Importance of floral scent compounds for the interaction between *Silene latifolia* (Caryophyllaceae) and the nursery pollinator *Hadena bicruris* (Lepidoptera: Noctuidae)

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1. General Introduction

Worldwide, at least thirteen nursery pollination systems, in which pollinators reproduce within the flowers they pollinate are known (nursery pollination, Dufay and Anstett, 2003). The most popular interactions are the insect-plant relationships between yucca and yucca moths (Riley, 1892), and fig and fig wasps (Anstett et al., 1997; Wiebes, 1979). In all these systems there is a conflict between the partners, because increasing fitness of one partner is often correlated with decreasing fitness of the other partner (Dufay and Anstett, 2003). Therefore, these insect-plant relationships are very interesting from an evolutionary point of view. A system, that has been studied as early as the 70ies (Brantjes, 1976b, c) and has received also recent attention (Bopp, 2003) is the interaction between the noctuid moth *Hadena bicruris* Hufn. and the nocturnal Caryophyllaceae *Silene latifolia* Poir. ssp. *alba* (Mill.) Greut. & Burdet. *Hadena bicruris* is one out of 143 described *Hadena* species. In general, larvae of this genus feed on plants of the family Caryophyllaceae, especially on their seeds. At least for central European species it is known that the moths are very specific in the choice of food plants (Steiner and Ebert, 1998). *Hadena bicruris*, which is distributed in Europe and Northern Africa (Hacker, 1996), deposits its eggs only in few *Silene* species, and in *Saponaria officinalis* L. However, the main host plant of this noctuid is *S. latifolia* (reviewed in Bopp and Gottsberger, 2004; Steiner and Ebert, 1998). *Silene latifolia* is a perennial dioecious weed, growing often along waysides. It is naturally distributed in Northern Africa, Europe, and Asia (Seybold, 1990). About 200 years ago, it was accidentally introduced to North America followed by rapid expansion throughout the whole country (McNeill, 1977). Therefore, *H. bicruris* and *S. latifolia* have overlapping, but not identical distribution areas. *Silene latifolia* is night-flowering and emits a strong scent in the night (Jürgens et al., 2002a) to attract pollinators (Brantjes, 1976b, c, d). The most important pollinators, or at

least flower visitors are various nocturnal Lepidoptera species, among them *H. bicruris* (Altizer et al., 1998; Ellis and Ellis-Adam, 1993; Jürgens et al., 1996; Young, 2002). Larvae of *Hadena bicruris* can be found in about 90% of European populations of *S. latifolia*, and they have dramatic effects on the fitness of the plant, because they destroy about 25% of fruits/seeds produced (Wolfe, 2002). The relationship between *H. bicruris* and *S. latifolia* can be seen more as parasitism than as mutualism, because *S. latifolia* exhibits a low degree of specialisation for nursery pollination. For instance, *S. latifolia* offers considerable amounts of nectar to generalist co-pollinators, and it lacks mechanisms regulating larval development to control the numbers of *H. bicruris* (Dufay and Anstett, 2003). On the other hand, *H. bicruris* is highly specialised and the adaptation to *S. latifolia* is quite good:

(1) the phenology of the moths overlapps to a large extent with the flowering period of *S. latifolia* (Bopp and Gottsberger, 2004), (2) the moths are characterised by a long proboscis compared to other *Hadena* and noctuid species and can therefore reach the nectar at the calyx base of the long-tubed *S. latifolia* flowers (Jürgens et al., 1996), (3) female moths are able to distinguish between female and male flowers, and lay their eggs, mostly one per flower, only in female flowers (Bopp, 2003; Brantjes, 1976a, b, c), (4) female moths drink nectar before each oviposition; the flowers are thereby effectively pollinated, and the larvae feed on the growing seeds (Brantjes, 1976b, c), (5) in comparison to other *Hadena* species, female moths have a long ovipositor (Hacker, 1996), which enables them to attach the eggs within the long-tubed flowers of *S. latifolia* to the ovary (Brantjes, 1976b), (6) seeds of *S. latifolia* are more profitable for nutrition of *H. bicruris* larvae than those of other Caryophyllaceae species (Bopp and Gottsberger, 2004; P. Oberpaul, personal communication).

Since Brantjes (1976b, c) it is known that the night-active *Hadena bicruris* is attracted to *S. latifolia* by the floral scent. Floral scents are often

complex mixtures of small (C5-C20), volatile organic compounds, belonging to several chemical classes, especially fatty acid derivatives, benzenoids, phenylpropanoids, isoprenoids, nitrogen- and sulphur-bearing compounds (Dudareva et al., 1999; Knudsen et al., 1993). They can be emitted from nearly any floral tissue (see Raguso, 2001), and different biosynthetic pathways are known for the production of volatiles. Fatty acid derivatives are synthesised via the Lipoxygenase pathway, aromatics via the Shikimate pathway, and isoprenoids via the Rhomer (monoterpenoids) and the Mevalonate (sesquiterpenoids) pathway (Dudareva et al., 1999; Raguso, 2001). Floral scents are typical secondary plant metabolites, indicating that they are, in contrast to the primary plant products, not essential for plant growth and development (Schoonhoven, 1972). Floral scents are important signals for chemical communication between flowering plants and animal pollinators, and are thought to be ancient attractants. It is assumed that they have originally evolved to deter insects feeding on reproductive parts of the plants (Pellmyr and Thien, 1986). However, if the insects accidentally pollinated the flowers, and the benefit of this process outweighed the disadvantages of their feeding activity, selection would have modified production and effect of repellants, thus changing their function from repellance to attraction, resulting in an increase of insect attraction optimal to the plants (Pellmyr and Thien, 1986). Floral scents can be used by pollinators for distance orientation, approach, landing, feeding, and associative learning (see reviews by Dobson, 1994; Raguso, 2001). However, little is known about how insects respond to individual components found in floral scents, and detailed knowledge of composition of floral scents coupled with behavioural assays on pollinators are needed (Dudareva and Pichersky, 2000; Pichersky and Gershenzon, 2002).

Jürgens et al. (2002a) studied the floral scent emitted by *S. latifolia*, and found monoterpenoids and benzenoids dominating the scent. Characteristic compounds were e.g. lilac aldehyde isomers, benzyl

acetate, and veratrole. However, the importance of single substances for the attraction of *H. bicruris* is still unknown. Only two plant specimens were analysed in the latter (Jürgens et al., 2002a) study, and nothing is known about the variability in the scent of *S. latifolia*. In general, little data about the variability of floral scents are available (see Knudsen, 2002). Nevertheless, such data are necessary to understand the dynamics of plant-pollinator adaptations (Knudsen et al., 1993).

The present work comprises four main chapters. (1) The geographic variability in flower scent of *S. latifolia* was determined. To this aim, the scent emitted from single flowers of 98 *S. latifolia* specimens from 15 European and 19 North American populations was analysed using a highly sensitive method for headspace analyses. Of special interest was the determination of variability within and between populations as well as within and between European and North American populations. The variability in floral scent was calculated using the dissimilarity index CNESS, and visualised using nonmetric multidimensional scaling (NMDS).
(2) Antennal (GC/EAD) and behavioural (wind tunnel) responses of *Hadena bicruris* to different headspace extracts respectively to scent of single flowers of specimens that originated from seeds of different populations of *S. latifolia* were recorded. The question arose whether the moths can electrophysiologically distinguish between flower scent of different populations, and whether *H. bicruris* is equally effectively attracted to flowers originating from different populations. Furthermore, electrophysiological and behavioural responses of *H. bicruris* to an extract respectively to a single flower of *S. vulgaris* (Moench) Garcke were studied, and compared with results obtained from *S. latifolia*. *Silene vulgaris* is emitting similar flower scent compounds as *S. latifolia* (Jürgens et al., 2002a), but is only rarely used by *H. bicruris* for oviposition.
(3) Antennal and behavioural responses of *H. bicruris* to single floral scent compounds, especially of *S. latifolia*, were analysed. The aim was to unveil the importance of single compounds of the complex floral scent of

S. latifolia for attraction of *H. bicruris*. In a first step a list of potential compounds was evaluated from the complex natural scent blend by using GC-FID/EAD or GC-MS/EAD methods. Further, neurologically active compounds were tested in wind tunnel bioassays.

(4) The spatial pattern of floral scent differences in female and male flowers of *S. latifolia* was investigated. Females of *H. bicruris* lay their eggs only in female flowers of *S. latifolia*, and volatiles may be cues for discrimination between female and male flowers. To determine the parts of the flowers responsible for scent emission, volatiles from attached intact flowers were collected, and then single flower parts were progressively removed. After each preparation step, volatiles were collected from the remaining "flower".

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2. Geographic variation of flower scent in *Silene latifolia*

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Abstract

The variability of the floral scent of 98 specimens of nocturnal *Silene latifolia* belonging to 15 European and 19 North American populations was determined. The floral scent was collected using dynamic headspace methods, and analysed by Micro-SPE and GC-MS methods. The variability in the floral scent was very high and we found different chemotypes characterised by specific scent compounds. The variation within populations was significantly lower than variation between populations. The differences in floral scent composition between European and North American populations are small. A tentatively negative correlation between the floral scent and the distance matrix in Europe indicates that closely located populations have quite different scent profiles comparing to far distant populations. In North America the spatial distribution of the populations has no effect on the fragrance profiles. Typical compounds were isoprenoids like lilac aldehyde isomers, or E-ocimene, and benzenoids like benzaldehyde, phenylacetaldehyde, or veratrole. Some of these compounds are known to attract nocturnal Lepidoptera species. The high variability is discussed in relation to

pollination biology of *S. latifolia*, and the results are compared with other studies investigating intraspecific variability of flower scent.

Key words: Caryophyllaceae, CNESS, flower scent, GC-MS, intraspecific variation, Micro-SPE, *Silene latifolia*, White campion.

Introduction

Floral odours are important signals for chemical communication between flowering plants and animal pollinators (Pellmyr and Thien, 1986), and may be of importance for reproductive isolation among sympatric, closely related species (e.g. Knudsen, 1999; Levin et al., 2001). Scent is particularly important in night-blooming species when visual cues become inefficient due to darkness (Jürgens et al., 2002a; Knudsen and Tollsten, 1993; Miyake et al., 1998; Raguso et al., 2003; Raguso and Pichersky, 1995). *Silene latifolia* Poir. ssp. *alba* (Mill.) Greut. & Burdet (Caryophyllaceae) is a dioecious European native perennial and produces flowers that open at dusk and close soon after dawn. Typical floral scent compounds of *S. latifolia* are lilac aldehyde isomers, veratrole, and benzyl acetate (Jürgens et al., 2002a). At least the lilac compounds and benzyl acetate are known to attract noctuid moths alone, or together with other compounds, and are known as effective antennal stimulants in electroantennographic detections (Meagher, 2002; Plepys et al., 2002a; Raguso and Light, 1998; Raguso et al., 1996).

Several attributes of this plant species make it an interesting subject for the study of floral scent. The dominant flower visitors in Europe are nocturnal Lepidoptera (Altizer et al., 1998; Ellis and Ellis-Adam, 1993; Jürgens et al., 1996), and at least one moth species is specialised on *S. latifolia*. *Silene latifolia* is the most important host plant for the noctuid moth *Hadena bicruris* Hufn. (Bopp, 2003; Brantjes, 1976b, c). The female moths lay their eggs in the female flowers of *S. latifolia*, thereby pollinate

the flowers, and the larvae feed on the developing seeds (Bopp, 2003; Brantjes, 1976b, c). This animal-plant interaction is one of the 13 known nursery pollination systems, where pollinators reproduce within the inflorescence, respectively flower, they pollinate (Dufay and Anstett, 2003). *Hadena bicruris* has dramatic effects on the fitness of its host plant in Europe and is responsible for the destruction of about 25% of all *S. latifolia* fruits produced (Wolfe, 2002). *H. bicruris* is attracted to *S. latifolia* by its flower scent, which is emitted at night (Brantjes, 1976b, c).

Yet, *S. latifolia* and its specialised pollinator and seed predator have very different global geographic distributions. The plant was accidentally introduced to North America (McNeill, 1977) about 200 years ago and has subsequently spread throughout most of the continent. *Hadena bicruris*, however, did not accompany its host plant and is still only found in Europe and Northern Africa (Hacker, 1996). In the introduced range the most important pollinators of *S. latifolia* are (similar to its original range) several noctuids, geometrids, and sphingids (Altizer et al., 1998; Young, 2002), but we find different taxa in Europe and North America. Given that floral scent patterns within and among species are likely the result of natural selection by different pollinators, the question remains to be addressed whether there has been a shift in odour compounds in *S. latifolia* in the introduced range. In other words, has there been evolutionary change in these characters? It is of interest to point out that there has been genetically based change in other characters in this species since its introduction. For the most part, North American individuals of *S. latifolia* germinate faster, display greater growth rates, and have higher fecundities than do the Europeans (L. Wolfe, personal communication).

The main purpose of this study was to determine if *S. latifolia* odour profiles have differentiated between Europe and North America. An additional goal was to determine patterns of variation within and among populations in the two continents. In most of the floral odour studies in literature, typically very few specimens are sampled, and there are only few studies analysing intraspecific variation of floral odours (summarised

in Knudsen, 2002). For example, floral scent composition of *S. latifolia* has been determined only in two specimens (Jürgens et al., 2002a). In most of the intraspecific studies the variability of floral scent of several specimens of different populations was high (Azuma et al., 2001; Galen and Kevan, 1980; Moya and Ackerman, 1993; Tollsten and Bergström, 1993; Tollsten and Ovstedal, 1994; Whitten and Williams, 1992). Here, results of headspace volatile collections from single flowers of 98 *S. latifolia* specimens from 15 European and 19 North American populations are presented. The prediction was that variation within populations would be less than between populations, and that North American specimens differ from European specimens due to their isolation and due to their different pollinator spectrum.

Material and methods

Plant material

Floral scent samples of *Silene latifolia* were collected from 41 European and 57 North American specimens belonging to 15 European and 19 North American populations. The localities of the different populations are shown in Figure 1 and Figure 2.

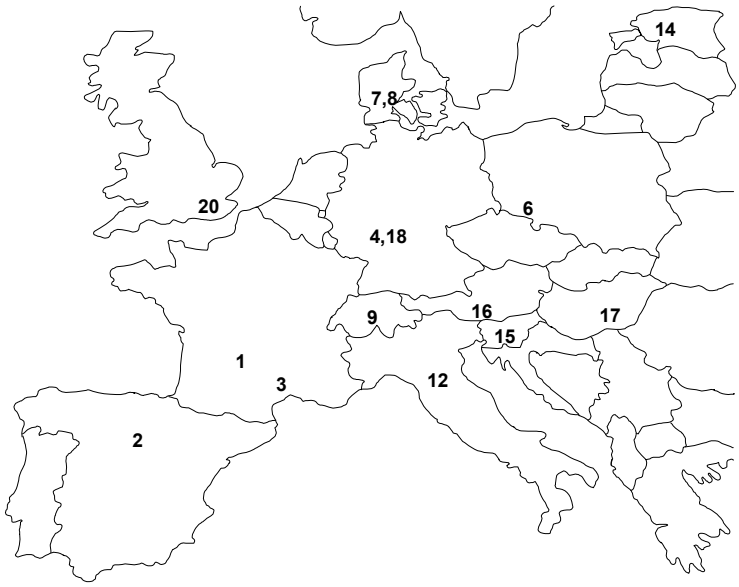

Figure 1: Geographic origin of the 15 analysed European populations. One specimen of population 12, two specimens of populations 8, 16, 17, 18, 20, three specimens of populations 1, 3, 4, 9, 14, 15, and four specimens of populations 2, 6, 7 were studied.

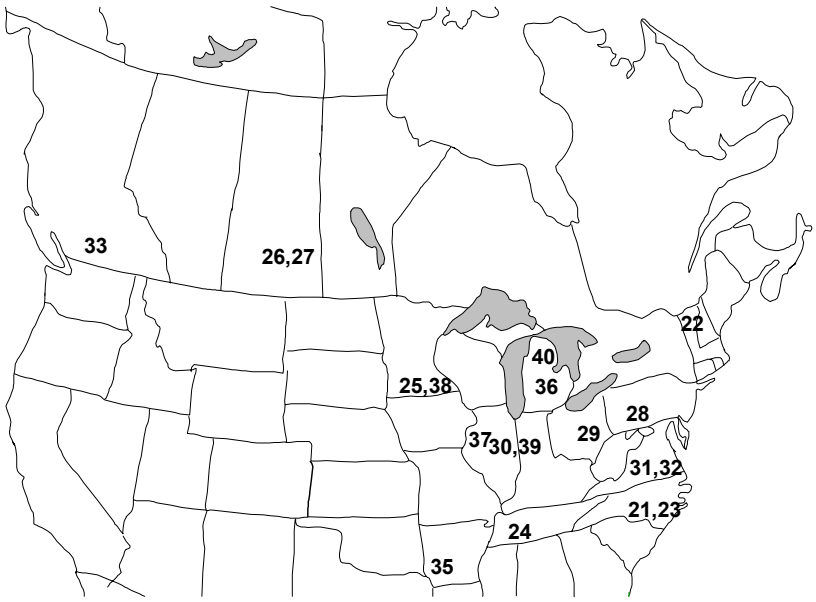

Figure 2: Geographic origin of the 19 analysed North American populations. One specimen of populations 23, 30, 32, 36, two specimens of populations 25, 29, 33, 38, three specimens of populations 26, 35, four specimens of populations 21, 28, 31, 37, 39, 40, five specimens of populations 24, 27, and 6 specimens of population 22 were studied.

