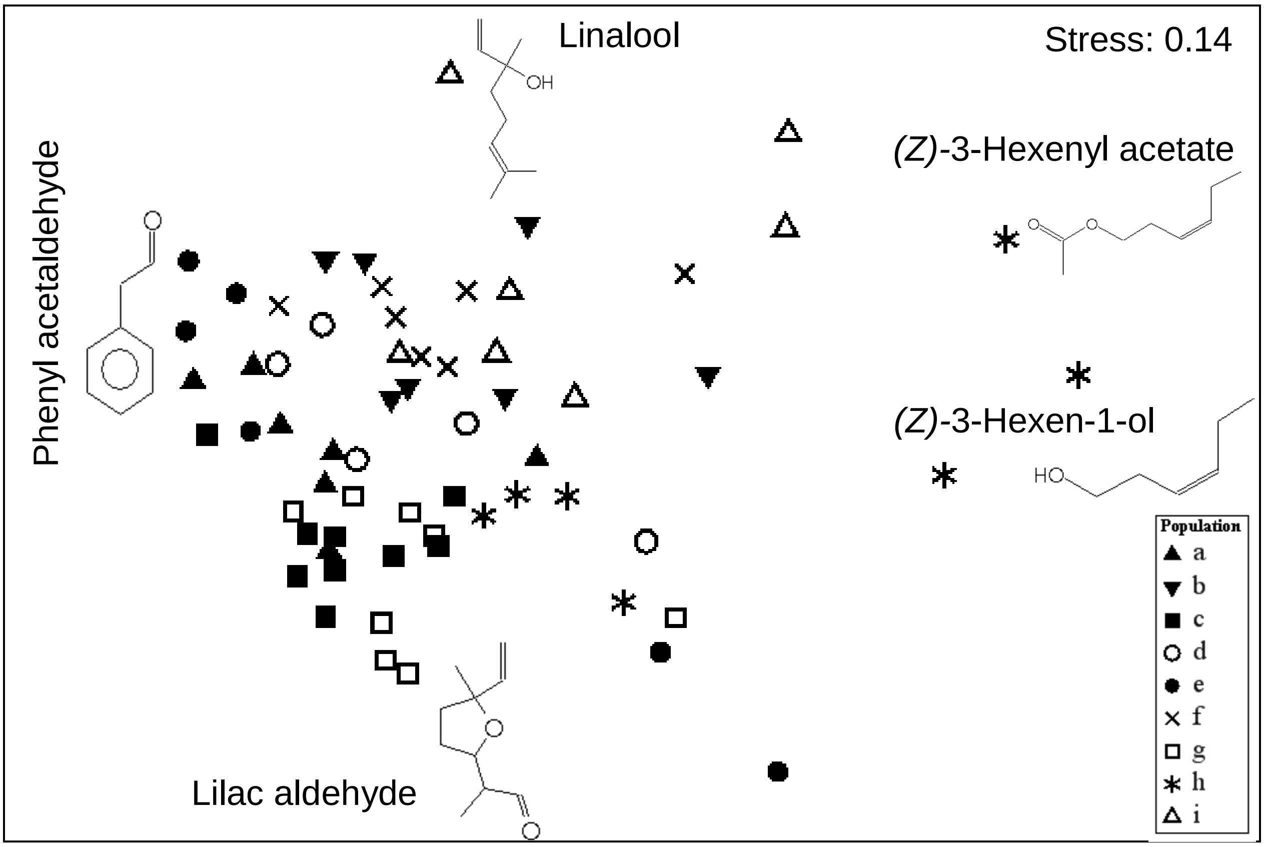

Linalool
OH
Stress: 0.14
(Z)-3-Hexenyl acetate
(Z)-3-Hexen-1-ol
HO
Phenyl acetaldehyde
Lilac aldehyde
Population
▲ a
▼ b
■ c
○ d
● e
× f
□ g
∗ h
△ i

Fig. 3.

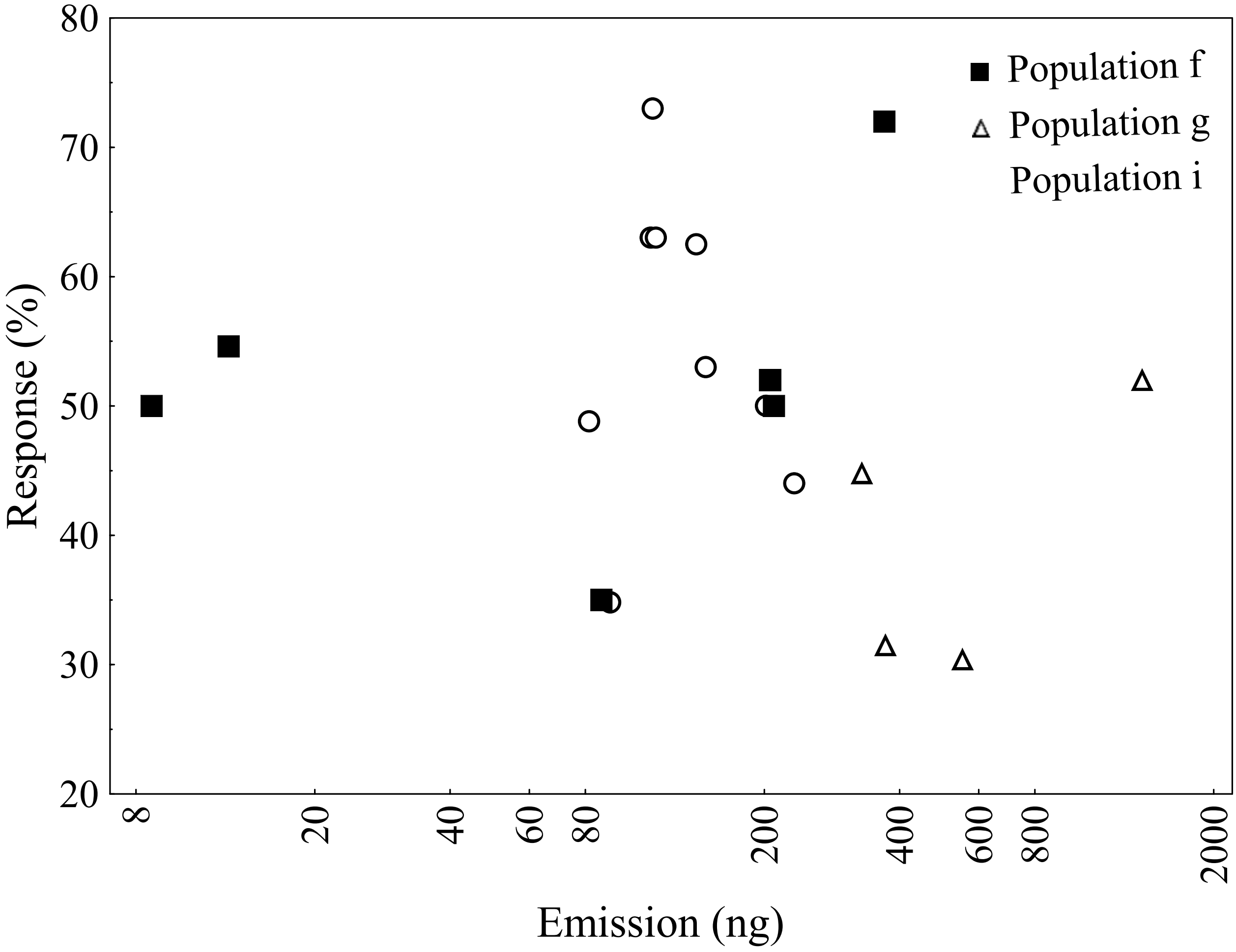
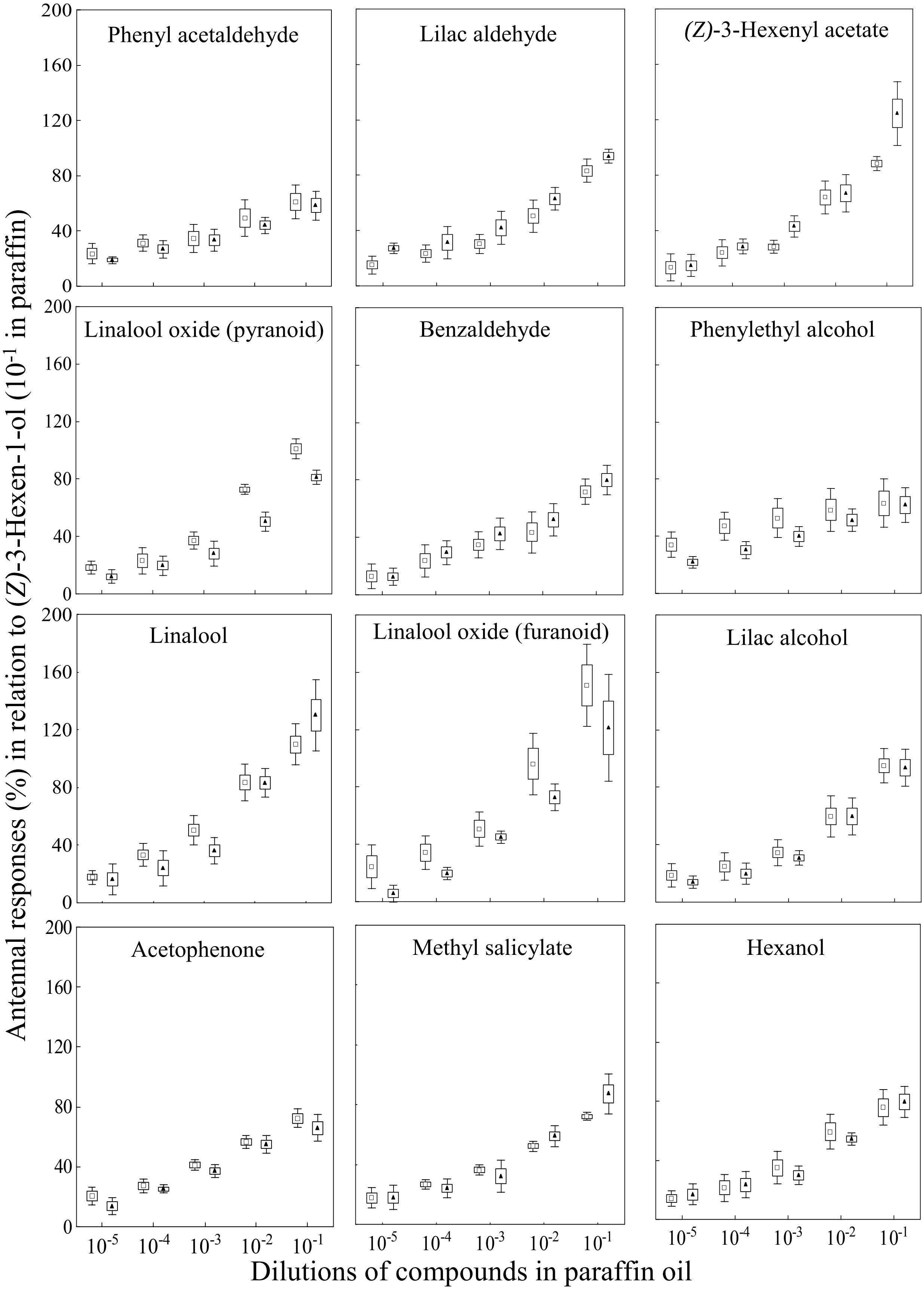

Response (%)
80
70
60
50
40
30
20
Emission (ng)
8
20
40
60
80
200
400
600
800
2000
Population f
Population g
Population i

Fig. 4.


Phenyl acetaldehyde
Lilac aldehyde
(Z)-3-Hexenyl acetate
Linalool oxide (pyranoid)
Benzaldehyde
Phenylethyl alcohol
Linalool
Linalool oxide (furanoid)
Lilac alcohol
Acetophenone
Methyl salicylate
Hexanol
0
40
80
120
160
200
10-5
10-4
10-3
10-2
10-1
Antennal responses (%) in relation to (Z)-3-Hexen-1-ol (10-1 in paraffin)
Dilutions of compounds in paraffin oil

Running title: *Silene otites* volatiles as attractants for mosquitoes

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Type of article: Original research paper

Electrophysiological and behavioural responses of mosquitoes to volatiles of *Silene otites* (Caryophyllaceae)

(Accepted, minor revision: Arthropod-Plant Interactions)

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Abstract

To flourish the understanding about the biological significance of flower odour for attraction of mosquitoes, electrophysiological responses to headspace flower odour samples of *Silene otites* (L.) Wibel were investigated on *Culex pipiens pipiens* biotype *molestus* Forskal 1775 and *Aedes aegypti* L. using coupled gas chromatographic–electroantennographic detection (GC-EAD). No remarkable differences in antennal responses to the odour compounds have been found between these two mosquito species. Further, the behavioural attractiveness of the electrophysiologically active compounds, singly or as multiple odour mixtures, was evaluated with bioassay experiments with *C. pipiens molestus*. In bioassays, *C. pipiens* responded to 14 electrophysiologically active compounds in different magnitudes (65-20%) and acetophenone, linalool oxide (pyranoid), phenyl acetaldehyde and phenylethyl alcohol were found as more attractive in comparison to the least attractive compound, hexanol. In two-stimulus choice test, mosquitoes were significantly more attracted to the mixture of the four most attractive compounds compared to the mixture of all 14 compounds. The results of present study render the general notion of floral odour as attractive cues for mosquitoes.

Key words *Aedes aegypti*, attraction, behavioural response, bioassay, *Culex pipiens molestus*, GC-EAD, electrophysiological response, flower odour, *Silene otites*.