The plants were grown in the greenhouse for about two months until they built up a rosette, and the pots were then placed in flowerbeds in the field. Vouchers of all studied populations are housed in the herbarium of the University of Bayreuth (UBT).

Volatile collection

Floral scent was collected using dynamic headspace methods. A single, newly opened flower was enclosed within a polyethylene oven bag (Toppits®) and the emitted volatiles were trapped in an adsorbent tube through the use of a membrane pump (ASF Thomas, Inc.). The flow rate was adjusted to 200ml/min using a power supply and a flow meter. Samples were collected for 2min in the night, when *S. latifolia* is emitting most of its floral volatiles (S. Dötterl, unpublished data). ChromatoProbe quartz microvials of Varian Inc. (length: 15mm; inner diameter: 2mm) were cut at the closed end, filled with a mixture (1:1) of 3mg Tenax-TA (mesh 60-80) and Carbotrap (mesh 20-40), and used as adsorbent tubes. The adsorbents were fixed in the tubes using glass wool. Simultaneous collections of the surrounding air were used to distinguish between floral compounds and ambient contaminants.

Chemical analysis

The samples were analysed on a Varian Saturn 2000 System using a 1079 injector that had been fitted with the ChromatoProbe kit. This kit allows the thermal desorption of small amounts of solids or liquids contained in quartz microvials (Micro-SPE, Amirav and Dagan, 1997; Wilkinson and Ladd, Varian Application note), or in the present case the thermal desorption of the trapped volatiles. The adsorbent tube was loaded into the probe, which was then inserted into the modified GC injector.

The injector split vent was opened (1/20) and the injector heated to 40°C to flush any air from the system. The split vent was closed after 2 minutes

and the injector was heated at 200°C/min, then held at 200°C for 4.2min, after which the split vent was opened (1/10) and the injector cooled down. A ZB-5 column (5% phenyl polysiloxane) was used for the analyses (60m long, inner diameter 0.25mm, film thickness 0.25µm, Phenomenex). Electronic flow control was used to maintain a constant helium carrier gas flow of 1.8ml min^{-1}. The GC oven temperature was held for 7min at 40°C, then increased by 6°C per min to 250°C and held for 1min. The MS interface was 260°C and the ion trap worked at 175°C. The mass spectra were taken at 70eV (in EI mode) with a scanning speed of 1 scan s^{-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; Davies, 1990). Identification of individual components was confirmed by comparison of both mass spectrum and GC retention data with those of authentic standards. Relative quantities (percentage) were used for further analyses, because the total amount of emitted volatiles varied greatly during the year, depending on season (S. Dötterl, unpublished data).

Statistical analyses

The CNESS (chord-normalised expected species shared) distance index, ranging between 0 and the square root of 2, was used to determine the differences between the single samples (Trueblood et al., 1994). CNESS is a metric version of Grassle and Smith´s (1976) NESS similarity index, which was originally built to compare faunal samples. NESS and CNESS can be regarded as more generalised forms of the Morisita index (Morisita, 1959). Wolda (1981) investigated several quantitative similarity indices and found that all but one, the last called Morisita index, were strongly influenced by sample size and diversity. The only disadvantage of the Morisita index is the high sensitivity to changes in the abundance of the most abundant species (Wolda, 1981), or in the actual case the most abundant compounds. However, this “problem” can be solved by using the

similar indices NESS or CNESS, which can be adjusted, by altering the sample size NESS-m, to emphasise the importance of rare species (or minor compounds respectively) in the data (Wolda, 1983). It was shown in a recent study that this method is very useful for analysis of floral (anther) scent data (Jürgens and Dötterl, accepted). The CNESS indices were calculated using the updated version of the COMPAH (Combinatorial Polythetic Agglomerative Hierarchical Clustering) program (Boesch, 1977), provided by Gallagher at UMASS/Boston (http://www.es.umb.edu/edgwebp.htm).

The significance of the correlation between the distance matrix (km) of European respectively North American populations and the CNESSm1-distance matrices was tested using the Mantel nonparametric test (Mantel, 1967) calculator (10,000 permutations) written by Liedloff (1999). Mean relative amounts of compounds for populations with more than one sample were used before determining the CNESS matrices for European and North American samples separately.

Nonmetric multidimensional scaling (NMDS) in the STATISTICA package (StatSoft Inc., 2003) was applied to detect meaningful underlying dimensions and to visualise similarities between samples (see Borg and Lingoes, 1987). 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 distance matrix to the observed distance matrix (Clarke, 1993).

A variance component analysis was used in the STATISTICA package (StatSoft Inc., 2003) to estimate the contribution of single compounds to the obtained total variation between all populations and between European and North American samples.

The Mann-Whitney U-Test was carried out to compare the variation in the chemical profiles within populations to between populations. Therefore, the pair-wise dissimilarities (CNESS) between individual samples within populations were compared to those between samples among populations. This test was also served to compare the mean relative

abundances of the compounds most responsible for the variation between European and North American samples. The p-values of the latter tests were Bonferroni corrected (sequential Bonferroni procedure: Hochberg, 1988) to assess the significance of differences.

Kruskal-Wallis-ANOVA was used to compare the pair-wise dissimilarities within European and North American populations, and between European and North American populations. The Tukey-Kramer test for non-parametric data was used as post-hoc test (Siegel and Castellan, 1988).

Results and discussion

The chemical composition of the flower scent of *Silene latifolia,* found in 15 European, and 19 North American populations, is summarised in Table 1. The compounds listed in Table 1 are ordered in classes, which to some degree reflect their biosynthetic origin (see Knudsen et al., 1993). Altogether, 57 compounds were detected and 52 volatiles could be identified. Most of these substances were also found in the specimens of *S. latifolia* studied by Jürgens et al. (2002a), and the results of the two studies are generally consistent. Dominant compound classes were isoprenoids, and benzenoids. Additionally, fatty acid derivatives, phenylpropanoids and nitrogen-containing compounds were found. The most commonly occurring compounds found in all samples were lilac aldehyde isomers and benzaldehyde. Commonly occurring compounds were also the fatty acid derivative *cis*-3-hexenyl acetate, the benzenoids phenyl acetaldehyde and methyl salicylate, and the isoprenoid *trans*-β-ocimene. Most of these compounds are again and again found in species pollinated by noctuids or hawkmoths (Jürgens et al., 2002a; Kaiser, 1993; Knudsen and Tollsten, 1993; Miyake et al., 1998), and at least for some of these components it is shown that they attract potential *S. latifolia* pollinators, especially nocturnal Lepidoptera species (for details see Chapters 3 and 4; Bruce and Cork, 2001; Fraser et al., 2003; Haynes et al., 1991; Heath et al., 1992; Meagher, 2001, 2002; Plepys et al., 2002b).

Plepys et al. (2002b) e.g. tested authentic samples of methyl salicylate and lilac aldehydes on the generalistic noctuid moth *Autographa gamma* L. and found both substances to be attractive. Moreover, responses of *Autographa gamma* to a mixture of lilac aldehyde isomers were similar as to a mixture of in total 9 attractive compounds (including lilac aldehydes), and the lilac aldehydes elicited significantly more responses than any other compound tested (Plepys et al., 2002b). *Autographa gamma* can often be found drinking nectar on *S. latifolia* (Jürgens et al., 1996; S. Dötterl, personal observation) and may be attracted to the flowers by the lilac aldehydes. The lilac aldehydes were also proved to be the most attractive compounds for *H. bicruris*, the nursery pollinator of *S. latifolia* (for more details see Chapter 4).

Table 1: Occurrence and relative abundance of compounds found in 98 flower scent samples of 15 European populations, and 19 North American (NA) 19 populations.

		Occurrence		Relative abundance (%)					
				Europe			NA		
Compound[a]	R_I[b]	Europe	NA	Median	Quartile	Min-Max	Median	Quartile	Min-Max
		Number of populations (samples)							
Fatty acid derivatives									
cis-3-Hexenol	860	14 (32)	19 (48)	0.45	0.04-0.70	0-10.97	0.31	0.10-1.29	0-6.32
cis-3-Hexenyl acetate*[c]	1016	15 (38)	19 (57)	2.66	1.07-5.24	0-26.02	3.70	1.72-6.48	0.19-24.52
2-Hexenol acetate	1025	13 (21)	18 (41)	0	0-0.34	0-1.60	0.12	0-0.36	0-4.44
4-Oxoisophorone	1159	-	1 (2)	-	-	-	0	0-0	0-0.76
Benzenoids									
Benzaldehyde*	982	5 (41	19 (57)	1.33	0.62-2.45	0.14-74.28	2.04	1.04-4.36	0.17-64.41
Benzyl alcohol*	1050	14 (37)	19 (56)	0.32	0.15-1.00	0-5.45	0.59	0.28-1.17	0-7.56
Phenyl acetaldehyde*	1060	15 (38)	19 (54)	3.36	0.52-8.57	0-47.24	2.16	0.68-23.66	0-83.13
2-Methoxy phenol*	1102	14 (34)	19 (54)	0.75	0.07-1.79	0-10.02	0.91	0.35-2.09	0-9.96
Methyl benzoate	1107	7 (13)	15 (25)	0	0-0.10	0-5.46	0	0-0.50	0-28.69
2-Phenylethanol	1129	14 (30)	18 (46)	0.05	0-0.40	0-9.07	0.12	0.03-0.58	0-3.80
Veratrole*	1153	12 (22)	10 (16)	0.06	0-14.41	0-38.61	0.00	0-0.04	0-23.22
Benzyl acetate*	1174	5 (9)	8 (15)	0	0-0	0-39.94	0	0-0.03	0-23.22
Methyl salicylate	1208	15 (35)	18 (52)	1.16	0.25-2.48	0-14.64	1.04	0.32-2.94	0-14.91

Table 1 continued

Compound	R_I	Occurrence		Relative abundance (%)					
		Europe	NA	Europe			NA		
		Number of populations (samples)		Median	Quartile	Min-Max	Median	Quartile	Min-Max
2-Methyl benzaldehyde	1217	1 (3)	-	0	0-0	0-0.40	-	-	-
Benzyl isobutanoate	1306	-	1 (1)	-	-	-	0	0-0	0-0.04
Dimethyl salicylate	1347	3 (3)	3 (3)	0	0-0	0-0.04	0	0-0	0-0.50
Benzyl benzoate*	1789	15 (39)	19 (56)	0.47	0.09-1.27	0-13.03	0.76	0.13-1.56	0-11.82
Phenylpropanoids									
Benzenepropanal	1178	2 (3)	7 (8)	0	0-0	0-5.02	0	0-0	0-1.70
Benzenepropanol	1243	7 (12)	11 (18)	0	0-0.03	0-4.53	0	0-0.30	0-13.87
trans-Cinnamaldehyde	1287	12 (23)	16 (41)	0.03	0-0.25	0-2.05	0.13	0-0.73	0-7.04
trans-Cinnamyl alcohol	1319	10 (17)	15 (34)	0	0-1.07	0-8.41	0.26	0-1.98	0-20.90
Benzenepropyl acetate	1380	1 (1)	4 (5)	0	0-0	0-0.67	0	0-0	0-0.56
Methyleugenol	1408	-	1 (1)	-	-	-	0	0-0	0-0.09
Cinnamyl alcohol acetate	1454	2 (3)	7 (8)	0	0-0	0-0.23	0	0-0	0-0.22
trans-Isoeugenol*	1463	-	1 (1)	-	-	-	0	0-0	0-0.05
Nitrogen-bearing compounds									
3-Methyl-butyl aldoxime*	858	6 (9)	12 (21)	0	0-0	0-3.84	0	0-0.54	0-5.17
3-Methyl-butyl aldoxime*	870	6 (8)	11 (17)	0	0-0	0-2.30	0	0-0.14	0-3.23
Indole*	1307	3 (5)	7 (15)	0	0-0	0-6.09	0	0-0.01	0-3.36

Table 1 continued

Compound	R_I	Occurrence		Relative abundance (%)					
		Europe	NA	Europe			NA		
		Number of populations (samples)		Median	Quartile	Min-Max	Median	Quartile	Min-Max
Monoterpenoids									
α-Pinene*	957	15 (39)	18 (51)	0.22	0.11-1.30	0-6.41	0.35	0.10-0.74	0-5.90
β-Pinene*	995	15 (35)	17 (45)	0.19	0.07-0.69	0-5.94	0.25	0.03-0.57	0-5.69
B-Myrcene*	1001	-	1 (3)	-	-	-	0	0-0	0-6.09
trans-β-Ocimene*	1058	11 (27)	6 (39)	1.91	0-6.94	0-25.84	3.24	0-8.90	0-22.68
Lilac aldehyde A*	1154	15 (41)	19 (57)	18.28	12.41-22.37	2.22-29.71	14.19	10.20-19.04	0.18-31.88
Lilac aldehyde B+C*	1163	15 (41)	19 (57)	28.41	18.61-33.56	3.88-41.22	22.45	15.68-28.75	0.23-40.33
Lilac aldehyde D*	1178	15 (41)	19 (56)	5.19	4.51-6.77	1.12-11.36	5.56	3.19-7.28	0-10.20
Lilac alcohol A*	1211	15 (40)	19 (54)	0.24	0.09-0.57	0-5.01	0.15	0.09-0.26	0-2.87
Lilac alcohol B+C*	1219	14 (37)	19 (52)	0.2	0.12-0.32	0-1.29	0.22	0.12-0.39	0-1.66
Lilac alcohol D*	1232	14 (37)	19 (51)	0.12	0.04-0.23	0-1.04	0.11	0.06-0.20	0-3.59
Lilac degradation	1341	15 (40)	18 (48)	0.21	0.15-0.26	0-0.39	0.18	0.10-0.26	0-0.41
Lilac degradation	1352	15 (41)	19 (57)	6.23	4.31-7.66	0.82-10.21	5.13	3.48-6.54	0.09-9.26
Lilac alcohol formate	1360	4 (5)	2 (2)	0	0-0	0-0.15	0	0-0	0-0.13
Sesquiterpenoids									
α-Longipinene	1377	2 (2)	3 (4)	0	0-0	0-0.30	0	0-0	0-0.16
Longicyclene	1401	2 (2)	3 (4)	0	0-0	0-0.17	0	0-0	0-0.15
α-*cis*-Bergamotene	1430	-	1 (1)	-	-	-	0	0-0	0-0.34

Table 1 continued

Compound	R_I	Occurrence		Relative abundance (%)					
		Europe	NA	Europe			NA		
		Number of populations (samples)		Median	Quartile	Min-Max	Median	Quartile	Min-Max
ST	1441	12 (18)	14 (31)	0	0-0.02	0-0.23	0.01	0-0.06	0-0.35
trans-β-Caryophyllene*	1450	5 (5)	4 (7)	0	0-0	0-0.36	0	0-0	0-0.30
α-*trans*-Bergamotene	1452	-	1 (1)	-	-	-	0	0-0	0-0.02
(*E,E*)-α-Farnesene	1512	3 (4)	6 (10)	0	0-0	0-0.64	0	0-0	0-2.30
α-Selinene	1521	-	1 81)	-	-	-	0	0-0	0-0.04
ST	1535	-	2 (2)	-	-	-	0	0-0	0-0.06
δ-Cadinene	1543	1 (1)	-	0	0-0	0-0.13	-	-	-
7-epi-α-Selinene	1548	-	2 (2)	-	-	-	0	0-0	0-0.06
trans-Nerolidol	1569	1 (1)	3 (6)	0	0-0	0-0.06	0	0-0	0-0.80
Dendrolasin	1582	2 (3)	4 (8)	0	0-0	0-0.18	0	0-0	0-0.29
Unknowns									
m/z 93,43,55,111,91,38	1067	11 (24)	15 (34)	0.12	0-0.41	0-1.70	0.09	0-0.46	0-1.71
m/z 93,43,55,111,91,77	1083	10 (12)	11 (17)	0	0-0.06	0-0.79	0	0-0.18	0-1.25
m/z 43,125,85,41,42,56	1145	13 (29)	16 (32)	0.28	0-0.71	0-1.46	0.10	0-0.46	0-1.30

[a] compounds within classes are listed according to Kovat´s retention index
[b] Kovat´s retention index
[c] compounds with asterisk were identified by comparison of mass spectrum and retention data with those of authentic standards

Variation between samples and populations

Differences of the chemical composition between individual samples, based on the CNESS index (m=1) are visualised using nonmetric multidimensional scaling (stress = 0.16) in Figure 3. Variation between the samples was very high and several chemotypes were found. The dominant chemotype was characterised by high amounts of lilac aldehyde isomers. However, some samples contained almost no lilac aldehydes, but high amounts of phenyl acetaldehyde (e.g. samples of population number 31). Some other samples were dominated by the benzenoids benzaldehyde (samples A27, B18), veratrole (e.g. samples B21, A3), or benzyl acetate (sample C4). Another group of samples was characterised by *trans*-β-ocimene (e.g. sample B15, D39) or cinnamyl alcohol (e.g. samples C35, B29). In general, variation within populations was significantly lower than between populations (U-Test: $Z_{df1=119;df2=4634}$= -7.05; $p<0.001$). The mean dissimilarity (CNESS) within populations was 0.56 (quartile: 0.40-0.80), and between populations 0.83 (quartile: 0.59-1.09). The contribution of single compounds to this observed variation between populations was estimated using a variance component analysis: seven compounds explained 96.2% of the observed total variation between populations. Phenyl acetaldehyde is the most variable compound and explains 58.8% of total variation. The lilac aldehyde isomers A, B, C explain together 15.0% of total variation. Veratrole is responsible for 8.3% of total variation; benzaldehyde and *trans*-β-ocimene are responsible for 5.5% each. Benzyl acetate explains 3.1% of total variation between populations.

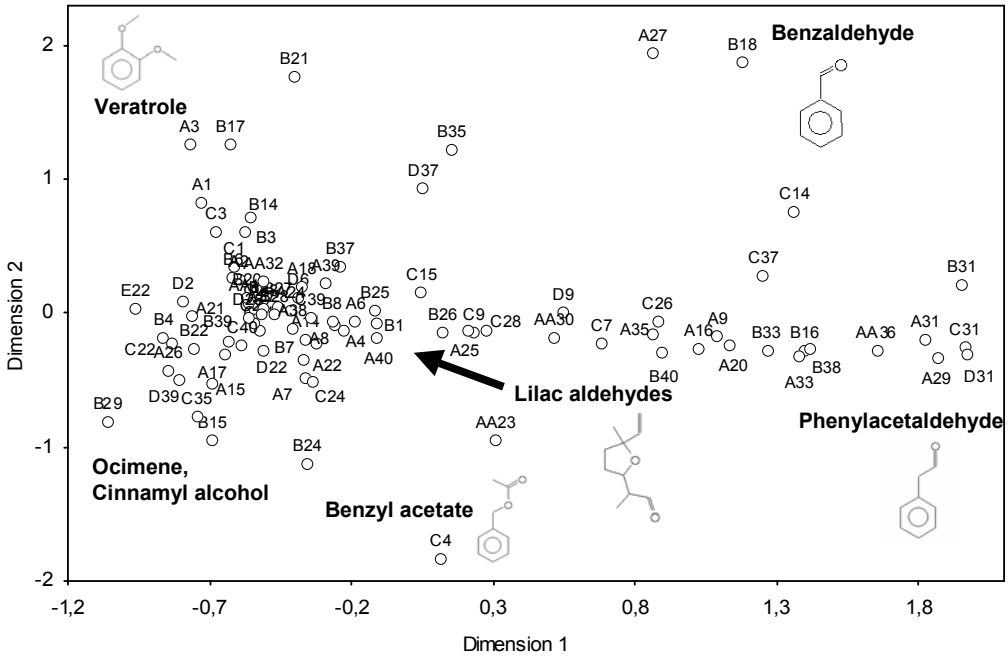

Figure 3: Nonmetric multidimensional scaling of the floral scent profile of 90 samples based on the CNESSm1 index. The numbers correspond to the population numbers presented in Figures 1 and 2. The letters indicate a specific sample of a population. "AA" before a number indicates that there is only 1 sample per population. For this analysis one sample of populations 6, 7, 21, 22, and two samples of populations 24 and 27 had to be omitted, because the software capacity was limited. Ocimene stands for *trans*-β-ocimene.

Variation within and between European and North American populations

Most compounds listed in Table 1, were found in both European and North American specimens, and only some minor compounds were detected either in European or North American specimens. Variation of samples within Europe was significantly lower than variation of samples within North America and also than variation between European and North American samples (Kruskal-Wallis-Anova: H (2;4753) = 39.1; p<0.001). Mean dissimilarity (CNESS) within European samples was 0.76 (quartile: 0.52-1.03), within North American samples 0.86 (quartile: 0.59-1.13), and between European and North American samples 0.82 (quartile: 0.59-

1.08). These results indicate that the variability in North America is higher than variability in Europe, and that there are in total no clear differences between European and North American samples. Otherwise a higher dissimilarity between European and North American samples would have been received.

Nevertheless, there is some variation between these two regions, and when comparing the relative abundances of compounds between European and North American populations in a variance component analysis, four substances were found, explaining 97% of total variation between these two regions: veratrole (37%), phenyl acetaldehyde (35%), the coeluting lilac aldehydes B and C (18%), and lilac aldehyde A (6%). Veratrole was especially characteristic for some European populations (see Figure 3) and was more abundant in Europe than in North America (U-Test: $Z_{df1=40,df2=56}$=2.7; p=0.007; see Figure 4). Phenyl acetaldehyde was frequently found in European and North American populations, and there was no difference between the mean abundances (U-Test: $Z_{df1=40,df2=56}$=-0.67; p=0.50). However, there were some North American samples where phenyl acetaldehyde was the most abundant compound, and it reached in these samples a relative content of up to 83% (e.g. samples of population 31; see also Figures 3 and 4). The lilac compounds lilac aldehyde A and the lilac aldehydes B and C were tentatively more abundant in Europe than in North America and these differences are nominal significant (U-Tests: $Z_{df1=40,df2=56}$=2.03; p=0.04 and $Z_{df1=40,df2=56}$=2.20; p=0.03, respectively).

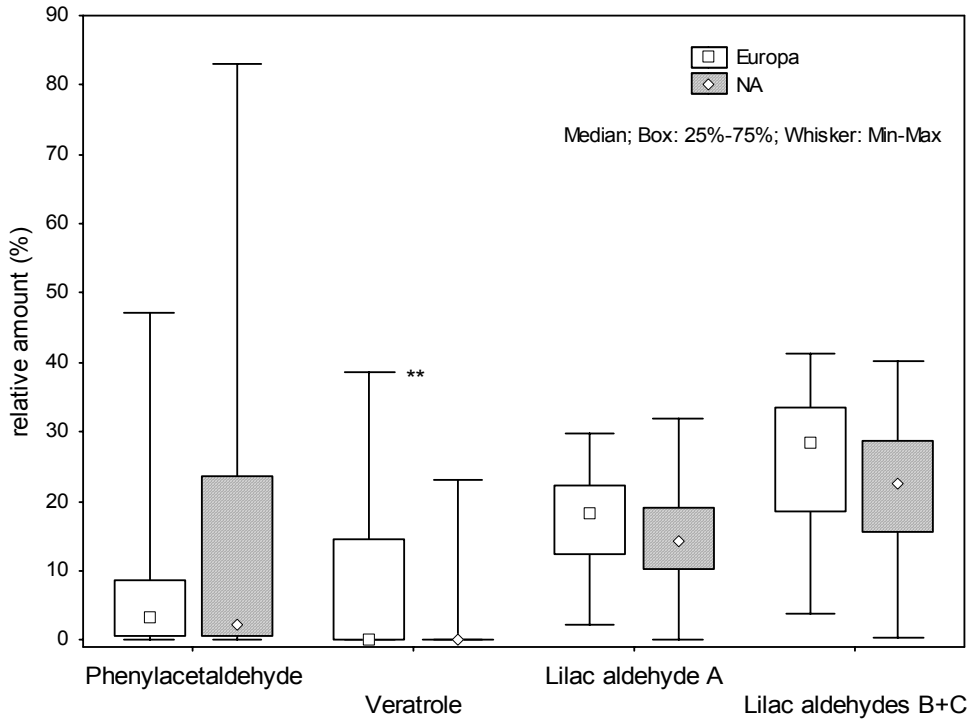

Figure 4: Compounds most responsible for variation between European and North American populations/samples (: U-Test, p<0.01).**

Mantel tests were performed in order to compare the European respectively North American floral scent matrices (CNESSm1) with the distance matrices (km) of the populations. A tentatively negative correlation (r= -0.244; p=0.057) was found between the floral scent and the distance matrix in Europe, and no association was detected between the two North American matrices (r= -0.007, p=0.475). This means there may be a trend in Europe that closely located populations have quite different scent profiles compared to distant populations. In North America the spatial distribution of the populations has no effect on the fragrance profiles. Especially the results of Europe are in contrast to other studies analysing the geographic variation of morphological and/or genetical characters of *S. latifolia* in Europe (Mastenbroek et al., 1984; Mastenbroek et al., 1983; Prentice, 1979; Prentice et al., 1984; Vellekoop et al., 1996). In all these analyses western and eastern races (clusters) of *S. latifolia*

were found. However, this west-east trend can not be found in the floral scent. The findings in *S. latifolia* are also in contrast to the findings of Knudsen (2002) at *Geonoma macrostachys* Mart. var. *macrostachys*. This plant shows a case of clinal variation of its floral scent within its distribution area.

The observed high variability of the flower scent of *S. latifolia* is comparable to other plants, like the Orchidaceae species *Platanthera bifolia* (L.) Rich. and *P. chlorantha* (Custer) Reichb. (Tollsten and Bergström, 1993), *Epidendrum ciliare* L. (Moya and Ackerman, 1993), and several *Stanhopea* Frost ex Hook. species (Whitten and Williams, 1992), or the Magnoliaceae *Magnolia kobus* DC. (Azuma et al., 2001), the Polemoniaceae *Polemonium viscosum* Nutt. (Galen and Kevan, 1980), and the Apiaceae *Conopodium majus* (Gouan) Loret (Tollsten and Ovstedal, 1994). Different explanations were offered for this variability; it was discussed that different chemotypes may be equally effective in attracting pollinators (Azuma et al., 2001; Whitten and Williams, 1992), that different chemotypes may be an adaptation to different pollinators (Galen and Kevan, 1980; Tollsten and Bergström, 1993; Tollsten and Ovstedal, 1994; Whitten and Williams, 1992), or that different chemotypes may be the result of random genetic drift in isolated populations (Tollsten and Bergström, 1993). It was also argued that high variability in floral scent chemistry might be due to the importance of visual cues in the reproductive biology of the studied species (Azuma et al., 2001). In the case of the non-rewarding moth-pollinated orchid *Epidendrum ciliare* L., it was discussed that the high variability is caused by a pollination strategy that disrupts associative learning processes and inhibits pollinators ability to recognise non-rewarding flowers (Moya and Ackerman, 1993).
Important pollinators or at least flower visitors of *S. latifolia* in Central Europe are different noctuids, like *Hadena bicruris*, *Autographa gamma*, *Diachrysia chrysitis* L., and *Cucullia umbratica* L., or different sphingids, such as *Deilephila porcellus* L., *D. elpenor* L., and *Proserpinus proserpina*

Pall. (Jürgens et al., 1996). North American *S. latifolia* specimens are pollinated or visited at night by noctuids like *Amphipoea americana* Speyer, *Autographa californica* Speyer, *A. precationis* Guenée, *Hadena variolata* Smith, and *Leucania multilinea* Walker, by sphingids such as *Hyles lineata* (Fabricius), *H. gallii* Rottenburg, and *Sphinx kalmiae* J.E. Smith, and by geometrids like *Euchlaena serrata* (Drury), and *Itame pustularia* Guenée (Altizer et al., 1998; Young, 2002). However, data about the electrophysiological or behavioural attractivity of single floral scent compounds of *S. latifolia* are only for three of these pollinators available. *Hadena bicruris* and *Autographa gamma* are strongly attracted to lilac aldehydes (for more details see Chapters 3 and 4; Plepys et al., 2002b), and *Hyles lineata* electrophysiologically responds to several volatiles (Raguso et al., 1996) occurring in *S. latifolia*. Nothing is known about the importance of single floral scent compounds of *S. latifolia* for the attraction of all other observed pollinators and/or flower visitors.

One explanation for the variability of the floral scent in *S. latifolia* thus may be, that different moth species are attracted by different scent compounds of *S. latifolia,* and that different chemotypes are therefore equally effective in attracting pollinators. The importance of different chemotypes for the interaction between *S. latifolia* and *H. bicruris* is shown and discussed in Chapter 3 and Chapter 6.

The flower visitors in North America are differing from the flower visitors in Europe, and it would be very interesting to know, whether North American flower visitors of *S. latifolia* are more effectively attracted by phenyl acetaldehyde than by veratrole or the lilac aldehydes, because phenyl acetaldehyde is very abundant in some North American populations, and veratrole and lilac aldehydes are (tentatively) more abundant in Europe than in North America (see Figure 4). These differences may be the result of interaction with different flower visitors.

The variability pattern in Europe, where the differences between closely located populations are tentatively stronger than the differences between distant populations, could be a strategy to disrupt associative learning

processes of the seed predator *Hadena bicruris*. A lot of insect pollinators are known to be flower constant (e.g. Chittka et al., 1999; Gegear and Laverty, 2001) and it may be that specific *H. bicruris* specimens learn the scent of one chemotype and do not visit *S. latifolia* flowers with another chemotype. Thus a particular *S. latifolia* individual or population is only attractive to part of the *Hadena* population and the predation pressure decreases. The fitness of plants differing in their chemotype from nearby located specimens could thereby increase.

Methodological considerations

It would not have been possible to realise this study without using a cost effective and highly sensitive method for analyses of headspace volatiles. To my knowledge, this is the first study using a MicroSPE (SPE: solid-phase extraction) tube similar to that described by Gordin and Amirav (2000) in combination with Varian´s ChromatoProbe, a direct sample introduction devise for GC-MS analyses (see Amirav and Dagan, 1997), for analyses of flower volatiles. This method allows the thermal desorption of volatiles trapped on the MicroSPE tube directly in the injector of a gas chromatograph.

In general, the advantage of thermal desorption methods (e.g. SPE, SPME) compared to solvent extraction methods is the loss of the elution step with a solvent, and therefore the loss of a dilution effect resulting in higher sensitivity (see also Amirav and Dagan, 1997; Jürgens and Dötterl, accepted; Raguso and Pellmyr, 1998). Thus sampling time of volatiles can dramatically be reduced. In consequence, it was possible to sample the volatiles emitted by a single flower of *S. latifolia* only for two minutes, an not for half an hour to several hours using solvent extraction methods (S. Dötterl, unpublished data). Such a high temporal resolution was reached never before in any floral scent analysis.

A mixture of Tenax TA and Carbotrap, two often used adsorbents in dynamic headspace analyses of floral volatiles (see Raguso and Pellmyr,

1998), was used as adsorbent, because both are known to be thermally stable (see De Bortoli et al., 1992), an important assumption for adsorbents used for thermal desorption. Furthermore, loaded tubes can be stored in a freezer (e.g. in small vials) for several weeks until analysis, without significant alterations (see De Bortoli et al., 1992), and for several days even at room temperature (A. Jürgens, personal communication).

Summary and perspectives

It was shown that the geographic variability in the floral scent of *S. latifolia* is very high. However, the factors responsible for this variability are not yet clear. Pollinators as well as genetic factors could influence the floral scent. Behavioural studies with pollinators testing the attractivity of different floral scent chemotypes (see Chapter 3), and analyses combining floral scent data with genetic data would be very helpful to understand the variability of floral scent.

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3. Antennal and behavioural responses of the nursery pollinator *H. bicruris* to various chemotypes of its most important host plant, *Silene latifolia*, and to *S. vulgaris*

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Abstract

Several available EAD and behavioural studies have analysed responses of herbivores to host plant odours, and the olfactory physiology of pollinators often remains unstudied. Here, antennal and behavioural responses of the nursery pollinator *H. bicruris* to various floral scent chemotypes of its most important host plant *S. latifolia*, and to *S. vulgaris* were characterised using GC-FID/EAD and GC-MS/EAD methods. All tested extracts elicited significant signals in the antennae of *H. bicruris*, and the moths distinguished electrophysiologically between most chemotypes. Main compounds in the tested extracts often elicited main signals in the antennae (e.g. lilac aldehydes, phenyl acetaldehyde). *Hadena bicruris* detects the compounds of the most common chemotypes of *S. latifolia* very sensitively, whereas compounds of less abundant chemotypes are less sensitively detected. The moth was effectively

attracted by floral scent blends that were dominated by lilac aldehydes or phenyl acetaldehyde. Furthermore, the moth can electrophysiologically and behaviourally distinguish between *S. latifolia,* its main host plant, and the similarly scented *S. vulgaris,* another rarely used larval host plant, only by their floral scent.