Introduction

Mosquitoes of three genera, *Culex, Anopheles* and *Aedes,* have been proven feeding on plant tissues as a source of regular diet (Müller and Schlein, 2005). They especially visit flowers of diverse plants for nectaring (Grimstad and DeFoliart, 1974), and some are even known to feed plant juices from leaves (Abdel-Malek and Baldwin, 1961). However, little is known about the role of flower visiting mosquitoes in pollination in most plants species known to be visited by mosquitoes. Only in the orchid *Habenaria* (*Platanthera*) *obtusata* (Banks ex Pursh) Richardson (Stoutamire, 1968), and in the Caryophyllaceae *Silene otites* (L.) Wibel (Brantjes and Leemans, 1976), mosquitoes have been proven to be pollen agents. However, *Platanthera* sp. and *Silene* spp. are also known to be pollinated by Lepidoptera (Nilsson, 1978; Brantjes & Leemans, 1976).

For finding the nectar host-plants mosquitoes (Bowen, 1991; Foster and Hancock, 1994; Jepson and Healy, 1988; Mauer and Rowley, 1999) as well as other flower visiting insects (Dobson, 2006; Kelber and Pfaff, 1997; Weiss, 2001) are known to rely on floral scents and/or on visual advertisements of flowers. Especially for night-active visitors, flower scents are essential for host-plant finding as visual floral cues become often inefficient due to darkness (e.g. Balkenius et al., 2006; Dötterl et al., 2006). In mosquitoes both night-active (Jhumur et al., 2006; Mauer and Rowley, 1999) as well as day-active (Jepson and Healy, 1988) species are known to be attracted by floral scents. Nevertheless, day active mosquitoes, such as *Aedes aegypti* (Taylor, 1969) might rely on both vision and olfaction, whereas, crepuscular and night active mosquitoes, such as *C. pipiens molestus* (Kawada et al., 2006) are more dependent on olfaction.

Culex pipiens molestus has been shown to visit *Silene otites*, which emits strong floral scent at night (Jürgens et al., 2002; Jhumur, unpublished data). This mosquito is known to be not only a flower visitor but also a pollinator of this Caryophyllaceae

(Brantjes and Leemans, 1976). Recent studies demonstrated that both sexes of *C. pipiens molestus* were attracted by the floral scent of *S. otites* plants of different geographic origin. Mosquitoes were strongly attracted by phenyl acetaldehyde, a dominant scent compound of *S. otites*, and they have olfactory receptors for the most common inflorescence scent compounds of *S. otites* (Jhumur et al., 2006; Jhumur et al., 2007). It is unclear, whether, besides phenyl acetaldehyde, also other compounds are behavioural active and responsible for attraction of *C. pipiens* to the flowers of *S. otites*. Not only main and most common compounds tested in electroantennographic studies (EAG) by Jhumur et al. (2007), but also minor compounds may be important attractants. Therefore, bioassays with single floral scent compounds found in *S. otites* are needed to fully understand the chemical basis for attraction of mosquitoes to *S. otites*. Since it is time-consuming to test all the 35 compounds identified in the inflorescence scent samples of *S. otites* (Jhumur et al., 2007), electrophysiological data may provide important information, limiting the number of compounds to be tested in field or lab experiments. A useful tool for this purpose is gas chromatography coupled to electroantennography (GC-EAD, Arn et al., 1975). Because behavioural active compounds generally elicit signals in such kind of analyses (Schiestl and Marion-Poll, 2001), once active volatiles have been identified, their function in modifying insect behaviour (e.g., as attractants or deterrents) can be explored using behavioural tests (e.g. Dötterl et al., 2006).

In the current study, the inflorescence odour samples of *S. otites* were measured on both male and female *C. pipiens molestus* antenna in GC-EAD analyses. Further, compounds eliciting signals in mosquito antennae in present study or described as antennal stimuli in the previous study (Jhumur et al., 2007) were used, as mixtures or singly, to evaluate the behavioural attractiveness of mosquitoes in wind tunnel bioassays. Therefore, in comparison to the study of Jhumur et al. (2007), where of the most common compounds were tested on the antennae of *C. pipiens molestus* in a EAG study, and where the attractivity of whole inflorescences was determined, here, headspace samples of *S. otites* were tested in a GC-EAD study, and the attractivity of single compounds as well as compound mixtures was determined. Additionally, the odour samples of *S. otites* were tested on the antennae of the day active *Aedes aegypti* L. to compare the antennal sensitivity of a night-active mosquito with that of a day-active mosquito. Night-active visitors have to use floral scent as cues for nectar plant finding because visual cues are insufficient, while day-active visitors not solely need to rely on floral scents for host-plant finding; additionally they may use visual advertisements. Therefore, it is hypothesised that night-active *C. pipiens molestus* more strongly responds to floral scents than day-active *A. aegypti*.

Materials and methods

Plant samples and volatile collection

Plants of *Silene otites* were grown from seeds of six different populations in the flower beds of the Department of Plant Systematics, University of Bayreuth. Floral scent was collected from eight males and three female specimens of *S. otites*. For collecting odours, potted plants were put under the extractor hood in the laboratory and the inflorescences were enclosed within a polyester oven bag (20 × 8 cm; Toppits). For each sample, floral scent was collected at night from 1–4 inflorescences for about 10 h using a dynamic headspace method. The emitted volatiles were trapped in an adsorbent tube using a membrane pump (ASF Thomas, Inc.) with a flow rate of 50 ml/min. The adsorbent tubes were filled with a mixture (1:1) of 20 mg Tenax-TA (mesh 60–80) and Carbotrap (mesh 20–40). Volatiles were eluted with 50–70 µl acetone (SupraSolv, Merck KgaA, Germany) to get the samples for GC-EAD analyses. Ten samples were tested on the antenna of *C. pipiens* and two on the antennae of *A. aegypti*.

Insects

Culex pipiens pipiens biotype *molestus* Forskal 1775 and *Aedes aegypti* L. were reared according to Jhumur et al. (2006) and Geier et al. (1999), respectively. For experiments we used flower inexperienced individuals. Adults had access to sugar solution (5%) on filter paper. For electrophysiological measurements regularly fed mosquitoes were used, while for bioassays the sugar supply was removed 61–63 h before the scheduled experiment.

Gas Chromatography coupled to Electroantennography (GC-EAD)

The GC-EAD system consisted of a gas chromatograph (Vega 6000 Series 2, Carlo Erba, Rodano, Italy) equipped with a flame ionization detector (FID) and an EAD setup provided by Syntech (Hilversum, Netherlands). One µl of an odour sample was injected splitless at 60° C. The split vent was opened after 1 min and then the oven was heated at a rate of 10° C/min to 200° C. The end temperature was held for 5 min. A ZB-5 column was used for the analyses (length 30 m, inner diam 0.32 mm, film thickness 0.25 µm, Phenomenex). The column was split at the end by the four-arm flow splitter GRAPHPACK 3D/2 (Gerstel, Mülheim, Germany) into two pieces of deactivated capillary (length 50 cm, ID 0.32 mm) leading to the FID and to the EAD setup. Helium (He) was used as makeup gas that has been introduced through the fourth arm of the splitter at a rate of 16 ml/min.

Four to five days old *C. pipiens molestus* and *A. aegypti* were used for measurements. The head of a mosquito was excised from the thorax and the postoccipital region was subsequently placed in a glass capillary electrode containing insect ringer (8.0 g/l NaCL, 0.4 g/l KCL, 0.4 g/l $CaCL_2$). The tip of one antenna was cut off and placed in another glass capillary electrode containing also insect ringer. The electrodes were connected to silver wires. Only compounds eliciting signals in at least two runs were treated as electrophysiologically active. Antennae of 9 males and 24 females, and 20 males and 6 females of *C. pipiens* and *A. aegypti*, respectively were measured on the inflorescence samples while 10 and 8, and 7 and 19, respectively were measured on the authentic standards.

Authentic standard compounds and bioassays

We evaluated the behavioural attraction of *C. pipiens molestus* to 14 floral scent compounds of *S. otites*, from which distinct antennal responses had been recorded in this study and/or in a previous study (Jhumur et al., 2007). The compounds (isomers were pooled), provided by Karlheinz Seifert (KS) or purchased from Sigma-Aldrich (SA) or

Wako (W), were tested as mixtures in different concentrations (10^{-1}, 10^{-2}, 10^{-3}) or singly (10^{-1}). According to their mean ratios in scent profiles of *S. otites,* mixtures of phenyl acetaldehyde (SA, 90%), lilac aldehyde (KS, >99%), (Z)-3-hexenyl acetate (SA, 98%), linalool oxide (pyranoid) (W, 98%), (Z)-3-hexen-1-ol (SA, 98%), benzaldehyde (SA, 99%), phenylethyl alcohol (SA, 99%), linalool (KS, >99%), linalool oxide (furanoid) (SA, 97%), lilac alcohol (KS, >99%), acetophenone (SA, 98%), methyl salicylate (SA, 98%), benzyl alcohol (SA, 99%), hexanol (SA, 98%) = 35.7 : 26.5: 7.1 : 7.1 : 6.1 : 5.1 : 3.1 : 3.1 : 2 : 1 : 1 : 1 : 1 : 0.2, were prepared in paraffin oil (Uvasol, MERCK, Damstadt, Germany). The single compounds and the mixtures were tested in a two-choice test against paraffin. Additionally, a mixture of the four most attractive compounds, corresponding to the ratios in natural samples (phenyl acetaldehyde, linalool oxide (pyranoid), phenylethyl alcohol, acetophenone = 76 : 15 : 7 : 2; 10^{-1}), was prepared. This mixture was used in two-stimulus choice assays against the mixture of 14 compounds, and also against phenyl acetaldehyde. For bioassays, the same wind tunnel and procedure as described by Jhumur et al. (2006) was used. In all bioassays, 10 µl compound (single or mixed) or paraffin oil (treated as blank) was applied to the rubber GC-septum and tested for their attractiveness to female mosquitoes.

Statistical analyses

We used the STATISTICA program package (StatSoft, Inc., 2004) for statistical analyses. For each group, consisting of five simultaneously tested mosquitoes, the relative proportion of responding mosquitoes was determined. The proportions, derived from the replicates, were used for further analyses. One-way ANOVA was used to compare the responses of naïve mosquitoes to the mixed odours of different dilutions. ANOVA was also used to compare the attraction of naïve mosquitoes to the 14 odour compounds.

Unequal N HSD was used as post-hoc test in case of significant F-test. Normality was tested using the Kolmogorov-Smirnov test, homogeneity of variances was tested using the Hartley test. The Chi-square observed vs. expected test was used to assess the differences in attractiveness in two-stimulus choice tests. For all statistical analyses, an alpha-level of 0.05 was assessed.

Results

Antennal responses of mosquitoes to volatile samples of *Silene otites*

Males and females of both *C. pipiens molestus* and *A. aegypti* responded to 12 compounds of the *S. otites* volatile samples (Table-1). *C. pipiens* responded further to acetophenone and linalool. *Silene otites* samples containing these two substances were not tested on *A. aegypti,* however, also this species responded to authentic standards of these compounds. In summary, both species responded in these GC-EAD analyses to several benzenoids and oxygenated monoterpenoids (linalool and derivatives thereof).

Attraction of *Culex pipiens molestus* to the mixed scent compounds of *Silene otites*

When testing the mixture of 14 compounds found to be electrophysiologically active in *S. otites*, 55%, 57%, and 36% mosquitoes were attracted to 10^{-1}, 10^{-2}, and 10^{-3} dilution, respectively (Fig. 1). Though less than 40% mosquitoes were attracted by the mixture in 10^{-3} dilution, there were no overall significant differences in attractiveness among the different dilutions (ANOVA: F=2.9; df=2, 37; P=0.07).

Attraction of *Culex pipiens molestus* to the single odour compounds of *Silene otites*

Mosquitoes were attracted to all tested 14 compounds (Fig. 2), however, some compounds were more attractive than others (ANOVA: F=2.3; df=13,157; P<0.001). While about 65% mosquitoes were attracted by acetophenone, only 20% mosquitoes were found to be attracted by hexanol. In a post-hoc test, the four compounds (acetophenone, linalool oxide (pyranoid), phenyl acetaldehyde, phenylethyl alcohol) attracting more than 56% mosquitoes each, were found to be significantly more attractive than hexanol. The other compounds were found to attract 33% (linalool) up to

55% (lilac aldehyde) mosquitoes. However, no further significant differences were found in the multiple comparisons.

Mosquitoes attraction in two-stimulus choice tests

When testing the mixture of the 14 compounds against the mixture including only the four most attractive compounds in a two-stimulus choice assay (Fig. 3), significantly more mosquitoes were attracted by the mixture containing only four compounds (Chi-square obs vs. exp.: $X^2_{df=1}=4.50$, $P=0.03$). No differences were found (Fig. 3) when testing the four most attractive compounds against phenyl acetaldehyde (Chi-square obs. vs. exp.: $X^2_{df=1}=1.29$, $P< 0.26$).

Discussion

This study has demonstrated that night-active *Culex pipiens molestus* and day-active *Aedes aegypti* were similarly sensitive to the odour compounds of night-flowering *S. otites* and that all electrophysiologically active compounds were attractive to *C. pipiens molestus*, though some compounds were more attractive than others. Although it was not possible to evaluate behavioural attractiveness of electrophysiologically active compounds to *A. aegypti* in the present experiment, we hypothesize that also this mosquito is attracted by the volatiles of *S. otites*. Indeed, when offering naïve *A. aegypti* inflorescences of *S. otites* they immediately fly towards them, land on them, and suck nectar (K. Jahreiß, personal communication). Further, ca. 40% of tested males and females of *A. aegypti* were attracted to *S. otites* by floral scent when visual signals were hidden (K. Jahreiß, personal communication). It seems that day- as well as night-active mosquitoes rely on floral scents for finding nectar host-plants. This result is contrary to a recent study testing scent on a day- and a night-active hawkmoth (Balkenius et al., 2006). The authors found the night-active moth more strongly responding to floral scent whereas the day-active moth more strongly responded to visual signals.