Key words: Caryophyllaceae, CNESS, floral scent, GC-FID/EAD, GC-MS/EAD, *Hadena bicruris*, Noctuidae, *Silene latifolia.*

Introduction

The interaction between the dioecious *Silene latifolia* Poir. ssp. *alba* (Mill.) Greut. & Burdet and the noctuid moth *Hadena bicruris* Hufn. is one of the known insect-plant relationships, where pollinators reproduce within the flowers/inflorescences they pollinate (see Chapter 1, and Dufay and Anstett, 2003). Female and male moths use the flowers of both sexes to drink nectar, and the females additionally use the female flowers to oviposit (Brantjes, 1976b).

Brantjes (1976b) studied the senses involved in flower visiting by *H. bicruris* and found that the moths are attracted to the flowers of *S. latifolia* by their scent, which is emitted at night. Scent alone triggers orientation towards the flowers and also landing on the flowers, when testing naïve moths. Typical floral scent compounds of this Caryophyllaceae are different lilac aldehyde isomers, veratrole, and benzyl acetate (Jürgens et al., 2002a). Previously, the flower scent of 98 specimens belonging to 34 European and North American populations was investigated and different chemotypes with a high variability in scent composition were found (see Chapter 2). Compounds abundant in a particular chemotype were only minor compounds or even absent in other chemotypes. However, it is neither known whether *H. bicruris* can distinguish electrophysiologically

between different chemotypes nor whether *H. bicruris* is equally attracted to different chemotypes.

A useful tool for the determination of volatile compounds eliciting neuronal signals in the antennal olfactory receptor neurons is gas chromatography coupled with electroantennographic detection (Arn et al., 1975; Weißbecker et al., 1997). Compounds eliciting a signal in the antennae of *H. bicruris* are most likely of biological significance for the moth. In general, it seems that the intensity of EAD responses is correlated to behavioural effectiveness (see review by Schiestl and Marion-Poll, 2001). However, there are also examples known where EAD responses were not necessarily correlated with behavioural responses (e.g. Honda et al., 1998; Omura et al., 1999b; Schütz et al., 1997). Most available EAD studies have analysed responses of herbivores to host plant odours, and the olfactory physiology of pollinators often remains unstudied (see review by Raguso, 2001).

In the present study coupled gas-chromatographic-electroantennographic detections of *H. bicruris* moths to headspace extracts of different chemotypes of the *S. latifolia* flower scent were used. Additionally, an extract of *Silene vulgaris* (Moench) Garcke, a plant taxa which is emitting similar flower scent compounds as *S. latifolia* (Jürgens et al., 2002a), but which is only seldom used by *H. bicruris* for oviposition (see Bopp and Gottsberger, 2004; Pettersson, 1991a, b; Steiner and Ebert, 1998), was analysed.

The main question was whether *H. bicruris* can effectively detect and distinguish different chemotypes and whether the moths can electrophysiologically distinguish between the flower scent of *S. latifolia* and *S. vulgaris*. Furthermore, wind tunnel bioassays with single, differently scented flowers were carried out, to evaluate the attractiveness of different chemotypes of *S. latifolia*, and of *S. vulgaris*.

Material and methods

Plant material and volatile collection

Floral scent was collected from ten different specimens of *S. latifolia* and one specimen of *S. vulgaris*, and the obtained extracts were tested in a GC-FID/EAD respectively GC-MS/EAD with *H. bicruris*. The plant specimens originated from seeds of different European and North American populations, and were grown in the greenhouse until they built up a rosette, and were then placed in flowerbeds. For each sample floral scent was collected from 10 to 40 flowers for about three hours using dynamic headspace methods.

The living flowers were enclosed in glass cylinders and the emitted volatiles were trapped in an adsorbent tube (pasteur pipette, filled with 100mg of a 1:1 mixture of Tenax-TA 60-80 and Carbotrap 20-40) through the use of a membrane pump (ASF Thomas, Inc.). Samples were collected at night, when *S. latifolia* is emitting most of its floral volatiles (Jürgens et al., 2002a; S. Dötterl, unpublished data). Volatiles were eluted with 200 to 300 µl of acetone.

To determine the chemotype of the flowers used in the behavioural tests (see below), floral scent of single flowers was collected using dynamic headspace methods similar to that described in Chapter 2. A single flower was enclosed within a polyethylene oven bag (Toppits®) and the emitted volatiles were trapped in an adsorbent tube through the use of a membrane pump (ASF Thomas, Inc.). The flow rate was adjusted to 200ml/min using a power supply and a flow meter. ChromatoProbe quartz microvials of Varian Inc. (length: 15mm; inner diameter: 2mm) were cut at the closed end, filled with a mixture (1:1) of 3mg Tenax-TA (mesh 60-80) and Carbotrap (mesh 20-40), and used as adsorbent tubes. The adsorbents were fixed in the tubes using glass wool. The specimens used in this experiment differed from specimens used for the EADs. Surrounding air was collected simultaneously to distinguish between floral scent compounds and ambient contaminants. To get the mean amount of

emitted volatiles during the tests, samples were collected for 2 min before and after the bioassays (about two hours later).

Insects

A *Hadena bicruris* culture was established by collecting eggs laid in *S. latifolia* flowers in the surroundings of Bayreuth (Germany). A photoperiod of 18h light and 6h darkness, and a temperature of 26°C and 18°C, respectively, yielded at least three generations per year. The larvae were reared on freshly collected fruits of *S. latifolia* during all five larval stages or they were fed with artificial diet similar to that described by Shorey (1963) and Shorey and Halle (1965) from the beginning of the second larval stage. However, it was necessary to add dried fruits of *S. latifolia* to the "basic" medium; otherwise the mortality of second-instar larvae was high (e.g. 500g white beans, 280g dried fruits, 9g ascorbic acid, 12g methyl-*p*-hydroxy benzoate, 6ml formalin, 130g agar, 1.5l water). Two to seven day old naïve female and male moths were used for the experiments. Adults were provided with a sugar solution (30%, same amounts of fructose and glucose corresponding to *S. latifolia* nectar as described by Witt et al., 1999).

Chemical analysis

The composition of the solvent extracts (1µl per sample) for the electroantennographic detections was analysed on a Varian Saturn 2000 mass spectrometer and a Varian 3800 gas chromatograph fitted with a 1079 injector. The injector split vent was opened (1/10) and the injector heated initially with 150°C. The injection temperature increased during the injection with 200°C/min to 250°C and was held for 2min. A ZB-5 column (5% phenyl polysiloxane, 60m long, inner diameter 0.25mm, film thickness 0.25µm, Phenomenex), the same as described in Chapter 2, was used for the analyses. Electronic flow control was used to maintain a constant

helium carrier gas flow of 1.8ml min^{-1}. The GC oven temperature was held for 2 min at 40°C, then increased by 5°C per min to 240°C and held for 3min. The MS interface was 260°C and the ion trap worked at 175°C. The mass spectra were taken at 70eV (in EI mode) with a scanning speed of 1 scan s^{-1} from m/z 40 to 350.

The volatiles sampled from flowers used in the behavioural tests were analysed (Micro-SPE) using the GC-MS system as described above. However, the 1079 injector was fitted with the ChromatoProbe kit. This kit allows the thermal desorption of small amounts of solids or liquids contained in quartz microvials (Micro-SPE, Amirav and Dagan, 1997; Wilkinson and Ladd, Varian Application note), or in the present case the thermal desorption of the trapped volatiles. The adsorbent tube was loaded into the probe, which was then inserted into the modified GC injector. The injector split vent was opened (1/20) and the injector heated to 40°C to flush any air from the system. The split vent was closed after 2 minutes and the injector was heated at 200°C/min, then held at 200°C for 4.2min, after which the split vent was opened (1/10) and the injector cooled down. The GC oven temperature was held for 7min at 40°C, then increased by 6°C per min to 250°C and held for 1min. The MS interface was 260°C and the ion trap worked at 175°C. The mass spectra were taken at 70eV with a scanning speed of 1 scan s^{-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; Davies, 1990). Identification of individual components was confirmed by comparison of both mass spectrum and GC retention data with those of authentic standards.

Electrophysiological analysis

Experiments were performed with a GC-EAD/FID system and a GC-EAD/MS system with different floral scent extracts of *S. latifolia* and additional standard compounds. Standard compounds were chosen from the scent of *S. latifolia*, and also from compounds found by Jürgens et al. (2002a) and Jürgens et al. (2003) in the floral scent of other Caryophyllaceae species. Four females and one male were tested.

GC-FID/EAD system

The GC-FID/EAD system consists of a gas chromatograph (Vega 6300-01, Carlo Erba, Rodano, Italy), an EAD-interface (Syntech, Hilversum, Netherlands), and an EAG-amplifier (Prof. Koch, University Kaiserslautern). The GC is equipped with a capillary column (30m x 0.32mm I.D., DB-1), a flame ionisation detector (FID) and a split-splitless injector. The injector is operated in the splitless mode. Nitrogen was used as carrier gas at a pressure of 100kPa (gas flow 2,5ml min^{-1}, gas vector 50cm s^{-1} at 50°C). The following temperature program was employed: start at 70°C, ramp 10°C min^{-1}, end temperature 200°C. A split connection at the end of the capillary column allows division of the GC-effluent into two capillaries leading to the FID (35cm, 0.2mm I.D.) and the EAD (45cm, 0.32mm I.D.), respectively. This provides a split ratio of 1:5 (FID/EAD). The EAD-interface (Weißbecker et al., 1997) is under thermostat control and guides the capillary column through the wall of the GC-oven thus preventing condensation of the sample in the cooler segments of the column. The end of the column projects into a small chamber where the effluent is mixed with cold humidified air (400ml min^{-1}). A PTFE-clad flow tube leads the airflow to the detector cell. The flow tube is provided with an injection port for manual calibration pulses. A PTFE tube guides the tip of an injection needle into the centre of the flow tube. Side-port injection needles (Supelco, Deisenhofen, Germany) allow injection in parallel to the air flow. The antenna of the butterfly is housed in a detector cell modified according to Färbert et al. (1997) and Schütz et al. (1999). Both ends of

the antenna are contacted to Ag/AgCl electrodes via hemolymph Ringer solution (Kaissling and Thorson, 1980). EAG-potentials of the antenna are amplified by a factor of 100 and recorded with a Chromstar data acquisition system (Bruker, Bremen, Germany).

GC-MS/EAD system

The system (Weißbecker et al., accepted) is based on a GC-MSD produced by Agilent (Palo Alto, USA) consisting of a type 6890N gas chromatograph connected to a type 5973N quadrupole mass spectrometer. The GC is equipped with a type 7163 autosampler and a split/splitless injector. A J&W Scientific HP-5MS column (Agilent) is used (length 30m, ID 0.25mm, film thickness 0.25µm). The effluent from the column is splitted into two pieces of deactivated capillary using a GRAPHPACK 3D/2 flow splitter (Gerstel, Mülheim, Germany). One capillary (length 1m, ID 0.1mm) leads to the mass spectrometer, the other (length 1m, ID 0.15mm) to an “olfactory detector port” (ODP-2, Gerstel, Mülheim, Germany). The restriction of these capillaries results in an equal split of the gas flow into the two setups.

The ODP incorporates a flexible heating sleeve which guides the capillary out of the GC oven. When the volatiles elute from the end of the capillary they are enveloped by a flow of helium used as a make-up gas in order to prevent contact of the volatiles with the surfaces of the setup. The nose-adapter that normally belongs to the ODP is replaced by a mixing chamber in which the effluent of the capillary is mixed with humidified air. The airflow is directed vertically through the flow tube to the insect antenna which is housed in a detector cell made of PTFE. This setup is in the following referred to as EAD interface.

Analytical conditions

Samples are injected in a quantity of 1µl into the injector in the pulsed splitless mode (pulse pressure 150kPa until 1.5min) at a temperature of 250°C. The GC is operated in the following temperature program: start:

50°C, hold for 1.5min, ramp 6 C/min to 200°C, hold for 5min. The chosen temperature program is a compromise between optimal separation conditions and a moderate run time which is essential because of the limited lifetime of the employed antenna.
Helium (purity 99.999%) is used as carrier gas after passing through a combined adsorbent trap for removal of traces of water, oxygen and hydrocarbons ("Big Universal Trap", Agilent). The carrier gas flow is set to 1ml/min resulting in a gas vector of 24cm/s. The GC-MS interface is operated at a temperature of 280°C. The heating sleeve of the ODP is set to 230°C.
The mass spectrometer uses electron ionisation (EI) at 70eV and is used in the scan mode with a mass range from 35-300 mass units at a scan speed of 2.78 scans per second.

EAD

Excised antennae of *H. bicruris* are placed in an antenna holder (Prof. Koch, Kaiserslautern, Germany) modified from a model designed for a portable EAG-system (Färbert et al., 1997; Schütz et al., 1999). Within this holder the ends of the antennae are in touch with an electrolyte solution adapted to the insect's hemolymph (Kaissling and Thorson, 1980) which provides electrical contact to a pair of Ag/AgCl-electrodes. The antenna holder provides a stable support for the antenna in which its surface is freely accessible to the air flow from the EAD interface.
For amplification of the EAD potentials an electronic setup (Prof. Koch) is used that consists of a pre-amplifier, a main amplifier, a frequency filter and an adjustment amplifier. Pre-amplifier (input impedance 100MΩ) and main amplifier each provide amplification by a factor of 10, resulting in a total amplification of 100. The amplifier has a built-in low pass filter which is set to a cut-off frequency of 1Hz in order to suppress the ubiquitous mains frequency of 50Hz.
The amplified signal is recorded by the Agilent ChemStation software using the type 35900E A/D-converter (Agilent). The following steps were

taken in order to match the amplifier output to the input signal range of the A/D-converter (0-1V):
(1) The additional frequency filter is used as a high pass filter with a cut-off frequency of 0.01Hz to suppress the slow drift which is often observed in the EAD signal and can cover several Volts in the amplified signal.
(2) The adjustment amplifier is used to add a constant voltage of 0.5V to the amplified, high pass filtered signal in order to keep the signal in the center of the signal input range of the A/D-converter.

Calibration of the EAD

For a quick calibration of the EAD system a calibration port is installed into the flow tube of the EAD interface (Weißbecker et al., accepted). Odour standards are produced using dilution series of the respective compounds in paraffin oil (Uvasol-quality, Merck/VWR). Small pieces of filter paper (2cm²) are drenched with a small amount of the standard dilution (100µl). The filter paper is put into a 10ml glass syringe. Inside the air volume of the syringe the odour will accumulate in a concentration that is proportional to the concentration of the substance in the solution and its vapour pressure according to Henry's law (Schütz et al., 1997). By injecting a fixed volume (5ml) of air onto the antenna a reproducible stimulus can be supplied. C*is*-3-hexenol in a 10^{-3} dilution in paraffine oil is used as calibration standard, containing 4.5ng *cis*-3-hexenol ml^{-1} of air at 25°C.

Behavioural tests

A 160cm x 75cm x 75cm wind tunnel, similar to that described by Rojas (1999b), was used for behavioural tests. A Fischbach speed controller fan (D340/E1, FDR32, Neunkirchen, Germany) pushed air through the tunnel with an air speed of 0.35m/s. Four charcoal filters (145mm x 457mm, carbon thickness 16mm, Camfil Farr) cleaned the incoming air. A two choice assay was used to test the attractivity of differently scented flowers.

To eliminate visual stimuli from the scenting flowers, they were offered at the upwind end of the tunnel behind gaze and different metal grids, so that they were invisible to the moths. Single moths were put in the wind tunnel and released at the downwind end of the tunnel. Data were recorded for the so-called zigzagg flight, if the insects finally flew to within 5cm of one of the flowers. The experiments were performed in the night at red light illumination (<0.01µE) 1-3 hours after start of the dark period. The temperature was adjusted to 22-24°C, but the relative humidity remained uncontrolled.

Statistical analyses

The CNESS (chord-normalised expected species shared) distance index (Trueblood et al., 1994, for details see Chapter 2) was calculated to determine the differences between the single extracts and EAD signals using the updated version of the COMPAH program (Boesch, 1977), provided by Gallagher at UMASS/Boston (http://www.es.umb.edu/edgwebp.htm). For calculation of CNESS relative amounts of compounds and relative EAD signals were used, respectively.

Nonmetric multidimensional scaling (NMDS) in the STATISTICA package (StatSoft Inc., 2003) was used to detect meaningful underlying dimensions and to visualise similarities between samples and EAD runs (see Borg and Lingoes, 1987). The stress value was used to evaluate how well (or poorly) the particular configuration produces the observed distance matrix (see also Chapter 2).

The behavioural two choice tests were analysed applying the observed vs. expected frequency chi^2-test in the STATISTICA package (StatSoft Inc., 2003).