Apart from the investigation of Jhumur et al. (2007), the biological significance of mosquitoes with regard to floral volatiles is known only for four compounds, i.e. phenyl acetaldehyde (behavioural active: Howse, 2003; Jhumur et al., 2006), phenylethyl alcohol and benzyl alcohol (electrophysiological active: Mauer and Rowley, 1999) and linalool (behavioural active: Kline et al., 2003). These compounds as well as several other compounds including benzyl alcohol, the additional compound found to be electrophysiologically active in this study, were also tested in our study and found to be more or less attractive to *C. pipiens*. All of these compounds are known to elicit antennal responses and/or behavioural responses in other insects, e.g. Coleoptera, Diptera, Hemiptera, Homoptera, Hymenoptera, Lepidoptera, Neuroptera and Orthoptera (see Table-2). Interestingly, some of these compounds were found to repel other insects, i.e. phenyl acetaldehyde the ants *Solenopsis geminate* Fabricus and *Pheidole morrisi* Forel, linalool oxide (pyranoid and furanoid) the butterfly *Pieris rapae crucivora* Boisd, methyl salicylate the male butterfly *Pieris napi* and the aphids *Rhopalosiphum padi* and *Aphis fabae* Scop., acetophenone the beetles *Dendroctonus pseudotsugae* and *Dendroctonus brevicomis* LeConte, and linalool *Lygaeus kalmii* Stål and *Tetraopes tetrophthalmus* Forster (Table-2).

Differences in attraction of mosquitoes, found among the three dilutions of the mixture of 14 floral scent compounds, were not significant. The odour emission from the septum impregnated with the 10^{-1} dilution is comparable to the scent emission from 1-2 inflorescences of a *S. otites* plant at night, whereas, odour emission from 10^{-3} dilution is comparable to the scent emission of only a few flowers of *S. otites* (Jhumur, unpublished data). In conclusion, even single flowers of *S. otites* are capable of attracting mosquitoes though mosquitoes might be more effectively attracted by a plant having dozens of flowers and being fully in bloom.

The mixture of 14 compounds represent the floral scent composition of *S. otites* quite well as they account for about 98% of floral scent emitted. It is not surprising that the attractiveness of mosquitoes to *S. otites* inflorescences found by Jhumur et al. (2007) (50% mosquitoes attracted) is comparable to the attractiveness to a mixture of 14 compounds in 10^{-1} dilution, conducted here. The small difference in attractivity between these two experiments could be explained by the microclimatic conditions in the wind tunnel, such as temperature (ranged from 23-25°C), humidity and atmospheric pressure, which we were not able to control during experiments (see also Grimstad and DeFoliart, 1975).

Interestingly, in our study the mixture of 14 electrophysiologically active compounds was not more attractive than some single components and mosquitoes did not prefer a mixture of the four most attractive compounds (including phenyl acetaldehyde) over phenyl acetaldehyde indicating that there was no synergistic effect. Contrary, mosquitoes preferred a mixture consisting of the four most attractive compounds over a mixture of all 14 GC-EAD active compounds. The mixture of 14 compounds also contained these four compounds but in lower concentration due to the addition of the 10 other compounds. Nevertheless the compounds used in the four compound mixture were also dominating the composition of the 14 compound mixture. On the other hand, small changes in concentration were not found to strongly influencing the behaviour of mosquitoes (Fig. 1). Therefore, some of the compounds being present in the complete mixture but not in the reduced mixture may act as repellent in combination, though being attractive when testing them singly. Some compounds in the complete mixture are known as typical green leaf volatiles, e.g. hexanol and Z-3-hexenyl acetate. It is reasonable that mosquitoes in search for food are attracted by “vegetative” scents when there are no typical flower signals, but prefer and rely on typical flower scents over a mixture of flower and vegetative scents for effectively finding flowers.

The behavioural attractiveness of mosquitoes to few single odour compounds was not found to be congruent with the electroantennographic study of Jhumur et al. (2007). Three compounds, namely acetophenone, phenyl acetaldehyde and phenylethyl alcohol were found to elicit only weak antennal responses in that investigation, whereas, in the behavioural assays of present investigation mosquitoes were strongly attracted by these compounds. In case of linalool the opposite was true. Linalool oxide furanosides elicited strong EAG response in *Pieris rapae crucivora* Boisd., but acted as weak deterrent in PER (Proboscis Extension Reflex) and weak repellents in flower visiting tests (Ômura et al., 2000). One compound, dipropyl disulphide elicited weak EAG response in the female of *Acrolepiopsis assectella* Zell. but was attractive to this moth (Lecomte et al., 1998). All these data indicate that the antennal sensitivity is not always directly correlated with attractiveness.

With respect to the floral scent profile of *S. otites* not only the abundant compounds (phenyl acetaldehyde, 35%) attracted high numbers of mosquitoes (%) but also compounds occurring only in small relative amounts in (acetophenone, 1%) attracted similar number of mosquitoes (%). Altogether, there is no correlation between the attractiveness to mosquitoes and the relative amount of compounds in *S. otites* (P=0.16, R=0.39). Mosquitoes are very generalistic in respect to flower odour detection and attraction and are capable to use a wide array of odorant compounds or a complex mixture of compounds for finding flowers, although they are more attracted to some compounds than to others.

Our results show that most of the odour compounds of *S. otites* are electrophysiologically active to both *C. pipiens molestus* and *A. aegypti*. The behavioural attractive compounds, mixtures as well as single, might guide mosquitoes to find this nectar host plant. These compounds could be used to bait traps.

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Tables

Table 1

Gas chromatographic and electroantennographic detection (GC-EAD) of scent compounds of *Silene otites* by males (m) and females (f) of *Culex pipiens molestus* and *Aedes aegypti*. + = antennal response to the compound in volatile samples of *Silene otites*, +a = antennal response to authentic standards only, - = no antennal response, +J= compounds found to be electrophysiologically active in Jhumur et al., 2007. Volatile compounds were sorted according to their elution on a ZB-5 column.

Compounds	***Culex pipiens molestus***		***Aedes aegypti***	
	m	**f**	**m**	**f**
(Z)- 3-hexen-1-ol	+J	+a, +J	-	+a
Hexanol	+J	+J	-	+a
(Z)-3-hexenyl acetate	+J, +a	+a, +J	-	+a
Benzaldehyde	+, +a, +J	+, +a, +J	+, +a	+, +a
Benzyl alcohol	+, +a	+, +a	+, +a	+, +a
Phenyl acetaldehyde	+, +a, +J	+, +a, +J	+, +a	+, +a
Acetophenon	+, +a, +J	+, +a, +J	+a	+a
Linalool oxide (furanoid)	+a, +J	+a, +J	+a	+a
Linalool	+, +a, +J	+, +a, +J	+a	+a
Phenylethyl alcohol	+, +a, +J	+, +a, +J	+, +a	+, +a
Lilac aldehyde A	+, +a, +J	+, +a, +J	+, +a	+, +a
Lilac aldehyde B+C	+, +a, +J	+, +a, +J	+, +a	+, +a
Lilac aldehyde D	+, +a, +J	+, +a, +J	+, +a	+, +a
Linalool oxide (pyranoid)	+, +a, +J	+, +a, +J	+, +a	+, +a
Methyl salicylate	+, +a, +J	+, +a, +J	+, +a	+, +a
Lilac alcohol A	+, +a, +J	+, +a, +J	+, +a	+, +a
Lilac alcohol B+C	+, +a, +J	+, +a, +J	+, +a	+, +a
Lilac alcohol D	+, +a, +J	+, +a, +J	+, +a	+, +a

Table 2

Electrophysiological and/or behavioural responses of the odour compounds of *Silene otites* found to be electrophysiological and behavioural active to mosquitoes to other insects of different orders.

Compounds	Electrophysiogical active	Electrophysiological active and behavioural attractive(a^+)/behavioural attractive (a)	Repellent
Acetophenone	Hy[16]	D[15+]	C[17, 38]
Linalool oxide (pyranoid)	L[26-27, 34]		L[29]
Phenyl acetaldehyde	L[19, 26-27]	C[2+], L[8], Hy[21, 22], L[28+, 31+, 32, 33+]	Hy[10]
Phenylethyl alcohol	C[1], L[26-27], Hy[30], L[34]	C[2+], L[8], D[11], N[18], L[29]	
Lilac aldehyde	L[26, 34]	L[31+, 35]	
Linalool oxide (furanoid)	L[26-27, 34]	C[21]	L[29]
Benzyl alcohol	L[3,19, 26-27, 34]	L[8], C[21], L[33+]	
(Z)-3-Hexenyl acetate	C[1], D[4], C[5], Hy[16], L[19, 27]	He-C[6], Hy[9+], L[14], D[15+]	
(Z)-3-Hexen-1-ol	C[1], D[4], C[12], Hy[16], L[27, 34]	C[5+], He-C-Hy[6], C[7], Hy[9+, 25+]	
Lilac alcohol	L[26]		
Benzaldehyde	C[1], L[3], D[23], L[26, 31, 34]	He-C[6], D[12+], Ho-C-N-Hy[20+], Hy-C[21]	
Methyl salicylate	C[1, 12], Hy[16], L[19, 26-27], Hy[30], L[34]	He[6],C[6-7], C[18], L[35]	Ho[36, 37], L[39]
Linalool	C[1, 12], D[24], L[26-27, 34]	L[8], Hy[9+, 21, 25+,] L[29], Hy[40]	O[21]
Hexanol	D[4],C[5],D[12, 23], L[27, 31]		

C: Coleoptera; D: Diptera (excluding mosquitoes); He: Hemiptera; Ho: Homoptera; Hy: Hymenoptera; L: Lepidoptera; N: Neuroptera; O: Orthoptera. The numbers above the letters denote corresponding references chronologically:

[1] Ruther et al., 2000; [2] Lampman et al., 1987; [3] Hoballah et al., 2005; [4] Zhao and Kang, 2002; [5] Ruther et al., 2002; [6] James, 2005; [7] Barlet et al., 1997; [8] Landolt et al., 2001; [9] Du et al., 1998; [10] Blum et al., 1982; [11] Ishikawa et al., 1983 ; [12] Dickens, 1999; [13] Dougherty et al., 1999; [14] Reddy and Guerrero, 2000; [15] Birkett et al., 2004; [16] Light et al., 1992; [17] Pureswaran and Borden, 2004; [18] Zhu and Park, 2005; [19] Fraser et al., 2003; [20] Han and Chan, 2002; [21] Theis, 2006; [22] Meagher and Mitchell, 1999; [23] Malo et al., 2005; [24] Cośśe et al., 1995; [25] Wei and Kang, 2006; [26] Andersson, 2003; [27] Raguso et al., 1996; [28] Olsson et al., 2005; [29] Omura et al., 2000; [30] Eltz and Lunau, 2005; [31] Dötterl et al., 2006b; [32] Cunningham et al., 2004; [33] Olsson et al., 2006 ; [34] Honda et al., 1998; [35] Plepys et al., 2002 ; [36] Pettersson et al., 1994 ; [37] Hardie et al., 1994 ; [38] Erbilgin et al., 2007; [39] Andersson et al., 2000; [40] Borg-Karlson et al., 2003.

Figure legends

Figure 1.

Attractiveness of *Culex pipiens molestus* to a mixture of 14 compounds of *Silene otites* that are known to be electrophysiologically active. Mixtures were prepared according to their mean ratio in *S. otites* scent profiles. N= number of mosquito groups tested.

Figure 2.

Attraction of *Culex pipiens molestus* to 14 individual odour compounds. N= number of mosquito groups tested.

Figure 3.

The responses of *Culex pipiens molestus* in two-stimulus choice tests. A: A mixture of the four most attractive compounds (transparent) vs. a mixture of 14 compounds (black). B: A mixture of the four most attractive compounds (transparent) vs. phenyl acetaldehyde (black). N= number of mosquitoes attracted to the stimuli. Asterisks (*) denote significant differences and indicate a preference for one stimulus over another (chi^2 observed vs. expected).

Fig. 1

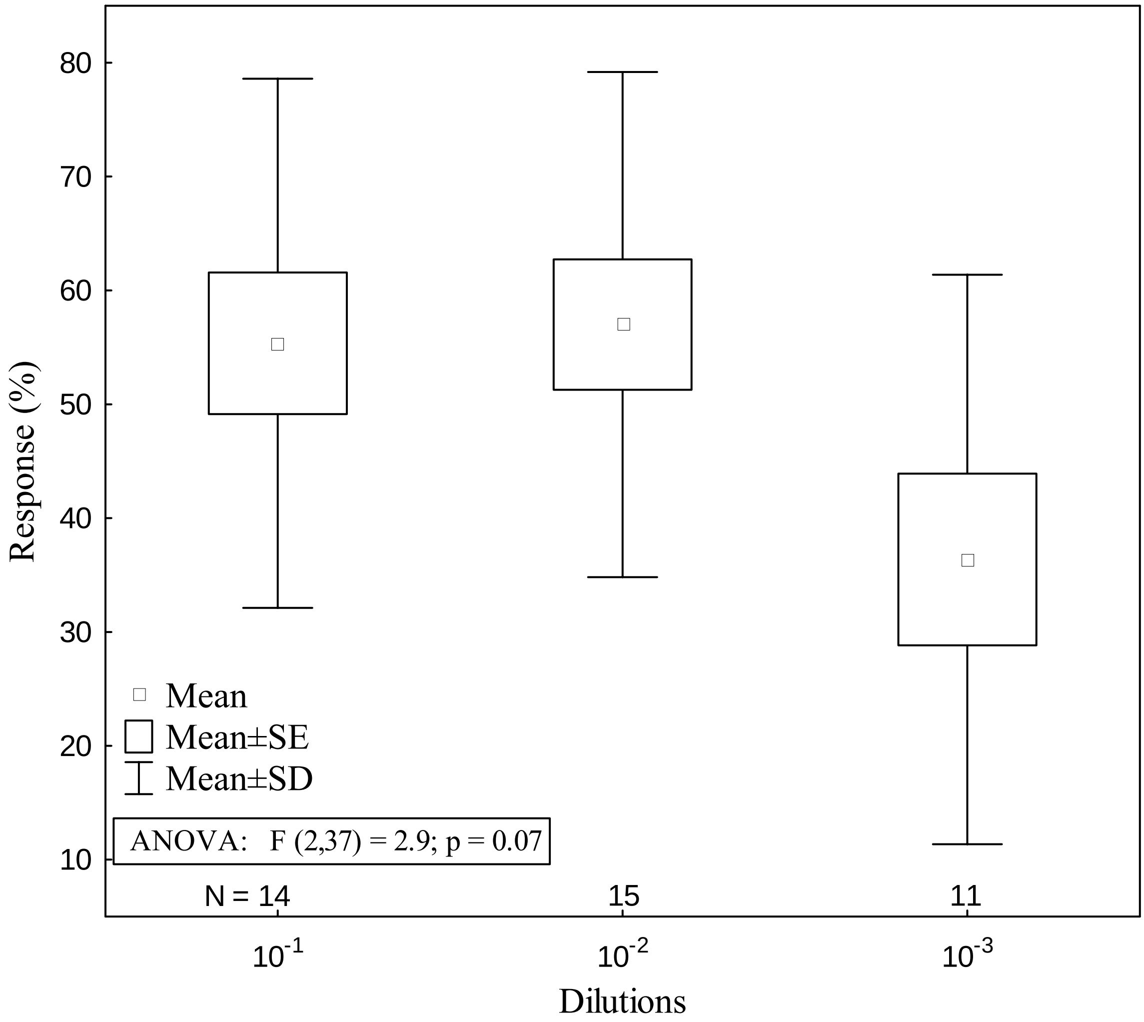

80
70
60
50
40
30
20
10
Response (%)
□ Mean
Mean±SE
Mean±SD
ANOVA: F (2,37) = 2.9; p = 0.07
N = 14
15
11
10-1
10-2
10-3
Dilutions