Results

Ten *S. latifolia* extracts and one *S. vulgaris* extract were analysed using GC-MS methods, and these extracts were used in GC-EAG and GC/MS-EAG detections, respectively. In total 45 scent compounds were found with benzenoids and isoprenoids as the main compound classes (see Table 1). Fatty acid derivatives, phenylpropanoids, and nitrogen-bearing compounds were also detected. The most common scent compounds found in at least 9 out of 11 samples were the benzenoids benzaldehyde, phenyl acetaldehyde, and 2-methoxy phenol, the isoprenoids *trans*-β-ocimene, different isomers of lilac aldehydes and lilac alcohols, and the fatty acid derivative *cis*-3-hexenyl acetate.

Table 1: Relative amount of floral volatiles identified in ten *S. latifolia* specimens (numbers 1-10) and one *S. vulgaris* specimen (number 11). Volatiles are arranged according to their elution on a ZB-5 column. Superscript letters refer to the EAD signals in Figure 2. (*:identification is based on injection of authentic standards; tr: <0.005%).

Compound	1	2	3	4	5	6	7	8	9	10	11
3-Methyl-butyl aldoxime*[a]	0.44	-	-	-	-	0.28	-	-	1.80	2.88	-
cis-3-Hexenol*[a]	-	0.10	0.01	0.01	-	0.01	0.07	-	-	-	-
3-Methyl-butyl aldoxime*[a]	-	-	-	-	-	-	-	-	0.01	0.33	-
α-Pinene*	-	-	-	0.19	0.07	0.33	0.01	-	-	0.02	-
Benzaldehyde*[b]	4.68	0.22	0.30	0.38	0.16	0.75	1.83	0.46	tr	0.45	0.08
β-Pinene*	-	-	0.07	0.13	-	0.17	0.00	-	-	0.03	-
β-Myrcene*[c]	-	-	0.01	0.01	-	0.02	0.00	-	31.85	0.02	0.64
cis-3-hexenyl acetate*[d]	0.58	1.78	2.72	2.05	0.64	2.62	3.74	3.70	0.14	2.21	1.02
2-Hexenol acetate	0.01	-	0.04	0.05	-	0.13	0.01	-	0.07	0.03	0.18
Benzyl alcohol*	-	0.01	-	0.01	-	0.01	0.27	-	-	0.02	-
trans-β-Ocimene*[e]	0.67	0.04	0.05	14.13	1.32	15.90	6.82	-	11.05	27.72	4.14
Phenyl acetaldehyde*[f]	1.28	0.68	8.53	-	0.07	16.56	48.94	95.84	-	5.92	4.14
2-Methoxy phenol*[g]	0.12	0.32	0.20	2.62	0.01	0.01	0.00	-	0.04	0.25	-
Methyl benzoate[g]	0.19	0.01	-	5.22	0.81	-	0.27	-	0.01	27.70	0.12

Table 1 continued

Compound	1	2	3	4	5	6	7	8	9	10	11
2-Phenylethanol[h]	-	0.00	0.01	-	-	0.17	2.38	-	-	-	-
Unknown	0.14	0.12	0.01	-	0.06	0.21	0.21	-	0.04	0.40	-
Veratrole*[i]	15.08	32.30	15.46	12.76	51.27	25.78	-	-	-	0.14	-
Lilac aldehyde A*[i]	15.95	21.13	15.46	12.76	10.29	6.50	7.01	-	10.26	6.93	15.21
Lilac aldehyde B+C*[i]	39.43	33.38	45.39	43.12	22.43	17.93	21.56	-	11.19	10.32	39.31
Benzyl acetate*[i]	-	-	-	-	-	-	-	-	3.11	-	-
Benzenepropanal[i]	-	0.01	-	-	-	-	-	-	1.31	-	-
Lilac aldehyde D*[i]	0.46	1.00	0.91	0.82	0.48	0.17	0.14	-	1.31	0.10	30.39
Methyl salicylate[j]	-	-	-	0.11	0.03	0.23	1.50	-	0.09	1.13	0.05
Lilac alcohol A*[j]	0.49	0.60	0.15	0.07	0.18	0.10	0.55	-	3.54	1.01	0.56
Lilac alcohol B+C*[j]	0.11	0.28	0.06	0.01	0.17	0.08	0.09	-	6.20	0.54	3.30
Lilac alcohol D*[j]	0.11	0.09	0.06	0.04	0.11	0.03	0.04	-	3.33	0.33	0.42
Benzenepropanol	0.01	4.97	-	-	-	-	-	-	0.28	-	-
(*E*)-Cinnamaldehyde	2.15	0.22	0.01	-	-	-	-	-	0.00	-	-
Benzyl isobutanoate	-	-	-	-	0.01	-	-	-	-	-	-
Indole*	-	-	-	-	0.01	-	-	-	-	-	-
Cinnamyl alcohol	17.15	0.53	-	-	-	-	-	-	-	-	-
α-Longipinene	-	-	-	-	-	-	0.26	-	-	1.79	-
Benzenepropyl acetate	0.06	1.97	-	-	-	-	-	-	0.27	-	-
Lilacalcohol formate	0.02	0.18	10.44	-	-	5.04	0.00	-	-	1.47	-
Longicyclene	-	-	-	-	-	-	0.40	-	-	0.07	-
ST	-	-	0.01	0.01	-	0.04	0.68	-	0.32	0.02	-
(*E*)-β-Caryophyllene*	-	0.05	0.05	0.01	0.08	0.01	0.01	-	-	0.02	-
(*E*)-α-Bergamotene	-	-	-	-	-	-	0.01	-	-	-	-
Cinnamyl alcohol acetate	0.88	0.02	-	-	-	-	-	-	-	-	-
(*E,E*)-α-Farnesene	-	-	0.04	0.16	0.10	-	0.01	-	10.99	-	0.42
ST	-	-	-	-	-	-	0.10	-	-	-	-
δ-Cadinene	-	0.01	-	-	-	-	-	-	-	-	-
7-epi-α-Selinene	-	0.01	-	-	-	-	-	-	-	-	-
Dendrolasin	-	-	-	-	0.05	-	0.01	-	1.56	-	-
Benzyl benzoate*	-	-	-	5.32	11.62	6.90	3.07	-	1.23	8.14	-

Four *S. latifolia* extracts (numbers 1-4), and the sample of *S. vulgaris* (11) were dominated by lilac aldehydes with relative contents between 56% and 85%. Lilac aldehyde isomers A, and B together with C dominated the *S. latifolia* extracts, while lilac aldehyde D was also abundant in the *S. vulgaris* extract. One sample was dominated by veratrole (5) with a relative content of 51%, and one sample was characterised by similar high amounts of veratrole and lilac aldehydes, and additional relatively high amounts of *trans*-β-ocimene and phenyl acetaldehyde (6). However, veratrole coeluted in the GC/EAD runs with the lilac aldehyde isomers, and it was therefore not possible to differentiate between the veratrole and the lilac aldehyde chemotypes.

Phenyl acetaldehyde reached a relative content of 49% in extract 7, and a relative content of 96% in extract 8. β-myrcene and (*E,E*)-α-farnesene were characteristic compounds in extract 9. Both, methyl benzoate and *trans*-β-ocimene reached relative contents of 28% each in extract 10.

To visualise the similarities and differences between both, the extracts and the EAD signals in one analysis, an ordination using nonmetric multidimensional scaling on the basis of the dissimilarity index CNESS was calculated (see Figure 1).

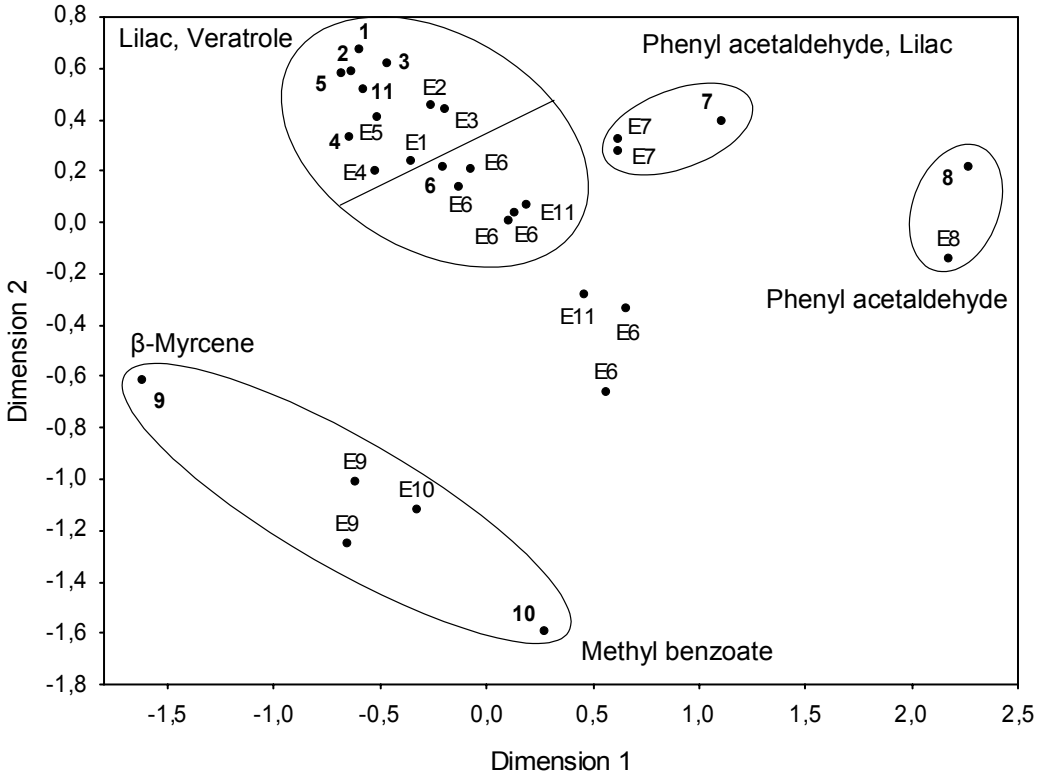

Figure 1: Nonmetric multidimensional scaling of the 11 floral scent extracts (numbers 1-11) and the corresponding EADs (E1-E11) based on the CNESSm1 dissimilarity index (stress=0.08; Lilac: Lilac aldehyde isomers).

All EAD signals of the five *S. latifolia* extracts, dominated by lilac aldehydes and/or veratrole, and grouping in Figure 1 above the plotted line (1-5), are located nearby the corresponding extracts, indicating the similarity between the extracts and the corresponding EADs. Main compounds in the extracts (lilac aldehydes, veratrole) elicited main signals in the antennae (52-72%) as shown for extract 3 in Figure 2A. The EADs of the *S. vulgaris* extract (11) dominated also by lilac aldehydes (85%) are not in this group, because the lilac aldehyde signals in the antennae were relatively small (31% and 37%).

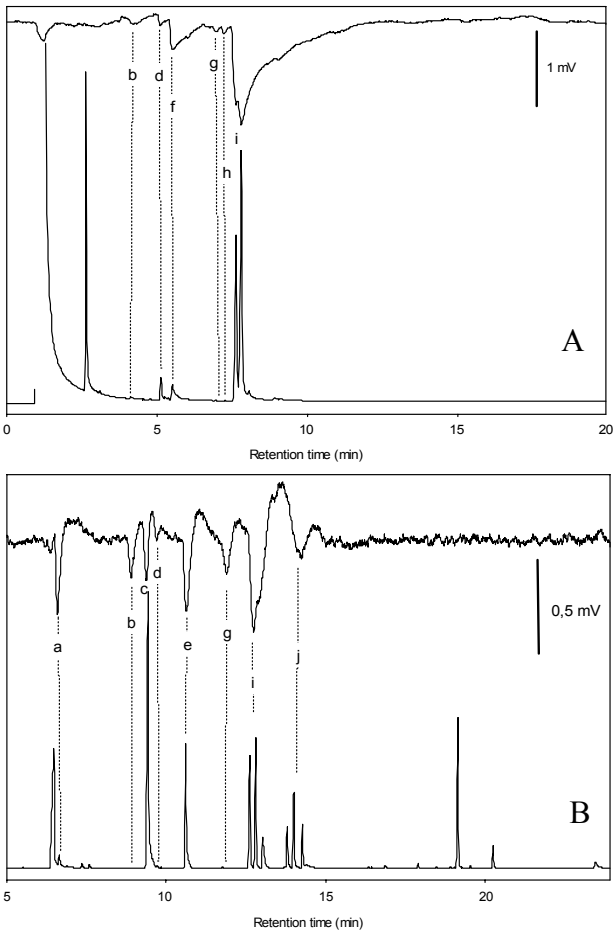

Figure 2: (A) GC-FID/EAD of a *S. latifolia* extract (number 3 in Table 1) dominated by veratrole and lilac aldehyde isomers, (B) GC-MS/EAD of a *S. latifolia* extract dominated by β-myrcene (number 9 in Table 1). Letters labelling the EAD positive compounds refer to compounds listed in Table 1. Peaks in (A) before retention time of 3min are resulting from the solvent.

Besides veratrole and lilac aldehydes, both *trans*-β-ocimene and phenyl acetaldehyde reached relatively high amounts in extract 6, and the antennae detected these differences. All six EADs of extract 6 grouped below the plotted line, four of them near to the extract, whereas two others were separated from the extract. The antennae in the latter cases responded very sensitively to 3-methyl-butyl-aldoxime and benzaldehyde, affecting a smaller relative veratrole-lilac aldehyde signal, though these

compounds were only found as minor components in the extract. Phenyl acetaldehyde elicited main or dominant signals in the EADs of extract 7 and 8, respectively, and the EADs were very similar to the corresponding extracts. The EADs of the β-myrcene dominated extract (9) are grouping far from the extract. Though β-myrcene dominated the extract, it elicited only a moderate signal (see Figure 2B). Furthermore, the abundant compound (*E,E*)-α-farnesene did not elicit any signal in the antennae of the moths, and 3-methyl-butyl aldoxime, reaching a relative content of only 1.8% in the extract, elicited a main signal in the antennae. This nitrogen-bearing compound was also in the EAD of the methyl benzoate characterised extract (10) a main signal (22%), and reached in this extract a relative content of 2.9%. On the other hand, methyl benzoate, a dominant compound, elicited together with 2-methoxy phenol only a minor EAD signal (2.5%). Therefore the corresponding EAD is separated from the extract.

In the first behavioural experiment two qualitatively similar, but quantitatively different scenting flowers with isoprenoids, and especially lilac aldehydes as main compounds were offered (see Figure 3A). The moths were significantly more attracted to the flower emitting a higher amount of volatiles. In the second experiment the moths had to choose between flowers of quite different chemotypes (see Figure 3B). One flower was strongly dominated by benzenoids, first of all phenyl acetaldehyde, the second flower emitted especially high amounts of lilac aldehyde isomers. The moths were equally attracted to both flowers, though the phenyl acetaldehyde flower emitted about twice the amount of volatiles. In the third two choice assay a *S. latifolia* flower was tested against a *S. vulgaris* flower. Dominant isoprenoids in both flowers were lilac aldehydes, and additionally *trans*-β-ocimene in the *S. latifolia* flower. Benzaldehyde was the main benzenoid compound, and *cis*-3-hexenyl acetate the main fatty acid derivative in both flowers. The moths significantly preferred the *S. latifolia* flower.

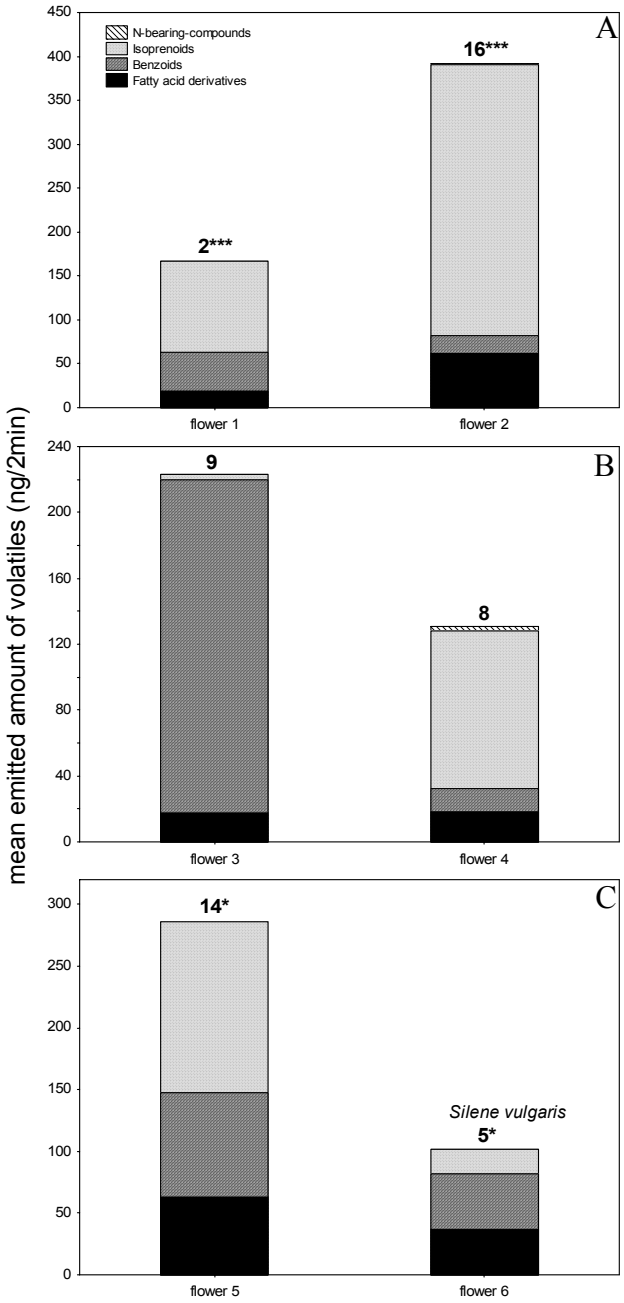

Figure 3: Attraction of *H. bicruris* to different scenting flowers of *S. latifolia* and to *S. vulgaris*. The numbers above the columns indicate the number of specimens attracted to a particular flower. 21 specimens were tested in experiment A and B, and 26 specimens in experiment C. (observed vs. expected chi^2-test; *: 0.01<p<0.05; ***: p<0.001).