Fig. 2

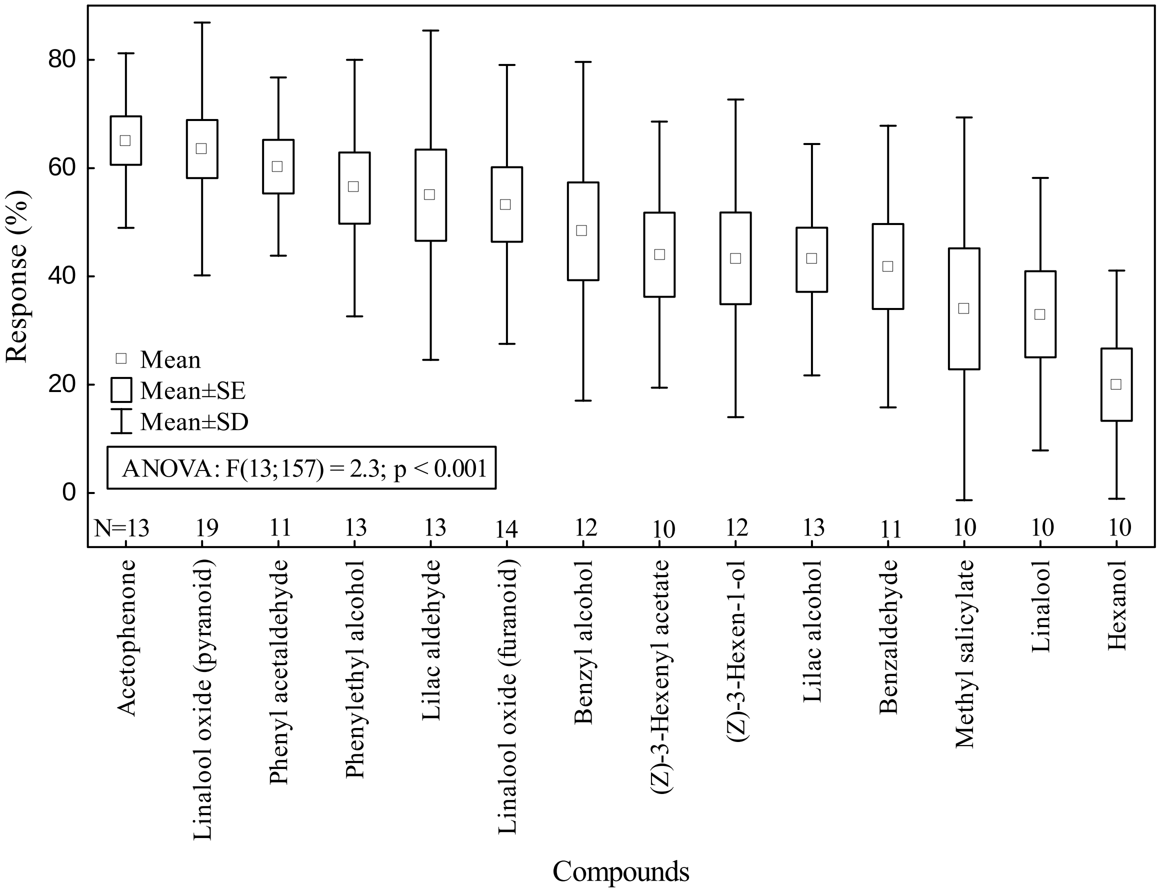
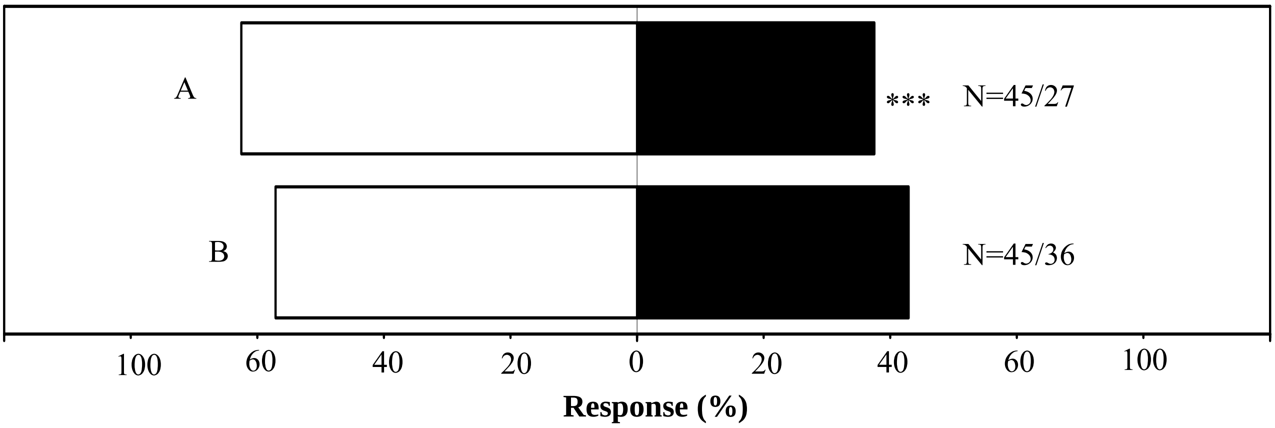

Response (%)
80
60
40
20
0
□ Mean
Mean±SE
Mean±SD
ANOVA: F(13;157) = 2.3; p < 0.001
N=13
19
11
13
13
14
12
10
12
13
11
10
10
10
Acetophenone
Linalool oxide (pyranoid)
Phenyl acetaldehyde
Phenylethyl alcohol
Lilac aldehyde
Linalool oxide (furanoid)
Benzyl alcohol
(Z)-3-Hexenyl acetate
(Z)-3-Hexen-1-ol
Lilac alcohol
Benzaldehyde
Methyl salicylate
Linalool
Hexanol
Compounds

Fig. 3



BEHAVIOR, CHEMICAL ECOLOGY

Naïve and Conditioned Responses of *Culex pipiens pipiens* Biotype *molestus* (Diptera: Culicidae) to Flower Odors

UMMA SALMA JHUMUR,[1] STEFAN DÖTTERL,[1,2] AND ANDREAS JÜRGENS[3]

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ABSTRACT Flower odors are important signals for chemical communication between plants and flower visitors. Here, we studied the naïve responses of *Culex pipiens pipiens* biotype *molestus* Forskal 1775 (Diptera: Culicidae) to typical flower odors and assessed the learning capacity of mosquitoes to floral volatiles. The odor compounds used in the bioassay, phenyl acetaldehyde, veratrole, and 2-methoxyphenol, are typically found in the floral odor of *Silene otites* (L.) Wibel, a plant that is pollinated by nectar-drinking mosquitoes and moths, and/or in other closely related *Silene* species. Wind tunnel bioassays with a mixture of these compounds revealed that attraction of mosquitoes to odors was positively correlated with time passed since the last feeding. In single component bioassays, mosquitoes showed strong innate responses to phenyl acetaldehyde and only moderate or weak responses to veratrole and 2-methoxyphenol. Furthermore, in comparison with naïve mosquitoes, conditioned mosquitoes were significantly more attracted to the mixture and single volatiles. These results indicate that naïve mosquitoes are effectively attracted by appropriate floral scent compounds and that learning can increase the attractiveness of these compounds.

KEY WORDS flower odor, *Culex pipiens pipiens molestus*, *Silene otites*, phenyl acetaldehyde, conditioned and naïve response

Mosquitoes, with >3,400 different species (Backer 1989), represent a significant threat to human health because of their ability to transmit pathogens that cause diseases, which afflict millions of people worldwide (WHO 1992, Pinheiro and Corber 1997, WHO/CTD 1998). Mosquitoes are the focal point of entomological studies worldwide, especially in connection with their human–medical significance as vectors of dangerous diseases, such as malaria, yellow fever, dengue fever, and filariasis.

For both autogenous mosquitoes (females can lay eggs without taking a bloodmeal) and anautogenous mosquitoes (females must take a bloodmeal to develop their eggs), carbohydrates are an important determinant of survivorship (Nayar and Sauerman 1971a,b). Most mosquitoes obtain carbohydrates primarily from floral nectars (e.g., Grimstad and DeFoliart 1974), but they also obtain carbohydrates from honeydew, plant phloem, or damaged and rotting fruit (Yuval 1992).

Many flower visitors are attracted to flowers by their scent (Dudareva and Pichersky 2000) and floral scent is also a cue for attraction of mosquitoes (Jepson and Healy 1988, Mauer and Rowley 1999). Nevertheless, few studies testing the attractiveness of synthetic floral scent compounds to mosquitoes have been published (Bowen 1992, Mauer and Rowley 1999, Howse 2003).

Because floral odors could be helpful for developing new pest control strategies, which are applicable not only in reproductive females but also in both male and female mosquitoes in different life stages, preferably as bait in traps, our objective was to identify highly effective nectar- or flower-related attractants. Based on the assumption that plant species not only visited but also effectively pollinated by mosquitoes are emitting more specific mosquito-attracting compounds than plants pollinated by other agents, we restricted our search for promising floral scent compounds on mosquito-pollinated plants, and plants closely related to such plants. However, so far the effective pollination by mosquitoes has been described only in two cases worldwide, in the orchid *Habenaria* (*Platanthera*) *obtusata* (Banks ex Pursh) Richardson (Stoutamire 1968) and in *Silene otites* (L.) Wibel (Caryophyllaceae) (Brantjes and Leemans 1976). Although species of *Aedes* have been reported as pollinators of the former species, *Culex pipiens* L. and *Culiseta annulata* Schrank were found as pollinators of the latter species. The mosquitoes are probably attracted to these plants by their scent; however, until now only floral scent of *S. otites* was analyzed (Jürgens et al. 2002). Nineteen floral volatile compounds were identified in the Jürgens et al. (2002) study, but it is still unclear, which of these compounds are responsible for attraction of the mosquitoes in particular.

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Furthermore, in other pollination systems it is known that previous experience strongly influences the attractiveness of floral scent. In the sexually deceptive orchid *Ophrys sphegodes* Miller, pollinated by males of the solitary bee *Andrena nigroaenea* (Kirby 1802), it has been shown that male bees learned the odor bouquets of that orchid during mating attempts and recognized them in later encounters (Ayasse et al. 2000). With regard to floral scent compounds, nothing was known about the learning ability of adult mosquitoes.

In the current study, we determined and compared the naïve responses of mosquitoes to typical flower odor compounds found in mosquito-pollinated *S. otites* and other *Silene* species, and we assessed the learning capacity of mosquitoes to these compounds. The findings might provide an important step toward the use of floral odors as attractants or baits in mosquito pest control.

Materials and Methods

Insects. We used the autogenous *Culex pipiens pipiens* biotype *molestus* Forskal 1775 (European strain) for experiments. Mosquitoes were reared in the climatic chamber under dark (9 h) and light (15 h) condition at 19.5 and 23.5°C, respectively. A temperature of 21–25°C was found to be useful for emergence and survival of *Cx. pipiens molestus* (Oda et al. 1980, 1999). The larvae were fed with TetraMin fish food (Tetra Werke, Melle, Germany) and reared until pupal stage in open trays (31 by 24 by 9 cm) containing tap water of a 5-cm depth. Emerging adults were separated in plastic boxes (46 by 37 by 23 cm) every day.

Volatile Compounds. The odor compounds phenyl acetaldehyde, veratrole, and 2-methoxyphenol (obtained from Sigma-Aldrich Chemie GmbH, Germany; purity 90–99%) were chosen to test their attractiveness to mosquitoes. Although phenyl acetaldehyde was found only in small amounts in the *S. otites* plants studied by Jürgens et al. (2002), this benzenoid is a main compound in flower scent of several European populations of this *Silene* species (U.S.J., unpublished data). Phenyl acetaldehyde is known to be attractive to *Aedes aegypti* (L.) (Howse 2003). 2-methoxyphenol and veratrole occur in closely related *Silene* species (Jürgens et al. 2002, Dötterl et al. 2005b) and are known to attract other flower-visiting insects such as moths (Dötterl et al. 2005a).