Discussion

All tested floral scent chemotypes of *S. latifolia* elicited significant signals in the antennae of *H. bicruris*, and the moths clearly distinguished electrophysiologically between most chemotypes. In most cases, main compounds in the tested extracts elicited also main signals in the antennae. However, also compounds abundant in the extracts, but resulting only in a minor signal in the EAD were found. On the other hand, there were compounds eliciting main signals in the antennae, though they were only minor components in the extracts. To my knowledge this is the first detailed study investigating and comparing antennal responses of an insect species to several floral scent chemotypes of its host plant, in this particular case a host plant that is both, nectar plant and larval food plant.

The most common chemotypes of the floral scent of *S. latifolia* are dominated by lilac aldehyde isomers, followed by phenyl acetaldehyde rich scent morphs (see Chapter 2). Interestingly, these compounds elicited the largest signals in the antennae of *H. bicruris* (when omitting signals elicited by different coeluting compounds; compare Chapter 4). Floral scent chemotypes dominated by methyl benzoate or especially β-myrcene are rarely found, and in the case of β-myrcene only in regions (North America) where *H. bicruris* is not distributed (Chapter 2). Interestingly, these compounds elicited only weak signals in the antennae. It seems that senses in *Hadena* have evolved to detect effectively the most typical and abundant compounds emitted by its important nectar and larval host plant.

Several other GC-EAD studies indicate that pollinators and/or herbivores are not always “tuned” to detect effectively the dominant scent compound(s) of host-plants (e.g. Baur et al., 1993; Fraser et al., 2003; Plepys et al., 2002a). Baur et al. (1993) e.g. found nonpolar monoterpenes as major volatiles in *Daucus carota* L., an Apiaceae species used as larval host plant by the black swallowtail butterfly, *Papilio polyxenes asterius* Stoll. However, none of these monoterpenes were found to be electrophysiologically very active. The most attractive compounds in

Baur´s study were minor compounds like sabinene hydrate isomers or 4-terpineol.

Nevertheless, there are data available on other systems, where most abundant compounds in host plants were responsible for the main signals in the antennae of the tested insects (e.g. Andersson, 2003a; Andersson and Dobson, 2003a) like in the case of *H. bicruris* and *S. latifolia*. Andersson and Dobson (2003a) studied antennal responses of the tropical nymphalid *Heliconius melpomene* L. to compounds of four plant species often used as nectar plants by butterflies. The authors found the main compounds detected exclusively in floral parts (e.g. linalool, oxoisophorone oxide, phenyl acetaldehyde) very effective in eliciting antennal responses. However, other main floral compounds, which were also present in vegetative parts (e.g. β-caryophyllene, α-humulene), failed to elicit antennal responses. Andersson and Dobson (2003a) discussed that the main exclusive floral compounds may represent adaptations by the plant to attract butterfly pollinators. A similar conclusion was drawn by Andersson (2003a) in the analyses of antennal responses in the butterflies *Inachis io* L., *Aglais urticae* L., and *Gonepteryx rhamni* L. to floral scent compounds of *Cirsium arvense* (L.) and *Buddleja davidii* Franch. cv. The strongest EAD responses were achieved by the exclusive floral scent compounds phenyl acetaldehyde, oxoisophorone oxide, and oxoisophorone emitted in high amounts, indicating again an adaptation to butterfly pollination, especially in *B. davidii*.

However, as these systems do not describe specific insect-plant interactions similar to *H. bicruris* and *S. latifolia*, it is not surprising that the insects are often adapted to minor extent to certain nectar or larval host plants. It is not necessary for more generalistic insects to find one particular plant species; they only have to find an appropriate one. From the view of the insects it should be of advantage to be able to detect a broad range of scents typically found in flowers. The situation is quite different for *Hadena bicruris*. *Silene latifolia* is the most important host plant for this moth species (Bopp and Gottsberger, 2004; see also

Chapter 1), and it is therefore very important for *H. bicruris* to find its nectar and larval food plant effectively. To ensure this, *H. bicruris* has to be adapted to the scent of its host plant by "tuning" the receptors to reliable components in the floral scent, and additionally, these electrophysiologically active compounds have to elicit behavioural responses. It seems that in general strongly EAD active compounds are also behaviourally active (see review by Schiestl and Marion-Poll, 2001), and in the case of *H. bicruris*, the floral scent compounds elicited the strongest signals in the antennae are pointing to their biological importance.

The wind tunnel bioassays with single flowers indicate that the scent of individual flowers, dominated either by lilac aldehydes or by phenyl acetaldehyde, effectively attracts *H. bicruris* (see Figure 3). Moreover, the lilac aldehydes alone were the most attractive compounds when testing them in the wind tunnel (for more details see Chapter 4). These isoprenoid compounds are only found in few plant species (Jürgens et al., 2002a; Knudsen et al., 1993) and the probability for *H. bicruris* to find *S. latifolia* when relying on these compounds is high. These compounds are also known to be most attractive for another noctuid moth. Plepys et al. (2002b) found the lilac aldehydes eliciting main signals in GC-EAD detections with *Autographa gamma* L., and the authors reported very sensitive olfactory receptor neurons responding to lilac aldehydes with a response treshold of <0.1ng in this noctuid (Plepys, 2001; Plepys et al., 2002a). Moreover, the lilac aldehydes were also in *A. gamma* the most attractive compounds in a wind tunnel biotest (Plepys et al., 2002b).

A plant species, which is also characterised by high amounts of lilac aldehydes (this study, and Jürgens et al., 2002a; Knudsen and Tollsten, 1993) and which is rarely used by *H. bicruris* as larval host plant (Steiner and Ebert, 1998), is *S. vulgaris*. Interestingly, the dominating lilac aldehydes in *S. vulgaris* elicited only relatively small EAD signals, and the different ratios for the isomers A, B, C, D are probably responsible for this phenomenon. Moreover, the wind tunnel biotest has shown that *H. bicruris*

distinguishes *S. latifolia* and *S. vulgaris* by their scent (see Figure 3C). The *S. latifolia* flower was much stronger scented than the *S. vulgaris* flower and this quantitative difference, besides qualitative differences, seems to be responsible for the decision of the moths. Plepys et al. (2002b) e.g. nicely demonstrated that the dose of the compounds is very important in bioassays. These authors found a particular dose most attractive for *Autographa gamma*, whereas smaller and higher doses inhibited the attraction.

Flowers dominated by phenyl acetaldehyde also attracted *H. bicruris* (see Figure 3B), and this benzenoid is known to be attractive for *H. bicruris* and other noctuid Lepidoptera species (see Chapter 4; Cantelo and Jacobson, 1979; Haynes et al., 1991; Heath et al., 1992; Meagher, 2001, 2002).

The receptors in the antennae of *H. bicruris* were very sensitive (or abundant) to the nitrogen-bearing compounds 3-methyl-butyl-aldoxime, and the benzenoid benzaldehyde in this study. These compounds did not reach high relative amounts in the extracts tested in the EADs. Compounds present only in minor amounts in the scent, but eliciting strong signals in the antennae are supposed to be of biological relevance (Gabel et al., 1992; Schütz et al., 1997). Benzaldehyde is a very widespread plant-derived compound (Knudsen et al., 1993), and is, like phenyl acetaldehyde, electrophysiologically and/or behaviourally active in various insects, e.g. in butterflies (Andersson, 2003a; Andersson and Dobson, 2003a; Omura et al., 1999a; Omura et al., 1999b), moths (Bruce and Cork, 2001; Haynes et al., 1991; Heath et al., 1992; Meagher, 2002), or beetles (Huber et al., 2000; Pierce et al., 1990). However, in case of *H. bicruris*, only the aldoxime compound, which is known from different nocturnal plant species (Kaiser, 1994; Knudsen et al., 1993), is attractive to *H. bicruris*, whereas benzaldehyde did not attract any specimen of *H. bicruris* (see Chapter 4).

Summary and perspectives

It was shown that *H. bicruris* is detecting the compounds of the main chemotypes of its most important host plant *S. latifolia* very sensitively, whereas compounds of less abundant chemotypes are less sensitively detected, indicating an adaptation of *H. bicruris* to the host plant scent. In contrast to other systems studied, the interaction between *H. bicruris* and *S. latifolia* is very specific and it seems that the antennae/receptors of *H. bicruris* are “tuned” to the most important compounds of its host, making sure to find *S. latifolia*. Furthermore, it was proven that *H. bicruris* is attracted to different chemotypes of *S. latifolia*, and that the moths distinguished its important host plant from another rarely used larval host plant only by their floral scent. To determine the attractivity of different chemotypes, different scenting flowers were used in two choice assays. However, as different flowers do not emit exact the same amount of compounds, it is difficult to interpret these data, because concentration is an important issue for attraction of moths (see also Chapter 4).

Therefore, it would be desirable to use quantitatively standardised mixtures (same total amount of emitted substances) of authentic standard compounds comparable to chemotypes of *S. latifolia* in wind tunnel tests. Furthermore, it would be very worthwhile to determine parasitism rates of *H. bicruris* in different *S. latifolia* chemotypes in the field.

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4. Antennal and behavioural responses of *Hadena bicruris* to floral volatiles of its main host plant, *Silene latifolia*

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Abstract

Since the 70ies it is known that the nursery pollinator *Hadena bicruris* is attracted to the flowers of its most important host plant, *Silene latifolia*, by their scent. However, it was not known which compounds are important for the attraction. To identify the attractive floral scent compounds, the GC-FID/EAD or the GC-MS/EAD method was used in a first step to detect compounds that elicit signals in the antennae of the moth. For further analyses, electrophysiologically very active compounds were tested in wind tunnel bioassays, and the attractivity of these compounds was compared to the attractivity of the natural scent of whole flowers of *S. latifolia*. The antennae of female and male moths detected substances of several compound classes such as monoterpenoids, benzenoids, fatty

acid derivatives, and nitrogen-bearing compounds. Phenyl acetaldehyde and lilac aldehyde isomers elicited the strongest signals in the antennae. Lilac aldehydes were the most attractive compounds in wind tunnel bioassays, and attracted 90% of the tested moths, as did the scent of single flowers. All other tested compounds attracted fewer individuals. Some compounds did not attract any moth, though they elicited significant signals in the antennae. In cases in which two different doses were tested, more moths were attracted to the higher dose.

Key words: attraction of moths, Caryophyllaceae, floral scent, GC-FID/EAD, GC-MS/EAD, *Hadena bicruris*, lilac aldehydes, *Silene latifolia.*

Introduction

Many Lepidoptera use flowers of some plant species to drink nectar and the foliage of others to deposit eggs. Important cues for nectar and larval host plant finding are volatiles, and the fragrance emitted from flowers is generally distinct from fragrance emitted from the vegetative plant parts (Dobson, 1994). It was shown that flower volatiles elicit foraging behaviour in Lepidoptera species (Andersson, 2003b; Andersson and Dobson, 2003b; Haynes et al., 1991; Heath et al., 1992; Omura et al., 2000a, b; Omura et al., 1999b; Plepys et al., 2002b), and that foliage compounds are used to find larval host plants (Fraser et al., 2003; Pivnick et al., 1994; Rojas, 1999a). In the case of *Hadena bicruris* Hufn. (Lepidoptera: Noctuidae) and the Caryophyllaceae *Silene latifolia* Poiret ssp. *alba* (Miller) Greuter & Burdet, the moths use the flowers for both, nectar drinking and oviposition (Brantjes, 1976b, c), therefore flower volatiles are possibly used for both, nectar and larval host plant finding. Floral volatiles are known to be important cues for *H. bicruris* to locate the flowers. Floral scent also triggers landing on the flowers (Brantjes, 1976b). However, it is not known which floral scent compounds are important for the attraction of

H. bicruris. Floral scent of *S. latifolia* is especially characterised by several fatty acid derivatives, benzenoids and monoterpenoids (Jürgens et al., 2002a). The most abundant compounds were lilac aldehyde isomers, veratrole and benzyl acetate. However, a study on the variability of floral scent in *S. latifolia* revealed different chemotypes (Chapter 2). Some compounds, e.g. lilac aldehyde isomers, phenyl acetaldehyde, veratrole, and benzyl acetate were abundant in particular chemotypes, but only minor compounds or even absent in other chemotypes. To prove the role of single compounds for the attraction of the moths, behavioural studies are needed. However, as it is impossible to test all compounds of typically complex scent blends, identification of potential active compounds is a necessary first step. A very useful method for this purpose is gas chromatography coupled to electroantennographic detection (GC/EAD, Arn et al., 1975; Weißbecker et al., 1997). Behaviourally active compounds in general elicit signals in GC/EAD analyses (Schiestl and Marion-Poll, 2001; Schütz, 2001), and electrophysiologically active compounds can be used for further biotests. This general procedure was successfully applied in previous studies (e.g. Omura et al., 2000b; Omura et al., 1999b; Plepys, 2001; Schiestl and Ayasse, 2000; Schiestl et al., 2000; Schütz and Weißbecker, 2003; Schütz et al., 1997).

In the present study, the GC-EAD/FID and GC-EAD/MS method (Weißbecker et al., accepted) was used for structural elucidation of floral scent compounds of *S. latifolia* eliciting signals in the antennae of *H. bicruris*. In addition, synthetic standard compounds found in other Caryophyllaceae (see Jürgens et al., 2002a, 2003; Knudsen and Tollsten, 1993) species were tested. Electrophysiologically very active compounds were tested in wind tunnel bioassays, and the attractivity of these compounds was compared to the attractivity of the scent of whole flowers of *S. latifolia*.

Material and methods

Plant material and volatile collection

Floral scent was collected from ten different specimens of *S. latifolia* and one specimen of *S. vulgaris*, and the obtained extracts were tested to *H. bicruris* in GC-FID/EAD respectively GC-MS/EAD analyses. The plant specimens originated from seeds of different populations, and were grown in the greenhouse for about eight weeks until they built up a rosette, and were then placed in flowerbeds. For each sample floral scent was collected from 10 to 40 flowers for about three hours using dynamic headspace methods.

The living flowers were enclosed in glass cylinders and the emitted volatiles were trapped in an adsorbent tube (pasteur pipette, filled with 100mg of a 1:1 mixture of Tenax-TA 60-80 and Carbotrap 20-40) through the use of a membrane pump (ASF Thomas, Inc.). Samples were collected at night, when *S. latifolia* is emitting most of its floral volatiles (Jürgens et al., 2002a; S. Dötterl, unpublished data). Volatiles were eluted with 200 to 300 µl of acetone.

Insects

A *Hadena bicruris* culture was established by collecting eggs laid in *S. latifolia* flowers in the surroundings of Bayreuth (Germany). A photoperiod of 18h light and 6h darkness, and a temperature of 26°C and 18°C, respectively, yielded at least three generations per year. The larvae were reared on freshly collected fruits of *S. latifolia* during all five larval stages or they were fed with artificial diet similar to that described by Shorey (1963) and Shorey and Halle (1965) from the beginning of the second larval stage. However, it was necessary to add dried fruits of *S. latifolia* to the “basic” medium; otherwise the mortality of second-instar larvae was high (e.g. 500g white beans, 280g dried fruits, 9g ascorbic acid, 12g methyl-*p*-hydroxy benzoate, 6ml formalin, 130g agar, 1.5l water). Two to

seven day old naïve female and male moths were used for the experiments. Adults were provided with a sugar solution (30%, same amounts of fructose and glucose corresponding to *S. latifolia* nectar as described by Witt et al., 1999).