The three compounds were tested both as mixture and singly. A ratio of 8:1:1 of phenyl acetaldehyde, veratrole, and 2-methoxyphenol was used for the mixture. The higher amount of phenyl acetaldehyde was used because this compound was found in high relative amounts in several *S. otites* populations (U.S.J., unpublished data).

Conditioning Procedure. For conditioning, groups of 25–30 3-d-old unfed mosquitoes were kept together in small cylindrical boxes (29 by 13 cm) and allowed access for 1 h to a filter paper (5 by 2 cm) soaked with 1,800 μl of a 5% (similar to Oda et al. 1980, and Geier and Boeckh 1999) sugar solution, in combination with a scent stimulus. The control groups of naïve mosquitoes were offered 1,800 μl of 5% sugar solution without a scent stimulus. Only individuals that had consumed sugar solutions (easily visible due to the distended abdomen after feeding) were used for further bioassays after a subsequent starvation period. This conditioning procedure differs somewhat from the classical conditioning protocol in the use of the conditioning stimulus (CS). Typically, the CS elicits a neutral or weak innate response (for review, see Smith et al. 2006); however, in our study especially phenyl acetaldehyde elicited a relatively strong innate response. The other two compounds used as CS elicited only moderate or weak innate responses (see Results). Nevertheless, the increased attractiveness after conditioning is indicating learning.

The threshold for nectar searching or the strength of the response to nectar-related compounds is probably dependent on the length of the starvation period. In a first experimental set (response of conditioned mosquitoes to a volatile mix after different starvation periods) we therefore aimed to identify the starvation period needed to get the most intense response of mosquitoes to floral volatiles. Therefore, mosquitoes were conditioned in a first step with the volatile mix, which was added to the 1,800-μl sugar solution. Thereafter, the mosquitoes were kept unfed for 1) 13–15 h, 2) 37–39 h, and 3) 61–63 h, before testing their attraction to the same volatile mix in wind tunnel bioassays. It was not possible to test mosquitoes after longer starvation periods, because they usually do not survive >3 d without carbohydrate. Furthermore, males have a shorter life span than females. Thus, both male and female mosquitoes were used 13–15 h and 37–39 h after conditioning, but only females were tested after 61–63 h.

We did not use the single compounds to determine the starvation period after which responsiveness is highest, because a similar result to that obtained by the mixture of compounds was expected. Sixty-one to 63 h after starvation, where conditioned response to the volatile mix was highest, the mixture of compounds also was used to determine the naïve response and thereby to test the effect of conditioning. In a second experimental set, naïve and conditioned responses to single compounds were determined. These tests were conducted after 61–63 h of starvation, because in the first experimental set mosquitoes showed strongest response in this period. Because male and female mosquitoes did not show significant differences in their response to scent mixtures after 13–15 and 37–39 h (see Results), only females were used for this second experimental set (responses of conditioned and naïve mosquitoes to single odor compounds). The preparation and conditioning of mosquitoes was done in the same way as for the test with scent mixtures.

Bioassays. A 160- by 75- by 75-cm wind tunnel (Dötterl et al. 2005a) was used for bioassays. A Fischbach speed controller fan (D340/E1, FDR32, Neunkirchen, Germany) continuously circulated the necessary air through the tunnel with an airspeed of 0.35 m/s. The incoming air was passed through four charcoal filters (145 by 457 mm), with a carbon thickness of 16 mm (Camfil Farr, Laval, Quebec, Canada). The temperature and humidity were adjusted to 22–24°C and 30–32%, respectively. Experiments were carried out during the first 3 h of the dark period, under dim red light. To allow the mosquitoes to adapt to the wind tunnel environment, they were kept in the wind tunnel room for ≈12 h before starting the experiment. All equipment was cleaned with ethanol, burned in flame, and then sterilized at 200°C. To avoid contamination, surgical gloves were worn during mosquito handling and bioassays.

A two-choice assay was used to investigate the attractiveness of odors. A blank rubber GC septum was used as control, and on a second septum an odor stimulus was applied. Blank septum and stimulus septum were alternatively offered from both left and right sides to control side biases. The septa were offered at the upwind end of the tunnel behind polyester gauze and metal grids, so that they were invisible to the mosquitoes. For the tests, mosquitoes were not used singly but as groups of several specimens (also see Jepson and Healy 1988, Geier and Boeckh 1999). Therefore, five randomly chosen male and/or female conditioned or naïve mosquitoes were released from a holding chamber (16 by 8 cm) at the downwind end of the tunnel, and their behavior was observed for 1 h. In experiments with mixed compounds, 22 males and 23 females, and 19 males and 45 females, respectively, were randomly used together in groups 13–15 and 37–39 h after conditioning. For the tests 37–39 h after conditioning, only four specimens were used in one of the 13 groups. The behaviors of mosquitoes were not found to be influenced by interaction between the sexes. The behavior of single mosquitoes was counted as attraction (response) to the odor when mosquitoes landed within a circle of 10 cm on the gauze in front of the odor source.

In bioassays with single compounds, the postchoice behavior of responding mosquitoes after landing on the gauze was furthermore classified into two types: "sitting" and "searching." Sitting was characterized simply as sitting without moving or doing anything on the gauze, and searching was characterized by excited movement of mosquitoes on the gauze and repeated penetration of gauze with their proboscis, presumably in search for a food source. Sitting and searching were recorded when the mosquitoes showed this postchoice behavior after landing on the gauze in front of the odor source. To avoid counting responding mosquitoes twice, they were immediately removed from the wind tunnel by an aspirator after the observation of a specific response.

Scent Stimuli Used for Conditioning and Bioassay. Mosquitoes were conditioned either with 5 μl of a single component or of the volatile mix, which was added to the 1,800-μl sugar solution on filter paper (5 by 2 cm), resulting in a scent emission comparable with the amount emitted by a *S. otites* plant (U.S.J., unpublished data). Thereafter, the attraction of the mosquitoes to 10 μl of the same pure single component, or the volatile mix applied to a rubber GC septum, was tested in wind tunnel bioassays. To standardize the amount of volatiles emitted during conditioning from the filter paper, and during the wind tunnel bioassays from the GC septum, a lower amount of substance was applied on the bigger filter paper compared with the smaller septum. Scent emission of 5 μl of single or mixed compounds, which was added to the 1,800-μl sugar solution on filter paper was comparable with 10 μl of the volatile(s) on GC septa used in wind tunnel bioassays (U.S.J., unpublished data).

Statistical Analyses. We used the STATISTICA program package (StatSoft, Inc. 2004) for statistical analyses. For each group, consisting of five simultaneously tested mosquitoes, the relative proportion of responding mosquitoes was determined. The proportions, derived from the replicates, were used without transformation for further analyses. One-way analysis of variance (ANOVA) was used to compare the responses of conditioned mosquitoes among different exposure periods to the mixed odors. ANOVA also was used to compare the attraction of either conditioned or naïve mosquitoes to the different odor compounds (phenyl acetaldehyde, veratrole, and 2-methoxyphenol). Unequal N honestly significant difference (HSD) was used as posthoc test in case of a significant F-test. A *t*-test for independent samples was used to compare the responses of conditioned and naïve mosquitoes to the mixture during the strongest response period. This test also was applied to evaluate the differences in attraction of conditioned and naïve mosquitoes to the single odor compounds. Normality was tested using the Kolmogorov–Smirnov test. Homogeneity of variances was tested using Hartley test. A chi-square test was used to assess the differences in attractiveness between males and females as well as to evaluate the post choice behavior (sitting and searching) in mosquitoes responding to single odor compounds. For all statistical analyses, an α level of 0.05 was used.

Results

Response of Conditioned and Naïve Mosquitoes to a Volatile Mix after Different Starvation Periods. The attraction (landing response) of conditioned mosquitoes to odors was found to be increased with time passed since the last feeding (Fig. 1). The attraction of mosquitoes to the odor source in the wind tunnel was 22, 60, and 81%, respectively, 13–15, 37–39, and 61–63 h after conditioning, and the observed differences were significant (ANOVA: $F = 16.73$; df = 2, 33; $P < 0.001$). Only a few mosquitoes were attracted to the mixture 13–15 h after conditioning, whereas significantly more mosquitoes were attracted 37–39 and 61–63 h after conditioning. Most mosquitoes responded 61–63 h after conditioning, and there was a nearly significant difference ($P = 0.06$) to the attraction of mosquitoes tested 37–39 h after conditioning. Moreover, 61–63 h after feeding significant more conditioned (81%) than naïve (50%) mosquitoes were

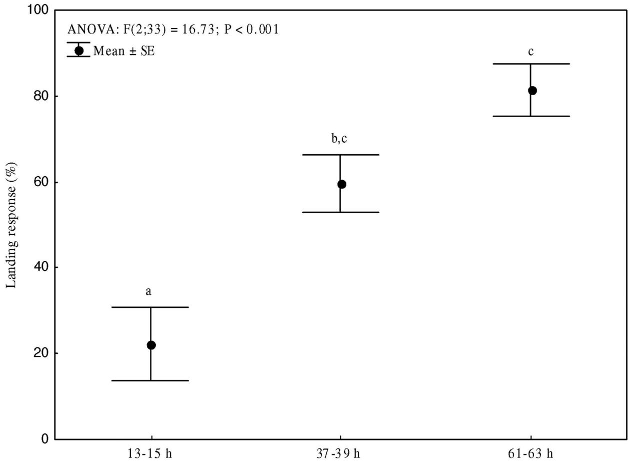

Fig. 1. Attraction (landing) of conditioned mosquitoes to a mixture of three odor compounds after different starvation periods. Different letters above whiskers denote significant differences between different treatments. Numbers of mosquito groups tested 13–15, 37–39, and 61–63 h after conditioning: n = 9, 13, and 14, respectively.

attracted (t = 3.8, df = 28, $P < 0.001$). There was no bias in side preferences of mosquitoes, and during all bioassays mosquitoes were never seen to land in front of the blank septum. No significant differences in attraction to the volatile mix were found between male and female mosquitoes after 13–15 h (chi-square test: $\chi^2 = 1.8$, df = 1, $P = 0.18$) and 37–39 h ($\chi^2 = 0.16$, df = 1, $P = 0.69$).

Responses of Naïve and Conditioned Mosquitoes to Single Odor Compounds. The mean response of naïve mosquitoes to phenyl acetaldehyde, veratrole, and 2-methoxyphenol was 63, 44, and 29%, respectively, compared with 80, 75, and 58% in conditioned mosquitoes. Naïve ($F = 7.4$; df = 2, 37; $P < 0.002$) as well as conditioned ($F = 3.9$; df = 2, 36; $P < 0.03$) mosquitoes were differentially attracted by single scent compounds. Mosquitoes showed the highest response to phenyl acetaldehyde followed by veratrole and 2-methoxyphenol (Fig. 2), and the differences were significant between phenyl acetaldehyde and 2-methoxyphenol in both naïve and conditioned mosquitoes. The differences in attraction of naïve and conditioned mosquitoes to the single compounds (effect of conditioning) were found to be significant for all tested compounds, i.e., phenyl acetaldehyde ($t = -2.1$, df = 27, $P = 0.047$), veratrole ($t = -4.0$, df = 24,

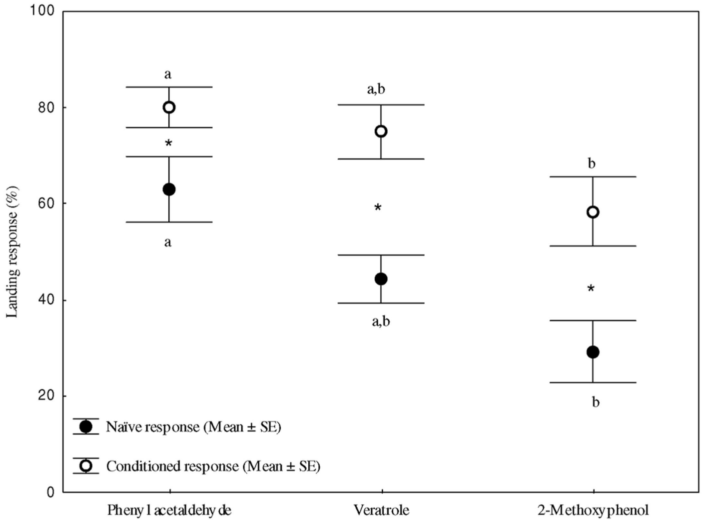

Fig. 2. Attraction of conditioned and naïve mosquitoes to single odor compounds. Asterisks (*) denote significant differences in attraction between conditioned and naïve mosquitoes to a single compound (t-test; $P < 0.05$); different letters above whiskers denote significant differences in attractiveness of different compounds to conditioned mosquitoes; different letters below whiskers denote significant differences in attractiveness of different compounds to naïve mosquitoes. Twelve to 15 groups of mosquitoes were used for each odor exposure.

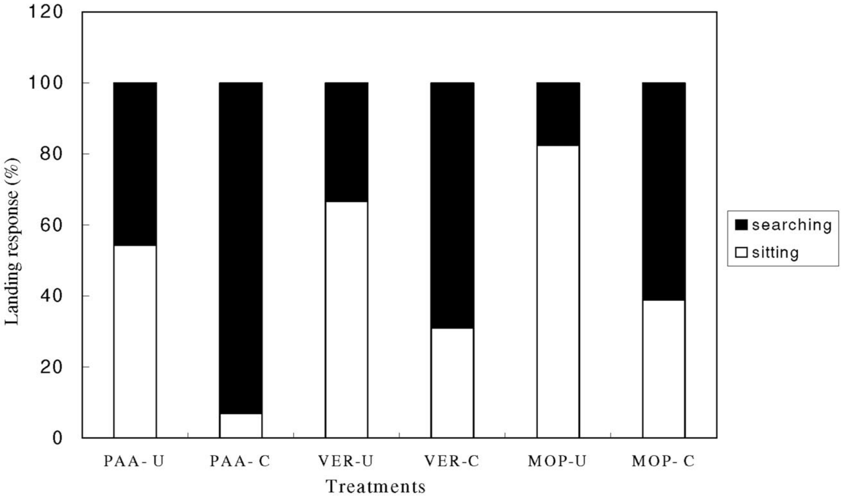

Fig. 3. Postchoice behavior of conditioned and naïve mosquitoes after attraction to single volatiles. U, naïve; C, conditioned; MOP, 2-methoxyphenol; PAA, phenyl acetaldehyde; and VER, veratrole. The percentage values of sitting and searching were calculated from the observations of landing mosquitoes.