Chemical analyses

The composition of the solvent extracts (1µl per sample) for the electroantennographic detections was analysed on a Varian Saturn 2000 mass spectrometer and a Varian 3800 gas chromatograph fitted with a 1079 injector. The injector split vent was opened (1/10) and the injector heated initially with 150° C. The injection temperature increased during the injection with 200°C/min to 250°C and was held for 2 minutes. A ZB-5 column was used for the analyses (60m long, inner diameter 0.25mm, film thickness 0.25µm, Phenomenex). Electronic flow control was used to maintain a constant helium carrier gas flow of 1.8ml min^{-1}. The GC oven temperature was held for 2 min at 40°C, then increased by 5°C per min to 240°C and held for 3min. The MS interface was 260°C and the ion trap worked at 175°C. The mass spectra were taken at 70eV with a scanning speed of 1 scan s^{-1} from m/z 40 to 350.

For quantification of compounds known amounts of lilac aldehydes, *trans*-β-ocimene, *cis*-3-hexenyl acetate, benzaldehyde, phenyl acetaldehyde, and veratrole were injected, and the mean response of these compounds was used for quantification.

Electrophysiological analysis

Experiments were performed with a GC-EAD/FID system and a GC-EAD/MS system with different floral scent extracts of *S. latifolia* and additional standard compounds. Standard compounds were chosen from the scent of *S. latifolia*, and also from compounds found by Jürgens et al. (2002a) and Jürgens et al. (2003) in the floral scent of other

Caryophyllaceae species. Seven females and two males of *H. bicruris* were tested.

GC-FID/EAD system

The GC-FID/EAD system consists of a gas chromatograph (Vega 6300-01, Carlo Erba, Rodano, Italy), an EAD-interface (Syntech, Hilversum, Netherlands), and an EAG-amplifier (Prof. Koch, University Kaiserslautern). The GC is equipped with a capillary column (30m x 0.32mm I.D., DB-1), a flame ionisation detector (FID) and a split-splitless injector. The injector is operated in the splitless mode. Nitrogen was used as carrier gas at a pressure of 100kPa (gas flow 2,5ml min^{-1}, gas vector 50cm s^{-1} at 50°C). The following temperature program was employed: start at 70°C, ramp 10°C min^{-1}, end temperature 200°C. A split connection at the end of the capillary column allows division of the GC-effluent into two capillaries leading to the FID (35cm, 0.2mm I.D.) and the EAD (45cm, 0.32mm I.D.), respectively. This provides a split ratio of 1:5 (FID/EAD). The EAD-interface (Weißbecker et al., 1997) is under thermostat control and guides the capillary column through the wall of the GC-oven thus preventing condensation of the sample in the cooler segments of the column. The end of the column projects into a small chamber where the effluent is mixed with cold humidified air (400ml min^{-1}). A PTFE-clad flow tube leads the airflow to the detector cell. The flow tube is provided with an injection port for manual calibration pulses. A PTFE tube guides the tip of an injection needle into the centre of the flow tube. Side-port injection needles (Supelco, Deisenhofen, Germany) allow injection in parallel to the air flow. The antenna of the butterfly is housed in a detector cell modified according to Färbert et al. (1997) and Schütz et al. (1999). Both ends of the antenna are contacted to Ag/AgCl electrodes via hemolymph Ringer solution (Kaissling and Thorson, 1980). EAG-potentials of the antenna are amplified by a factor of 100 and recorded with a Chromstar data acquisition system (Bruker, Bremen, Germany).

GC-MS/EAD system

The system (Weißbecker et al., accepted) is based on a GC-MSD produced by Agilent (Palo Alto, USA) consisting of a type 6890N gas chromatograph connected to a type 5973N quadrupole mass spectrometer. The GC is equipped with a type 7163 autosampler and a split/splitless injector. A J&W Scientific HP-5MS column (Agilent) is used (length 30m, ID 0.25mm, film thickness 0.25µm). The effluent from the column is splitted into two pieces of deactivated capillary using a GRAPHPACK 3D/2 flow splitter (Gerstel, Mülheim, Germany). One capillary (length 1m, ID 0.1mm) leads to the mass spectrometer, the other (length 1m, ID 0.15mm) to an "olfactory detector port" (ODP-2, Gerstel, Mülheim, Germany). The restriction of these capillaries results in an equal split of the gas flow into the two setups.

The ODP incorporates a flexible heating sleeve which guides the capillary out of the GC oven. When the volatiles elute from the end of the capillary they are enveloped by a flow of helium used as a make-up gas in order to prevent contact of the volatiles with the surfaces of the setup. The nose-adapter that normally belongs to the ODP is replaced by a mixing chamber in which the effluent of the capillary is mixed with humidified air. The airflow is directed vertically through the flow tube to the insect antenna which is housed in a detector cell made of PTFE. This setup is in the following referred to as EAD interface.

Analytical conditions

Samples are injected in a quantity of 1µl into the injector in the pulsed splitless mode (pulse pressure 150kPa until 1.5min) at a temperature of 250°C. The GC is operated in the following temperature program: start: 50°C, hold for 1.5min, ramp 6 C/min to 200°C, hold for 5min. The chosen temperature program is a compromise between optimal separation conditions and a moderate run time which is essential because of the limited lifetime of the employed antenna.

Helium (purity 99.999%) is used as carrier gas after passing through a combined adsorbent trap for removal of traces of water, oxygen and hydrocarbons (“Big Universal Trap”, Agilent). The carrier gas flow is set to 1ml/min resulting in a gas vector of 24cm/s. The GC-MS interface is operated at a temperature of 280°C. The heating sleeve of the ODP is set to 230°C.

The mass spectrometer uses electron ionisation (EI) at 70eV and is used in the scan mode with a mass range from 35-300 mass units at a scan speed of 2.78 scans per second.

EAD

Excised antennae of *H. bicruris* are placed in an antenna holder (Prof. Koch, Kaiserslautern, Germany) modified from a model designed for a portable EAG-system (Färbert et al., 1997; Schütz et al., 1999). Within this holder the ends of the antennae are in touch with an electrolyte solution adapted to the insect’s hemolymph (Kaissling and Thorson, 1980) which provides electrical contact to a pair of Ag/AgCl-electrodes. The antenna holder provides a stable support for the antenna in which its surface is freely accessible to the air flow from the EAD interface.

For amplification of the EAD potentials an electronic setup (Prof. Koch) is used that consists of a pre-amplifier, a main amplifier, a frequency filter and an adjustment amplifier. Pre-amplifier (input impedance 100MΩ) and main amplifier each provide amplification by a factor of 10, resulting in a total amplification of 100. The amplifier has a built-in low pass filter which is set to a cut-off frequency of 1Hz in order to suppress the ubiquitous mains frequency of 50Hz.

The amplified signal is recorded by the Agilent ChemStation software using the type 35900E A/D-converter (Agilent). The following steps were taken in order to match the amplifier output to the input signal range of the A/D-converter (0-1V):

(1) The additional frequency filter is used as a high pass filter with a cut-off frequency of 0.01Hz to suppress the slow drift which is often observed in the EAD signal and can cover several Volts in the amplified signal.
(2) The adjustment amplifier is used to add a constant voltage of 0.5V to the amplified, high pass filtered signal in order to keep the signal in the center of the signal input range of the A/D-converter.

Calibration of the EAD

For a quick calibration of the EAD system a calibration port is installed into the flow tube of the EAD interface (Weißbecker et al., accepted). Odour standards are produced using dilution series of the respective compounds in paraffin oil (Uvasol-quality, Merck/VWR). Small pieces of filter paper (2cm²) are drenched with a small amount of the standard dilution (100µl). The filter paper is put into a 10ml glass syringe. Inside the air volume of the syringe the odour will accumulate in a concentration that is proportional to the concentration of the substance in the solution and its vapour pressure according to Henry's law (Schütz et al., 1997). By injecting a fixed volume (5ml) of air onto the antenna a reproducible stimulus can be supplied. To compensate the interindividual variation in absolute response (mV) the signals were normalised (corrected) by use of a standard stimulus of *cis*-3-hexenol at a 10^{-3} dilution in paraffine oil offered before and after EAD runs. The EAD signals were normalised to the mean response of the standard stimulus (corrected signal). Responses were e.g. halved, if the amplitude of signals in specific antennae to *cis*-3-hexenol was double as high as the mean amplitude of *cis*-3-hexenol.

Authentic standard compounds, behavioural tests, and statistical analyses

A 160cm x 75cm x 75cm wind tunnel, similar to that described by Rojas (1999b), was used for behavioural tests. A Fischbach speed controller fan (D340/E1, FDR32, Neunkirchen, Germany) pushed air through the tunnel

with an air velocity of 0.35m/s. Four charcoal filters (145mm x 457mm, carbon thickness 16mm, Camfil Farr) cleaned the incoming air.

Authentic standard compounds (see Table 2) eliciting strong signals in the EAD study were used as olfactory stimuli. With the exception of the lilac aldehydes and 3-methyl-butyl-aldoxime, compounds with highest available purity were purchased from commercial suppliers. Dr. Roman Kaiser (Givaudan, Switzerland) provided 3-methyl-butyl-aldoxime, and lilac aldehydes were synthesised as follows (see also Figure 1):

6-Acetoxy-2,6-dimethyl-2,7-octadienal (**2**)

In 100ml of dioxane/H_2O (9:1) linalyl acetate (**1**, 39.2g; 0.2mol) and SeO_2 (22.2g; 0.2mol) were dissolved and stirred at 80°C for 5h. The reaction mixture was filtered through silica gel and the solvent was removed under reduced pressure. The residue was extracted three times with ether/petrolether (1:1). After removing the solvent further purification was carried out by column-chromatography (H/EE 9:1) to get **2** (13.0g; 61.9mmol; 31% yield). ^{1}H-NMR ($CDCl_3$) : δ 9.35 (s, 1H, H-8), 6.43 (m, 1H, J = 1.2/7.3 Hz, H-6), 5.92 (dd, 1H, J = 10.9/17.0 Hz, H-2), 5.16 (dd, 1H, J = 1.1/17.0 Hz, H-1), 5.14 (dd, 1H, J = 1.1/10.9 Hz, H-1), 2.32 (m, 2H, H-5), 2.05 (m, 1H, H-4), 1.99 (s, 3H, CH_3CO), 1.87 (m, 1H, H-4), 1.70 (s, 3H, 3H-9), 1.55 (s, 3H, 3H-10). ^{13}C-NMR ($CDCl_3$): δ 195.0 (C-8), 153.7 (C-6), 141.1 (C-2), 113.8 (C-1), 82.3 (C-3), 38.1 (C-4), 23.8 (C-10), 23.5 (C-5), 22.1 (C-9).

3,7-Dimethyl-1,6-octadien-3,8-diol (**3**)

To compound **2** (500mg, 2.38mmol) dissolved in 30ml of CH_2Cl_2 40ml of DIBAH (1.0 M, in CH_2Cl_2) was added dropwise at 0^{o}C. After 15min the solution was slowly poured into a mixture of 100ml of conc. HCl and 250g of ice. The organic layer was separated and the aqueous layer extracted two times with ethyl acetate. The combined organic layers were washed with sat. $KHCO_3$-solution, sat. NaCl-solution, dried with Na_2SO_4 and filtered through silica gel. After removing the solvent further purification was carried out by column-chromatography (H/EE 1:1) to get **3** (372mg;

2.19mmol; 92% yield). ^{1}H-NMR ($CDCl_3$) : δ 5.89 (dd, 1H, J = 10.6/16.9 Hz, H-2), 5.39 (m, 1H, J = 1.2/7.4 Hz, H-6), 5.20 (dd, 1H, J = 1.1/16.9 Hz, H-1), 5.05 (dd, 1H, J = 1.1/10.6, H-1), 3.95 (s, 2H, 2H-8), 1.64 (s, 3H, 3H-9), 1.26 (s, 3H, 3H-10).

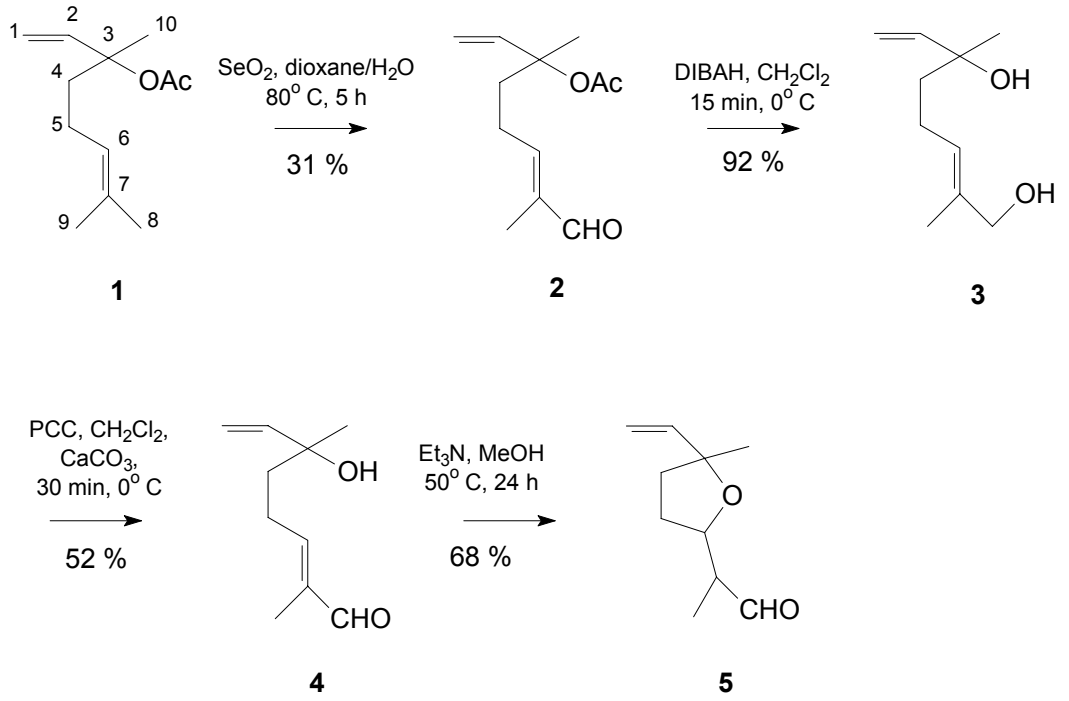
6-Hydroxy-2,6-dimethyl-2,7-octadienal (**4**)

To a stirred mixture of PCC (15.0g; 69.6mmol) and $CaCO_3$ (10.0g; 100mmol) in 250ml of CH_2Cl_2 compound **3** (5.6g; 32.9mmol) in 50 ml of CH_2Cl_2 was added dropwise and stirred for 30min at 0^{o}C. The reaction mixture was filtered through silica gel and the filtrate was washed with 300ml of H_2O. The organic phase was separated and the water phase was washed with 50ml of CH_2Cl_2 for two times. The combined organic layers were washed with sat. NH_4Cl-solution, sat. NaCl-solution, dried with Na_2SO_4 and filtered through silica gel. After removing the solvent further purification was carried out by column-chromatography (H/EE 1:1) to get **4** (2.86g; 17.0mmol; 52% yield). ^{1}H-NMR ($CDCl_3$) : δ 9.33 (s, 1H, H-8), 6.46 (m, 1H, J = 1.4/7.5 Hz, H-6), 5.88 (dd, 1H, J = 10.7/17.2 Hz, H-2), 5.20 (dd, 1H, J = 1.1/17.2 Hz, H-1), 5.07 (dd, 1H, J = 1.1/10.7 Hz, H-1), 1.69 (s, 3H, 3H-9), 1.29 (s, 3H, 3H-10).

Lilac aldehyde (**5**)

To a mixture of 25ml of MeOH and 5 drops of Et_3N compound **4** (5.66g; 33.66mmol) was added and stirred at 50^o C for 24 h. After removing the solvent the residue wad adsorbed on silica gel and further purified by column-chromatography (H/EE 12:1) to get **5** (3.8g; 22.75mmol; 68% yield). ^{1}H-NMR ($CDCl_3$) : δ 9.79 (d, 1H, J = 1.2 Hz, H-8), 5.80 (dd, 1H, J = 10.7/17.2, H-2), 5.13 (dd, 1H, J = 1.2/17.2, H-1), 4.96 (dd, 1H, 1.2/10.7, H-1), 1.26 (s, 3H, 3H-10), 1.08 (d, 3H, J = 7 Hz, 3H-9). MS m/z (%): 168 (3, $M^{+\bullet}$), 153 (26), 111 (95), 110 (46), 93 (100), 39 (33).

For comparison of the NMR data the numbering of linalyl acetate was used.