$P < 0.001$), and 2-methoxyphenol ($t = -3.0$, df = 24, $P = 0.006$).

Comparison of Postchoice Behavior in Naïve and Conditioned Mosquitoes. The conditioning of mosquitoes was found to affect the postlanding behavior, i.e., sitting and searching. Whereas most of the naïve mosquitoes were observed only sitting, the conditioned mosquitoes were mainly reported as searching after landing on the gauze (Fig. 3). These differences were significant for all three odor compounds (phenyl acetaldehyde: chi-square test: $\chi^2 = 18.2$, df = 1, $P < 0.001$; veratrole: $\chi^2 = 8.8$, df = 1, $P = 0.003$; and 2-methoxyphenol: $\chi^2 = 9.4$, df = 1, $P = 0.002$).

Discussion

Nectar is the most important carbohydrate source for mosquitoes (Nayar and Van Handel 1971, Grimstad and DeFoliart 1974), and it has been shown that flower scent is a cue for nectar plant finding. For *Aedes aegypti* L., it was shown that floral odors of ox-eye daisy, *Leucanthemum vulgare* L., in the absence of visual stimuli are attractive, although solvent extracts of this flower did not attract this mosquito species (Jepson and Healy 1988). *Cx. pipiens* was attracted to solvent extracts of common milkweed, *Asclepias syriaca* L., but synthetic blends of these two compounds failed to attract any mosquito in a dual-port olfactometer (Mauer and Rowley 1999). Minor compounds, to date unidentified, of the scent of *A. syriaca*, may therefore have been important for attraction of *Cx. pipiens* to the solvent extract. Bowen (1992) demonstrated that antennal receptors of *Cx. pipiens* responded to bicyclic terpenes, such as thujone, and verbenone. Thujone also attracted this species in a bioassay conducted in a small chamber, but this compound did not elicit any upwind flight in a dual-port olfactometer. Furthermore, a probing response assay to thujone demonstrated that it was dependent on both stimulus dose and time of food deprivation (Foster and Hancock 1994). Together, these studies show that the results are highly dependent on the methodology and that far more studies are needed to evaluate the exact role of floral odors in mosquito attraction and behavior. Our results demonstrate that naïve mosquitoes are effectively attracted to mixed as well as to specific flower scent compounds. Moreover, we could show that this attraction is significantly enhanced by learning. In this study, the attractiveness of phenyl acetaldehyde was higher than that of veratrole, followed by 2-methoxyphenol. Phenyl acetaldehyde is a very widespread floral scent compound (Knudsen et al. 1993) and an abundant floral scent compound of *S. otites* (U.S.J., unpublished data). Recently, this compound also was found to be attractive to another mosquito species, i.e., *Ae. aegypti* (Howse 2003). Furthermore, it is highly attractive to other insects, such as butterflies (Andersson 2003), moths (Cunningham et al. 2004, Dötterl et al. 2005a), and Brachycera (Howse 2003). Therefore, this benzenoid seems to be a very important attractant for many insects in search for nectar. Veratrole is also known to be attractive to moths (Dötterl et al. 2005a), locusts (Niassy et al. 1999), corn rootworms (*Diabrotica* spp.) (Lampman et al. 1987), and fruit flies (Metcalf et al. 1975).

In our study, conditioned *Cx. pipiens molestus* were more effectively attracted to the mixture and to single compounds compared with naïve mosquitoes. The significant differences in attraction of conditioned and naïve mosquitoes present evidence for learning capability of mosquitoes in relation to floral scent compounds and indicate that mosquitoes can retain their memory (acquisition of information for storage; see Thorpe 1963, Immelmann and Beer 1989) for at least 3 d. Interestingly, mosquitoes showed, after conditioning, similar ranking of preferences for the tested individual compounds compared with naïve mosquitoes, and it seems that conditioned responses are correlated with innate preferences.

The learning capability of mosquitoes also is described in other species, especially with regard to oviposition kairomones (McCall and Eaton 2001, Kaur et al. 2003); substances presented during the larval or pupal stages were preferred by adult mosquitoes (*Culex quinquefasciatus* Say and *Ae. aegypti*, respectively). It also was shown that host-seeking females of *Anopheles farauti* Laveran learned suitable breeding sites (Charlwood et al. 1988), and male *Aedes taeniorhynchus* (Wiedemann) (Nielsen and Nielsen 1953) and *Culex torrentium* Martini (Service 1994) repeatedly swarm at the same locations, suggesting a "spatial memory" in mosquitoes. Mwandawiro et al. (2000) found that three species of *Culex* had an overall tendency to return to the bloodmeal host species upon which they had fed previously. All these studies give evidence that mosquitoes have generally an excellent learning capability.

In our study, conditioned mosquitoes were not only more effectively attracted to floral scent compounds, they also showed a different behavior after landing on the gauze compared with naïve mosquitoes. Although in naïve mosquitoes the sitting response dominated, most conditioned mosquitoes actively searched for food and penetrated the gauze with their proboscis. This finding most strongly indicates that based on their previous experience, the conditioned mosquitoes have learned the odor as a signal for food. Interestingly, phenyl acetaldehyde, the compounds triggering most landings, also elicited most effectively the innate as well as conditioned searching response. Furthermore, the compounds eliciting a higher innate searching response also elicited a higher conditioned searching response.

In summary, different floral scent compounds have diverse effects as attractants to mosquitoes and learning strongly influences the behavior of mosquitoes, resulting in an increased attraction to these compounds after conditioning. Furthermore, the length of the starvation period plays an important role and modifies the strength of the response to floral scents, probably by shifting the threshold for nectar searching. By comparing the degree of attractiveness, we hypothesize that the foraging or host plant-seeking behavior of naïve mosquitoes is mediated by their innate attraction to floral scent compounds, whereas learning may help the mosquitoes in finding the most suitable host plant, which they visit more frequently thereafter. However, cross-comparisons among scents (e.g., conditioning with phenyl acetaldehyde and testing to veratrole) were not performed, to test for stimulus specificity. Therefore, further studies are needed to determine whether learning of floral scents in mosquitoes indeed results in acquired plant preferences as it has been shown for other insects (e.g., moths; Cunningham et al. 1998). Moreover, the attraction of *Cx. pipiens pipiens* biotype *molestus* to the tested odor compounds, especially phenyl acetaldehyde, suggests that they could be used as bait in traps to improve existing or to develop new control strategies. With improved knowledge by further analyses of these chemical cues as well as other flower scent compounds on different mosquito species, new methods of mosquito control might be implemented.

Acknowledgments

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Temporal variation of flower scent and flower visitors of “nocturnal” *Silene otites* (Caryophyllaceae)

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Abstract

The emission of floral odours is one of the important features in angiosperms for attracting pollinators and for determining sexual plant reproduction. Floral scent patterns are often interpreted in relation to the pollinating agents and their olfactory abilities and preferences. Pattern analysis of floral bouquets has been a fruitful approach to compare the complex patterns of species in relation to their pollination syndromes. Such studies revealed similarities in the floral odour composition of plants with similar types of pollinators. However, floral scent has a dynamic nature and many plant species show a rhythmic scent emission. Studies on the temporal dynamics in volatile emission in plant species can provide insights in the adaptive value of certain floral compounds for the attraction of pollinators and are of particular interest in plants with mixed pollination syndromes where the activity times of the pollinators and their olfactory preferences differ. In this paper we investigated the night-flowering species *Silene otites* (Caryophyllaceae) that emits a complex mixture of volatile components during day and night with an emission maximum in the early night. Flower visitor observations showed that *S. otites,* although regarded as adapted to microlepidoptera and mosquitoes, is visited by a wide range of insects during the night and day with almost 60 species of 5 insect orders. During day beetles, brachyceran flies and hymenopteran species were found, at night flowers were visited by nocturnal Lepidoptera, nematoceran flies, and Neuroptera individuals. Five different temporal emission patterns were found for the scent compounds. (1) Compounds being emitted only (mainly) at night, especially few hours after sunset (e.g., phenyl acetaldehyde, lilac aldehyde), (2) compounds emitted during the whole night (e.g., δ-3-carene, α-pinene), (3) compounds emitted mainly in the afternoon (e.g., hotrienol, linalool), (4) compounds emitted during the forenoon as well as the afternoon (e.g., (E)-β-ocimene), and (5) compounds emitted mainly during the afternoon, and the first half of the night (e.g., benzaldehyde). These changes in the floral odour composition during day and night are discussed in relation to the different insect visitors.

Key words: *Silene otites*, day-visitor, night-visitor, adaptation, temporal variations, flower odour, pollinator, selection.

Introduction

The complex features of angiosperm flowers are generally interpreted as adaptations for attracting and exploiting certain types of pollinators, and for excluding other types (Fenster et al. 2004). This implicates that flowering plants have developed adaptations that promote the interaction with flower visitors that are advantageous for them and suppresses the interactions with flower visitors that would negatively affect their reproduction. Floral odours play an important role for the chemical communication between plants and their pollinators (Pellmyr and Thien 1986; Dobson 2006; Knudsen et al. 2006) and may affect pollinator choices and behaviour, thus having subsequent effects on plant reproduction (Pellmyr 1986). The time of floral scent emission and the composition may be typical for certain groups of pollinators (Dobson 2006; Knudsen et al. 2006). Similarities in the composition of floral volatiles have been found for example in plants adapted to bats (Knudsen and Tollsten 1995; Bestmann et al. 1997), beetles (Thien et al. 1975; Yasukawa et al. 1992; Jürgens et al. 2000), flies (Kite 1995; Kite and Hetterschieid 1997; Jürgens et al. 2006), and moths (Knudsen and Tollsten 1993; Raguso and Pichersky 1995; Miyake et al. 1998; Jürgens et al. 2002; Jürgens et al. 2003).

Of particular importance for the attraction of pollinators are floral volatiles in night-flowering plants where olfactory signals, besides visual cues, significantly add to the distance attraction (Brantjes 1976, 1978). Floral fragrances of moth-pollinated flowers are often described as being dominated by oxygenated terpenes and aromatic esters (Loughrin et al. 1990; Knudsen and Tollsten 1993; Raguso and Pichersky 1995; Jürgens et al. 2002). However, the odour composition of flowers within a single species can vary in the presence, concentration and relative proportions of their constituents at different times of a day (Baldwin et al. 1997; Shaver et al. 1997; De Moraes et al. 2001). Plants pollinated by moths tend to have maximal scent production during the late evening or night (Matile and Altenburger 1988; Loughrin et al. 1990; Kolosova et al. 2001; Levin et al. 2001; Dötterl et al. 2005; Hoballah et al. 2005). For example in *Silene latifolia* a night-flowering species pollinated by settling moths (mainly Noctuidae) and hawkmoths (Sphingidae) (Jürgens et al. 2002), the emission of all scent compounds starts at dusk after flower opening, reaches a maximum between 2200 and 2400 h, decreases until the next morning, and starts again at dusk the following day (Dötterl et al. 2005). This nocturnal emission of floral volatiles is controlled by an endogenous circadian clock, and the rhythmicity is maintained upon exposure to continuous light or dark (Altenburger and Matile 1988; Matile and Altenburger 1988; Altenburger and Matile 1990; Loughrin et al. 1991).

Moreover, odours are complex blends of individual volatile components built from different biosynthetic routes, and a potentially different effect on insect behaviour (Pichersky and Gershenzon 2002).

In this paper we investigated the dynamics of floral volatile emission in *Silene otites* (L.) Wibel (Caryophyllaceae), a night-flowering dioecious species (Wringley 1986). However, *S. otites* is an exceptional case for a night-flowering plant. The flowers are small and white-greenish in terminal cymes and many insects including microlepidoptera and mosquitoes have been described as the flower visitors and/or pollinators of this plant (Brantjes and Leemans 1976). Thus, the flower morphology and the nocturnal pollinator-composition do not fit into the typical moth-pollination syndrome. Moreover, day active insects, mainly flies, wasps and small solitary bees have been reported as important flower-visitors in a *S. otites* population in southern Germany (K. Jahrreiß, unpublished data). Thus, based on the flower visitor observations *S. otites* seems to be rather a generalist.

Jürgens et al. (2002) and Jhumur et al. (2007b) analyzed the floral odours of *S. otites* and they found mainly different benzenoids and isoprenoid compounds. The dominating compounds (e.g., phenyl acetaldehyde, lilac aldehyde, cis-3-hexenyl acetate, benzaldehyde, linalool, 2-phenylethanol) are known to be antennal and/or behavioural attractive in nocturnal insects see (Raguso and Light 1998; Dötterl 2004; Jhumur et al. 2006). Attracting pollinators is especially crucial in dioecious plants like *S. otites*, where sexual reproduction depends on the pollination service by animal pollen vectors. In the night-flowering dioecious *S. otites* it can be assumed that strong selective pressures are working on the odour composition to optimize pollination. On the other hand, the association of this plant with a wide range of different types of flower visitors during day and night (all with different odour preferences) is a factor that might complicate the adaptation. One solution for this problem would be however, that *S. otites* has developed a 'fine tuned' odour emission so that different odours are produced at different times of the day and night. The objectives of this study were to test this hypothesis. To do this, we investigated the dynamics of the floral scent emission of *Silene otites* during the day and night to analyze whether the emission of the major floral scent compounds changes with the activity times of different types of flower visitors.

Materials and Methods

Plant Material and Volatile Collection

Plants used in this study were cultivated from seed provided by botanical gardens or collected from wild growing *S. otites* near Bamberg (Germany). Plants were grown in pots in the greenhouse until they built up a rosette, and the pots were then placed in flower beds in the field. Floral scent was collected in the field from three male and one female plant individual by dynamic headspace in order to determine temporal variation in scent over a day. Therefore, 1-3 inflorescences were enclosed within a polyester oven bag (20 × 8 cm; Toppits®, Germany) at beginning of measurements, and the oven bag was not removed before the end of the experiment. Volatiles were trapped for 2 min each hour. To avoid accumulation of flower scent from one measurement to the next, volatiles were sucked out of the bag by the use of a membrane pump (G12/01 EB, ASF Thomas, Inc.) between measurements. Volatiles were trapped in an adsorbent tube using the same pump as described above with a flow rate of 200 ml/min. The adsorbent tube was filled with a mixture (1:1) of 3 mg Tenax-TA (mesh 60–80) and Carbotrap (mesh 20–40). To distinguish between plant volatiles and ambient contaminants, surrounding air was collected for comparison. Further, scent from non-flowering plants were collected to discriminate between scent emitted by vegetative parts and flowers. Only scent emitted from flowers and not vegetative plant parts were included in that study.

Observation of Flower Visitors

The flower visitors of *S. otites* were recorded on a wild growing population near Bamberg (Pettstadt, Germany), and on plants cultivated in a flower bed of the

University of Bayreuth. In total, observations were made on seven days. Flower visitors were recorded from 8 am until midnight to get an idea on the temporal variation of flower visitors over a day. Insects were caught by an insect net for later identification in the lab or in some cases only determined on the level of insect order, super family or family (depending on insect group) in the field. Additionally, a BG Sentinel^{TM} trap was "lured" with *S. otites* plants in trapping experiments. This trap was placed near the *S. otites* flower bed at the University of Bayreuth and was used 8 times during night, and 2 times during the day. This trap was especially useful for trapping mosquitoes. *S. otites* containing air exited the trap through white gauze, and mosquitoes or other insects attracted by these compounds were sucked in the trap by use of an electrical fan (for more information see www.bg-sentinel.com). It was almost impossible to catch flower visiting (female) Culicidae using a net, because mosquitoes were strongly attracted to human beings. As soon as human beings were near the flowers mosquitoes were found "visiting" them instead of the *S. otites* flowers (especially in Pettstadt). However, many male and female mosquitoes were regularly observed visiting the flowers of *S. otites* in the flower bed next the Dept. of Plant Systematics (Univ. Bayreuth).

Results

Flower Visitors

Insect flower visitors could be observed during the day as well as at night on the inflorescences and flowers of *S. otites*. In total, almost 60 species of 5 insect orders were observed visiting the flowers (Table 1, Figure 1). During day beetles, brachyceran flies and hymenopteran species were found, at night flowers were visited by nocturnal Lepidoptera, nematoceran flies, and Neuroptera individuals. All species found on the inflorescences and flowers of *S. otites* are listed in Table 1. Most of the species occurred only in low abundances (1-5 individuals), and only few species were found numerously on the inflorescences.

The Coleoptera fauna observed during the day on the flowers was relatively diverse and species of several families were found. However, the abundance of most of the species was low. Similarly, most of the brachyceran fly species occurred only in low abundances, but many specimens of the syrphid fly *Episyrphus balteatus* were observed. Nematoceran flies were only observed during night at the flowers, and several nematoceran flies could be trapped in the trap "lured" with *S. otites* plants. The most abundant ones were unidentified chironomids and *Culex pipiens*/*torrentioum*.

Hymenopteran species were only observed during daytime, and most bees and wasps observed only occurred in low abundance. *Lasioglossum* species were the most frequently occurring bees. Among the nocturnal Lepidoptera found, several geometrids and pyralids occurred with low abundances. More frequently gelechoid moths visited the flowers for nectaring.

Temporal Variation of Floral Scent

The compounds found in the scent samples (isomers, e.g., different linalool oxide isomers, different lilac aldehyde isomers were pooled) are listed in Table 2. Especially monoterpenoids and terpenoids were identified. Most of the compounds occurred in all four samples, but some compounds were only found in one, two, or three individuals.
The total amount of scent emitted over a day was strongly variable (Figure 2). Most of the scent was emitted at the beginning of the night, and after reaching the maximum scent emission at 9 to 10 pm, the amount of scent decreased and was low in the morning and the forenoon. After midday the amount of scent increased continuously to reach the maximum at night.

Interestingly, there were big differences in the emission pattern of different compounds. Five different patterns were found. (1) Compounds being emitted only (mainly) at night, especially few hours after sunset (e.g., phenyl acetaldehyde, lilac aldehyde), (2) compounds emitted during the whole night (e.g., δ-3-carene, α-pinene), (3) compounds emitted mainly in the afternoon (e.g., hotrienol, linalool), (4) compounds emitted during the forenoon as well as the afternoon (e.g., (E)-β-ocimene), and (5) compounds emitted mainly during the afternoon, and the first half of the night (e.g., benzaldehyde).

Therefore, depending on day-time, the scent of *S. otites* inflorescences was dominated by different compounds. In the forenoon, (E)-β-ocimene, hotrienol, and benzyl alcohol reached percentage amounts of at least 10%. In the afternoon linalool and hotrienol contributed to the scent with at least 10% each. At night lilac aldehyde, benzaldehyde, phenyl acetaldehyde, and 2-phenylethanol dominated the scent spectrum, and in the morning after sunrise especially δ-3-carene, α-pinene, hotrienol, and 2-phenylethanol were found in the scent.

Discussion

Though *Silene otites* is described as being pollinated only during night (Brantjes and Leemans 1976) several insect species were found in this study visiting the flowers of *S. otites* during night as well as during day-time. During the day mainly brachyceran flies, hymenopteran and coleopteran species were found, and during night, similar to the study of Brantjes and Leemans (1976), nocturnal moths and nematoceran flies. Brantjes and Leemans (1976) found that anther dehiscence in this dioecious species takes place in the evening, and no pollen is available during day-time. Therefore, the authors concluded that *S. otites* is pollinated only during night, and observations on flower visitors were only conducted during night. We agree with the result of Brantjes and Leemans (1976) that anther dehiscence takes place in the evening, however, in contrast to their study we also found pollen being available during day-time (S. Dötterl, unpublished data), and therefore we argue that the day-active visitors found in this study are also potential pollinators of *S. otites*. Only during bad weather conditions, especially when it was very windy and rainy, no pollen was found on the flowers during day-time. Most day- as well as night-active flower visitors were observed drinking nectar, the most important floral reward of *S. otites*. This nectar was available even for insects with very short mouthparts, such as the syrphid *Episyrphus balteatus*, bees of the genus *Lasioglossun* and *Hylaeus*, and *Meligethes* beetles. Most flower visitors were observed visiting many flowers consecutively, and visiting male and female flowers, making them to potential pollen vectors. Therefore *S. otites* might not be pollinated only during night, but also during day-time.

The floral scent analyses support the hypothesis that *S. otites* is not only pollinated during night but also during day-time. Floral scent is a trait being strongly influenced by pollinator-mediated selection. As an example, plants being pollinated by a particular group of pollinators emit a similar spectrum of compounds independent of their phylogenetic relatedness (Dobson 2006; Knudsen et al. 2006). Further, not only the chemical composition is adapted to the olfactory abilities and preferences of the pollinators, but also the timing of floral scent emission is correlated with the activity of the pollinators. As an example, plants, such *Silene latifolia*, which are pollinated only by nocturnal moths, emit flower scent only at night and no scent at all is emitted during day-time (Dötterl et al. 2005). There are several advantages for nocturnal plants to have a precise timing of volatile emission: (1) it optimizes the metabolism in respect to energy and carbon expenditures (Dudareva and Pichersky 2000), (2) it synchronizes the activity of pollinators to a few hours in the evening and night, (3) it reduces the attraction of flower visitors that negatively affect plant reproductive success (Miyake et al. 1998), and (4) it reduces exposure to flower herbivores (Heath et al. 1992; De Moraes et al. 2001).

In present study it was demonstrated that *S. otites* emitted the most scent at night, but a smaller amount of scent was also emitted during day-time. Further, not only the amount of scent emitted in the course of a day, but also the chemical composition strongly differed. These differences in scent in the course of the day might be the results of pollinator mediated selection. Most scent was emitted immediately after darkness, during a time when most of the nocturnal visitors, i.e. moths and mosquitoes, were observed visiting the flowers. Between 11 pm and midnight almost no specimen were observed visiting the flowers, and we assume that flowers of *S. otites* are visited mainly before midnight, and that only few nocturnal insects visit the flowers after midnight though observations were not conducted during that time. After darkness, the scent of *S. otites* was characterized by high percentage amounts of the benzenoids phenyl acetaldehyde and 2-phenylethanol, and the monoterpenoid lilac aldehyde. All these compounds are known to be attractants for moths (Haynes et al. 1991; Heath et al. 1992; Meagher 2001; Meagher 2002; Plepys et al. 2002; Dötterl et al. 2006) and/or mosquitoes (Jhumur et al. 2007b; Jhumur et al. 2007a), the main flower visitors of *S. otites* at night.

In the afternoon, when especially bees and syrphids were frequently found, the flowers emitted several compounds known to be attractive for these insects. As an example, linalool, the most abundant compound in the afternoon is known as attractant for bees (Henning et al. 1992), and methyl salicylate, which is also emitted mainly in the afternoon, is known as attractant for syrphid flies (James 2005). Some compounds, e.g., δ-3-carene and α-pinene were emitted by the flowers during the whole night but not during day-time. It is unclear, whether these compounds play any role for attracting nocturnal visitors.

The different emission patterns of different compounds may not only be the result of pollinator mediated selection, but also be influenced by the biosynthetic relatedness of different compounds. Well studied are the compounds being involved in the biosynthesis of lilac aldehyde (Burkhardt and Mosandl 2003; Kreck and Mosandl 2003; Kreck et al. 2003), a monoterpenoid being emitted especially few hours after darkness in *S. otites*. One precursor of this compound is linalool, which also was found in the scent of *S. otites*. However, instead of lilac aldehyde, this monoterpenoid was emitted especially in the late afternoon. At night, when the amount of lilac aldehyde increased, the amount of linalool decreased. It is unclear, whether the decreasing amount of linalool is a result of less synthesis activity of linalool, or whether the rate of biosynthesis is constant, but instead of emitting linalool, it is used as precursor for the synthesis of lilac aldehyde. However, when having a look on the emission pattern of linalool and lilac aldehyde during a whole day, it seems that the decrease of linalool at night is indeed an effect of less biosynthetic activity, and therefore a result of pollinator mediated selection, because the amount of linalool does not increase in parallel with a decreasing amount of lilac aldehyde.

In summary the results demonstrate that *Silene otites* is not only visited by night-active but also by day-active insects, and both groups of insects may be important pollen agents. The ‘fine tuned’ odour emission of this plant seems to be an adaptation to the olfactory abilities and preferences of the day- and night-active pollinators, respectively. Pollination experiments are now needed to test, whether day-active visitors are indeed pollinators of this plant, and to determine the contribution of day- as well as night-active visitors to the reproductive success.

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Table 1: Flower visitors of *S. otites* in a flowering bed in Bayreuth and in a wild growing population near Bamberg (Pettstadt), and insects trapped with an automatic trap lured with *Silene otites*. The abundance of insects is given as 1, if the number of individuals observed was not more than 5, as 2, if between 6 and 19 individuals were observed, and as 3, if more than 20 individuals were found.

Order	(Super)Family	Genus	Species	Bayreuth	Pettstadt	Trap Bayreuth	Abundance
Coleoptera	Alleculidae	*Cteniopus*	*flavus* SCOPOLI, 1763		x		1
	Cantharidae	*Rhagonycha*	*fulva* (SCOPOLI, 1763)		x		2
	Cerambycidae	*Leptura*	*rubra* (LINNAEUS, 1758)	x			1
		Leptura	*sanguinolenta* LINNAEUS, 1761		x		1
	Chrysomelidae	*Zeugophora*	*flavicollis* (MARSHAM, 1802)		x		1
	Elateridae	*Lacon* LAPORTE DE CASTELNAU, 1838	spec.		x		1
	Mordellidae	Unidentified			x		1
	Nitidulidae	*Meligethes* STEPHENS, 1830	spec.	x	x		2
	Oedemeridae	*Oedemera* OLIVIER, 1789	spec.	x	x		1
Diptera B	Anthomyiidae	Unidentified			x		1
	Calliphoridae	*Lucilia ROBINEAU-DEVOIDY, 1830*	spec.	x			1
	Phoridae	Unidentified				x	1
	Sarcophagidae	*Sarcophaga MEIGEN, 1826*	spec.	x			1
	Calyptratae	Unidentified				x	1
	Syrphidae	*Episyrphus*	*balteatus* (DEGEER, 1776)	x	x	x	3
		Eristalis	*tenax* (LINNEAUS, 1758)	x			1
		Eupeodes	*corollae* (FABRICIUS, 1794)	x	x	x	1

Order	(Super)Family	Genus	Species	Bayreuth	Pettstadt	Trap Bayreuth	Abundance
		Scaeva	*pyrastri* (LINNAEUS, 1758)		x		1
		Sphaerophoria LEPELETIER and SERVILLE, 1828	spec.	x	x		2
		Syrphus	*vitripennis* MEIGEN, 1822	x			1
		Syritta	*pipie*ns (LINNEAUS, 1758)	x			1
	Tachinidae	*Eriothrix* MEIGEN, 1803	spec.	x			1
		Unidentified		x			1
Diptera N	Cecidomyiidae	Unidentified				x	2
	Ceratopogonidae	Unidentified				x	1
	Chironomidae	Unidentified				x	2
	Culicidae	*Aedes*	*vexans* (MEIGEN, 1830)			x	1
		Anopheles	*claviger* (MEIGEN, 1804)			x	1
		Ochlerotatus	*cantans* (MEIGEN, 1818)			x	1
		Culex	*pipiens pipiens* (LINNEAUS, 1758)			x	1
		Culex	*torrentium* MARTINI, 1925			x	1
		Culex	*pipiens/torrentium*			x	2
		Culex LINNAEUS, 1758	spec.			x	1
		Unidentified		x			2
	Mycetophilidae	Unidentified				x	1
	Sciaridae	Unidentified				x	2
Hymenoptera	Apidae	*Apis*	*mellifera* LINNEAUS, 1758			x	1
		Bombus	*lapidarius* LINNAEUS, 1758		x		1

Order	(Super)Family	Genus	Species	Bayreuth	Pettstadt	Trap Bayreuth	Abundance
	Colletidae	*Hylaeus*	*communis* NYLANDER, 1852	x			1
	Halictidae	*Lasioglossum*	*laticeps* (SCHENK, 1868)	x			1
		Lasioglossum CURTIS, 1833	spec.	x			2
	Chalcidoidea	Unidentified				x	1
	Ichneumonidae	Unidentified				x	1
	Vespidae	*Ancistrocerus*	spec.	x			1
	Eumeninae	Unidentified				x	1
Lepidoptera	Geometridae	*Idaea*	c.f. *humiliata* (HUFNAGEL, 1767)		x		1
		Idaea	c.f. *dimidiata* (HUFNAGEL, 1767)			x	1
		Scopula	*rubiginata* (HUFNAGEL 1767)		x		1
	Noctuidae	*Autographa*	*gamma* (LINNEAUS, 1758)			x	1
		Hoplodrina	*octogenaria* GOEZE, 1781		x		1
	Gelechoidea	Unidentified			x		2
	Pyralidae	*Crambus*	*lathionellus (*ZINCKEN, 1817)		x		1
		Endotricha	*flammealis* (DENIS and SCHIFFER-MÜLLER, 1775)			x	1
		Microstega	*pandalis* (HÜBNER, 1825)		x		1
		Pleuroptya	*ruralis* (SCOPOLI, 1763)			x	1
Neuroptera	Chrysopidae	Unidentified				x	1

Table 2: Mean (median) percentage and total amount of scent compounds emitted by 4 *S. otites* individuals over a day. The median value for a specific compound was built from 4 values, if the compound was present in all 4 individuals, and from 3 or 2 values, if the compound was resent in 3 or 2 individuals respectively. For compounds, which occurred only in 1 individual, the percentage amount found in that individual is presented. The mean value of two hours is presented here. N: number of individuals where a specific compound occurred; tr: the percentage amount was less than 0.5.

Time	**N**	**9am/ 10am**	**11am/ 12pm**	**1pm/ 2pm**	**3pm/ 4pm**	**5pm/ 6pm**	**7pm/ 8pm**	**9pm/ 10pm**	**11pm/ 12am**	**1am/ 2am**	**3am/ 4am**	**5am/ 6am**	**7am/ 8am**
ng/h*flower		8	11	38	61	81	77	124	56	30	16	10	21
Monoterpenoids													
α-Pinene	**4**	—	—	—	—	—	—	tr	tr	3	6	**12**	6
β-Pinene	**4**	—	—	—	—	—	—	tr	tr	1	3	7	5
δ-3-Carene	**2**	1	tr	tr	tr	—	tr	tr	1	3	7	**13**	8
Unknown furanoid	**2**	—	—	tr	tr	tr	tr	tr	tr	—	—	—	—
(E)-β-Ocimene	**4**	**11**	6	4	2	2	tr	—	tr	—	—	—	tr
Linalool oxide (furanoid)	**4**	tr	2	2	2	3	3	1	tr	tr	tr	—	tr
Linalool	**4**	2	7	**20**	**18**	**20**	**16**	4	1	1	tr	—	tr
Hotrienol	**4**	**24**	**17**	9	8	**11**	**10**	5	3	5	9	7	**13**
Unknown Pyranoid	**4**	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	—	tr
Myrtenol	**3**	1	tr	tr	tr	tr	tr	—	—	—	—	—	—
Lilac aldehyde	**4**	—	—	—	—	—	9	**16**	**13**	**11**	5	3	2
Linalool oxide (pyranoid)	**4**	6	8	7	7	8	8	4	4	4	4	5	4
Lilac alcohol	**4**	—	—	—	—	—	tr	2	1	1	tr	tr	tr
2,6-Dimethyl-1,7-octadiene-3,6-diol	**4**	4	tr	1	3	2	3	1	tr	tr	tr	tr	1
Benzenoids													
Benzaldehyde	**4**	7	9	7	7	8	9	9	**13**	9	9	6	8

Time	**N**	**9am/ 10am**	**11am/ 12pm**	**1pm/ 2pm**	**3pm/ 4pm**	**5pm/ 6pm**	**7pm/ 8pm**	**9pm/ 10pm**	**11pm/ 12am**	**1am/ 2am**	**3am/ 4am**	**5am/ 6am**	**7am/ 8am**
Benzyl alcohol	**4**	**12**	**14**	**10**	5	8	7	2	tr	tr	tr	2	2
Phenyl acetaldehyde	**4**	—	—	—	—	—	8	**34**	**28**	**17**	7	3	tr
Acetophenone	**4**	6	8	2	tr	1	1	1	1	1	2	3	tr
2-Phenylethanol	**4**	4	2	1	5	4	8	**13**	**20**	**20**	**20**	**12**	**31**
Benzyl acetate	**1**	—	—	tr	tr	tr	tr	tr	—	—	—	—	—
Methyl salicylate	**3**	tr	3	5	8	6	5	2	tr	tr	tr	1	1
N-containing compounds													
3-Methyl-butyl-aldoxime	1	—	—	—	—	tr	tr	tr	tr	tr	—	—	—

Figure legends:

Fig. 1

Insect flower visitors of *Silene otites* in the course of a day from 8pm to 12am. No observations were done between 1am and 7am.

Fig. 2

Emission pattern of the most abundant floral scent compounds of *Silene otites*. The median and quartile values for a specific compound were built from 4 values, if the compound was present in all 4 individuals, but for 2 values in case of δ-3-Carene, as this compound only occurred in 2 of the 4 individuals studied.

Fig. 1

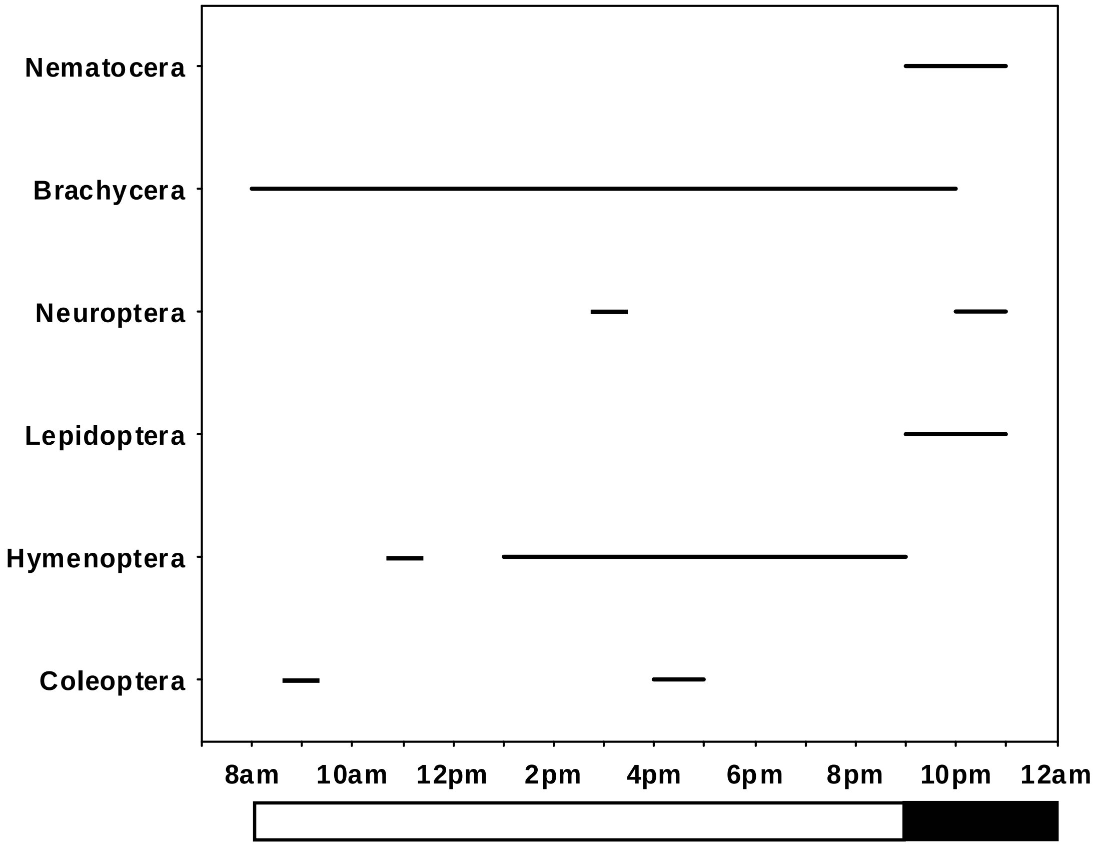
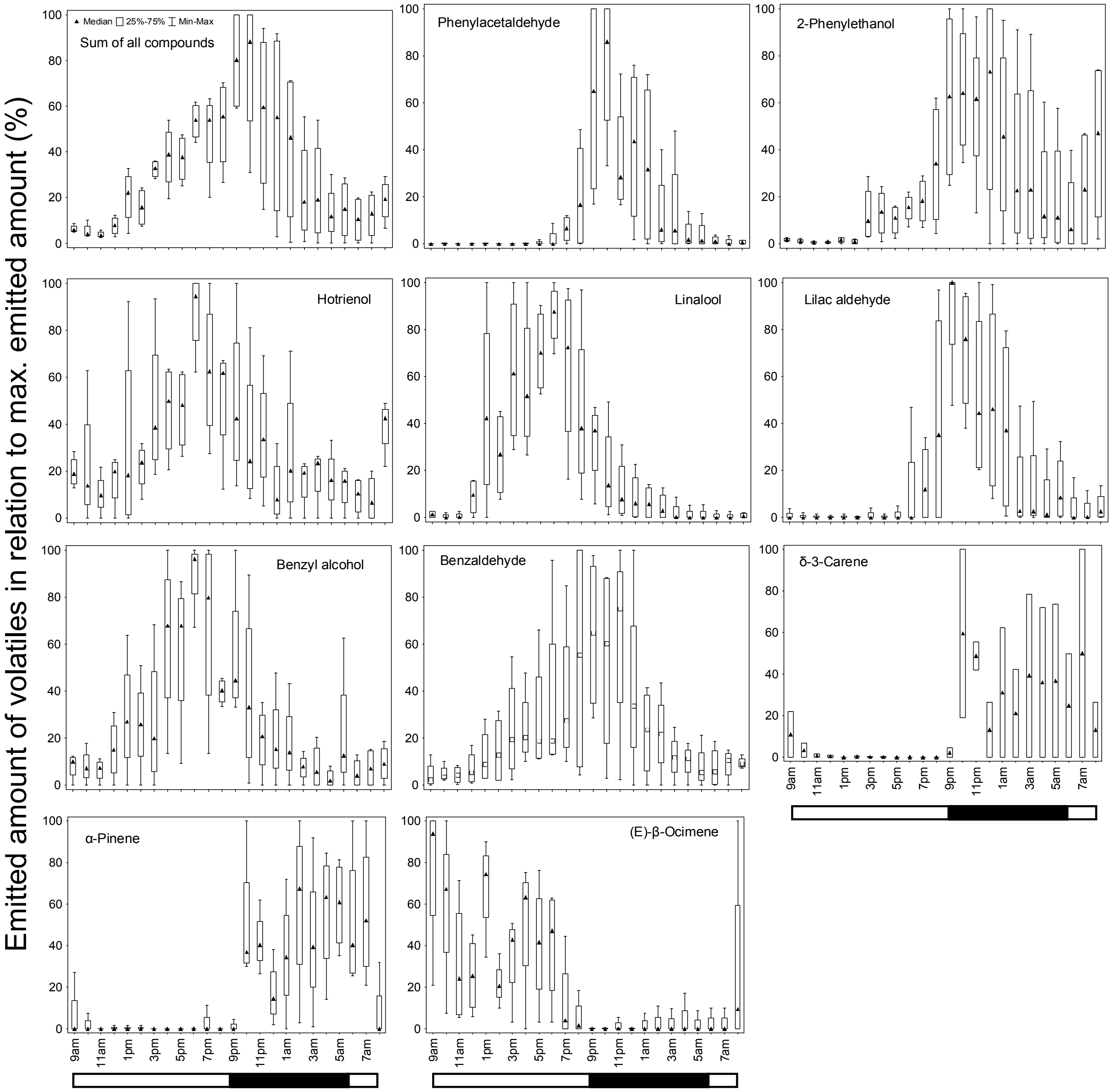

Nematocera
Brachycera
Neuroptera
Lepidoptera
Hymenoptera
Coleoptera
8am
10am
12pm
2pm
4pm
6pm
8pm
10pm
12am

Fig. 2


Emitted amount of volatiles in relation to max. emitted amount (%)
▲ Median ▭ 25%-75% ⌶ Min-Max
Sum of all compounds
Phenylacetaldehyde
2-Phenylethanol
Hotrienol
Linalool
Lilac aldehyde
Benzyl alcohol
Benzaldehyde
δ-3-Carene
α-Pinene
(E)-β-Ocimene
0
20
40
60
80
100
9am
11am
1pm
3pm
5pm
7pm
9pm
11pm
1am
3am
5am
7am

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ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst und dabei keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Ferner erkläre ich, dass ich diese Arbeit weder einer anderen Prüfungsbehörde vorgelegt noch anderweitig mit oder ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen.

Bayreuth, den September 2007

Umma Salma Jhumur