1 **2** **3**

4 **5**

Figure 1: Synthesis of lilac aldehyde isomers

Two different doses were used for the experiments: 4µg/2min, and 400µg/2min. The lower dose was obtained by diluting the compound 1:100 in paraffine (Uvasol) and applying 10µl of the paraffine dilution (Schütz et al., 1999) on filter paper (1x1cm). This dose is comparable to the scent emitted by two to ten strong scenting flowers of *S. latifolia* (S. Dötterl, unpublished data). For the higher dose 10µl of the undiluted standard compounds were applied on filter paper, resulting in a dose comparable to the flower scent emitted by a small population (200 to 1000 flowers) of *S. latifolia*. For the low dose experiments only compounds found in the scent of *S. latifolia* (as reported in Chapter 2) were used. Filter paper alone (high dose), or filter paper with 10µl paraffin (low dose) was used as control. The olfactory stimuli were offered behind gaze and different metal grids, so that the moths could not see the filter paper. In order to evaluate the attractivity of single compounds, the attractivity of single flowers of *S. latifolia* was measured for comparison. Therefore, 147 single naïve moths were tested in seven experiments to 14 flowers. Two single flowers were

offered to the moths in the experiments simultaneously. The flowers were offered at the upwind end of the tunnel behind gaze and different metal grids, so that they were invisible to the moths. “Orientation flight” was recorded if the moth flew till five to ten cm to the gaze at the upwind end towards the scent source, “landing” if the moth landed on the gaze, but only if this landing was preceded by orientation flight, and "unrolling the proboscis”, but only if this act was preceded by landing. A chi^2-test was used to compare between the attractivity of flowers and the attractivity of single standard scent compounds, and to compare the attractivity of different doses. The experiments were performed in the night at red light illumination (<0.01µE) 1-3 hours after start of the dark period. The temperature was adjusted to 22-24°C.

Results

In the electroantennographic study 58 floral scent compounds were tested on seven female and two male moths of *H. bicruris*. The antennae of the moths detected at least 18 different compounds, first of all monoterpenoids, benzenoids, fatty acid derivatives and nitrogen-bearing compounds (see Table 1), disregarding the sex of the moths. Some of the compounds coeluted, or eluted very close to each other, and it was not possible to differentiate electrophysiologically between these compounds (e.g. different lilac aldehyde isomers). The antennae of the moths did not significantly respond to any phenylpropanoid or sesquiterpenoid, or to the tested cyclic pinenes and cineoles.

The strongest signals in the antennae were elicited by phenyl acetaldehyde, the lilac aldehyde isomers, and by veratrole together with lilac aldehydes in case of coelution with an amplitude of up to about 2mV (see Figure 2). However, in most other cases, the signals were not larger than 0.5mV. In general, the EAD response increased with increasing dose of tested compound (dose-response-curve), though the variability of responses from different individuals was considerably (see Figure 2).

Table 1: Amount and electrophysiological detection of volatiles tested in 44 EAD runs to *H. bicruris*. Volatiles are arranged according to their elution on a ZB-5 column; tr: < 0.005 ng; compounds or EADs with the same superscript letter coeluted or resulted in one EAD signal respectively; *: identification is based on the injection of authentic samples; [Ca] compounds found in other Caryophyllaceae species (see Jürgens et al., 2002a, 2003; Knudsen and Tollsten, 1993), but not in *S. latifolia*; ST: sesquiterpene; +: clear EAD signal; ?: electrophysiological detection possible, but verification needed.

	Occurrence	Amount (ng)			EAD
	Number of EAD runs	Median	Min	Max	
3-Methyl-butyl aldoxime*,[a]	14	0.99	tr	39.81	[a]+
cis-3-Hexenol*	15	0.03	tr	2.50	+
3-Methyl-butyl-aldoxime[a]	14	0.02	tr	0.29	[a]+
o-Xylene*	2	8.90	8.51	9.29	-
α-Pinene*	18	0.34	tr	20.55	-
Benzaldehyde*	29	1.15	tr	1115.17	+
β-Pinene*	15	0.20	tr	8.84	-
β-Myrcene*	6	3.15	0.14	704.02	+
cis-3-Hexenyl acetate*[b]	30	4.08	tr	771.37	+
2-Hexenol acetate[b]	14	0.32	tr	14.43	?
1,4-Cineole*,[Ca]	2	6.98	4.73	9.24	-
4-Methyl anisole*,[Ca]	1	38.72	38.72	38.72	-
p-Cymene*,[Ca]	1	13.89	13.89	13.89	+
D-Limonene*	18	0.07	tr	32.95	-
cis-β-Ocimene*	23	0.49	tr	333.89	+
Benzyl alcohol*	15	0.03	tr	5.21	-
1,8-Cineole*,[Ca]	2	1.03	0.65	1.40	-
trans-β-Ocimene*	33	19.13	0.02	840.96	+
Phenyl acetaldehyde*	26	19.92	0.02	1086.90	+
2-Methoxy phenol*	27	0.14	tr	831.60	+
Linalool*	3	12.35	9.56	12.35	+
Methyl benzoate[c]	16	0.58	0.01	79.62	[c]+
Nonanal[c]	15	0.18	tr	0.87	[c]+
2-Phenylethanol[c]	14	0.21	tr	80.33	[c]+

Table 1 continued

	Occurrence	Amount (ng)			EAD
	Number of EAD runs	Median	Min	Max	
Unknown	6	0.92	0.39	1.59	+
Veratrole*	21	31.01	0.03	984.42	+
Lilac aldehyde A*,[d]	33	26.76	0.01	286.67	[d]+
Lilac aldehyde B+C*,[d]	33	44.46	0.02	477.44	[d]+
Benzyl acetate*	8	38.51	0.01	764.92	+
Benzenepropanal	1	0.01	0.01	0.01	-
Lilac aldehyde D*	33	1.37	tr	307.21	[d]+
Decanal*	16	0.08	tr	28.37	+
Methyl salicylate[e]	21	0.27	tr	33.61	+
Lilac alcohol A[e]	26	1.78	tr	78.20	?
Lilac alcohol B+C	26	0.51	tr	748.26	?
Lilac alcohol D	26	0.23	tr	73.67	?
Benzenepropanol	4	6.21	0.01	12.25	-
trans-Cinnamaldehyde	5	0.03	0.01	4.82	-
Indole*	2	0.02	tr	0.04	-
trans-Cinnamyl alcohol	2	19.93	1.30	38.55	-
Lilac alcohol formate	19	0.20	tr	2.69	-
Lilac alcohol formate	19	0.33	tr	2.99	-
Lilac alcohol formate	20	0.29	tr	10.41	-
a-Longipinene	5	2.26	0.97	4.91	-
Benzenepropyl acetate	4	5.37	0.14	5.87	-
Cycloisosativene	5	1.12	0.18	1.23	-
Longicyclene	3	1.52	1.52	6.14	-
Longifolene	5	0.43	0.01	8.24	-
γ-Muurulolene	2	0.01	0.01	0.01	-
trans-β-Caryophyllene*	17	0.03	tr	7.16	-
Cinnamyl alcohol acetate	2	1.02	0.05	1.99	-
(*E*,*E*)-α-Farnesene	10	0.42	0.05	242.91	-
ST (91,93,119,107,105,71)	2	0.37	0.37	0.37	-
Germacrene	2	0.03	0.03	0.03	-
δ-Cadinene	1	0.01	0.01	0.01	-
7-epi-α-Selinene	1	0.01	0.01	0.01	-
Dendrolasin	6	0.16	0.03	34.56	-
Benzyl benzoate*	20	18.31	0.01	94.57	-

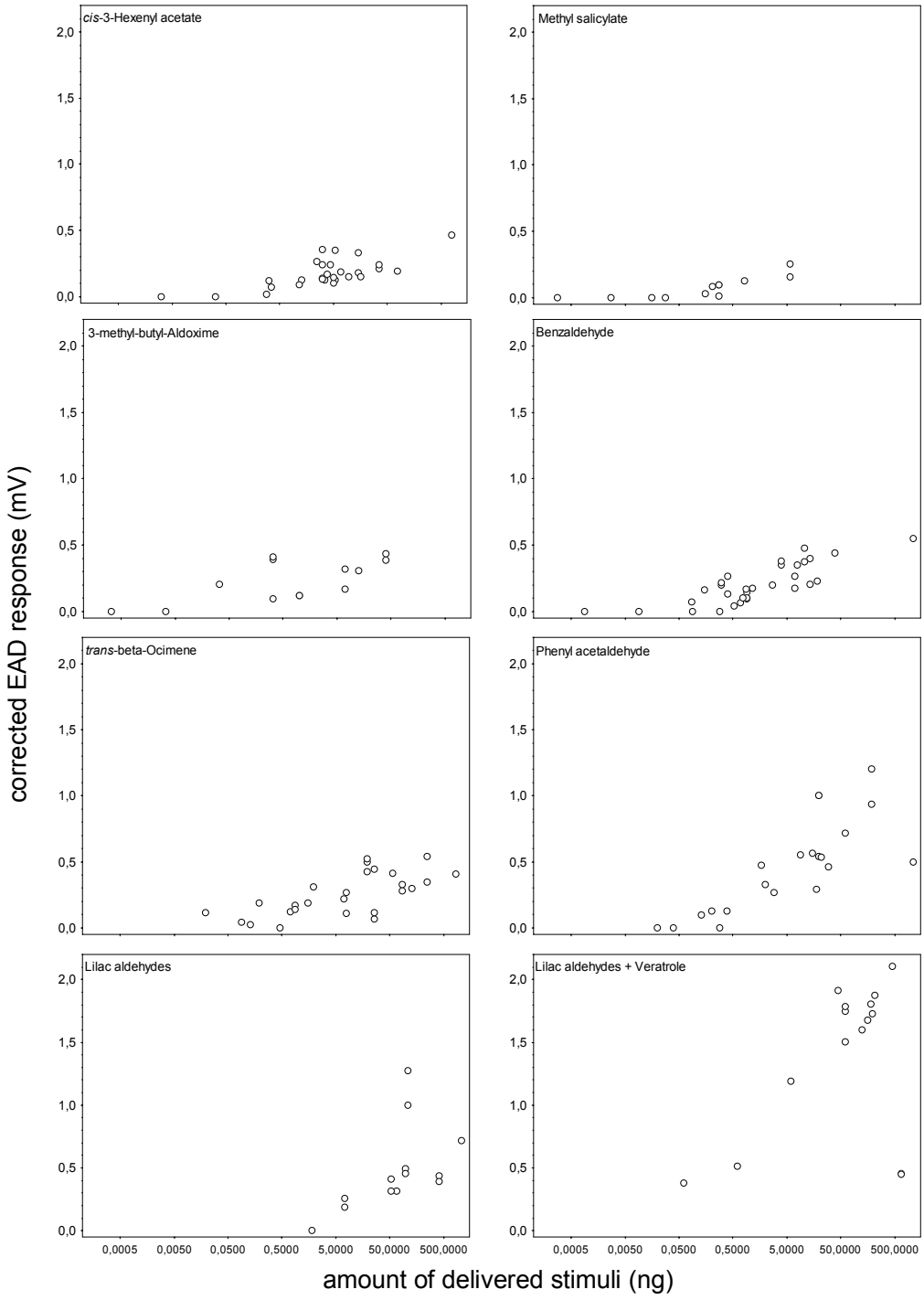


Figure 2: EAD signals of *H. bicruris* to different amounts of common floral scent compounds of *S. latifolia*. The EAD response was normalised to a standard stimulus of *cis*-3-hexenol in a 10^{-3} dilution in paraffine oil (see text).

Ten of the 18 electrophysiologically active compounds were tested in wind tunnel bioassays, some of them with two different doses (see Table 2). Seven out of ten compounds attracted moths (females and males), while benzaldehyde, *cis*-3-hexenylacetate and p-cymene did not elicit any reaction of *H. bicruris*.

Table 2: Authentic standard compounds and dose used for the wind tunnel biotests. (S): compound was found in present work or previous study (Jürgens et al., 2002a) in the scent of *Silene latifolia*; +: substance attracted moths; -: no specimen was attracted; the superscript digits indicate the number of individually tested naïve moths.

Compound	4µg/2min	400µg/2min
Benzaldehyde (S)		$-^{19}$
cis-3-Hexenyl acetate (S)		$-^{24}$
Decanal (S)		$+^{51}$
p-Cymene		$-^{19}$
Linalool		$+^{28}$
Guaiacol (S)	$+^{19}$	$+^{50}$
Phenyl acetaldehyde (S)	$+^{16}$	$+^{50}$
Veratrole (S)	$+^{20}$	$+^{50}$
3-Methyl-butyl-aldoxime (S)	$+^{20}$	
Lilac aldehydes (S)	$+^{20}$	

The most attractive compounds were lilac aldehyde isomers (see Figure 3A). These monoterpenoids attracted 90% of the tested moths, as did the scent of single flowers. All other behaviourally active compounds attracted significantly fewer individuals (11%-63%) compared to the scent of single flowers. In all three cases in which two different doses were tested, more moths were attracted by the higher dose. These differences

were significant in the case of veratrole and phenyl acetaldehyde, but not in the case of 2-methoxy phenol (guaiacol). The lilac aldehydes elicited with 65% responding moths more landings on the upwind end of the wind tunnel than any other compound tested. Moreover, they elicited even more landings than the scent of single flowers, though this difference is not significant (see Figure 3B). All other compounds elicited fewer landings than the scent of single flowers. Phenyl acetaldehyde in the high dose was the most attractive compound concerning the extension of the proboscis (see Figure 3C). 22% of the tested moths were attracted by this benzenoid, landed on the upwind end of the tunnel, and extended the proboscis. Only 5% of the tested moths unrolled the proboscis when offering the scent of single flowers. In contrast to the attraction and landing, this behavioural response was not elicited by all tested compounds.

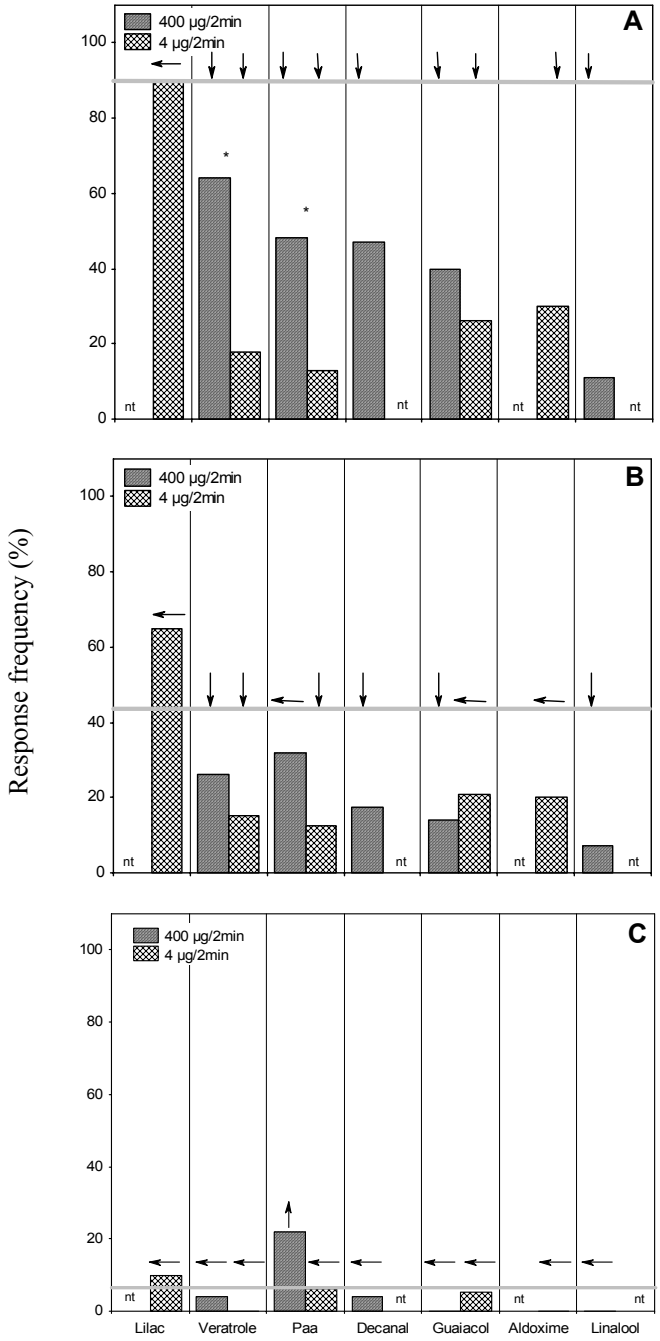


Figure 3: Attractivity of the compounds tested in a wind tunnel. A: orientation flight, B: landing, C: extension of proboscis. ——— Attractivity of scent of single flowers; ← the tested scent compound is as attractive as the scent of single flowers; ↑,↓ there are significant differences between the tested compound and the scent of single flowers (chi^2- test: $p<0.05$); *: there are significant differences in attractivity of different doses (chi^2-test: $p<0.05$); lilac: lilac aldehyde isomers; paa: phenyl acetaldehyde; aldoxime: 3-methyl-butyl-aldoxime; nt: not tested.

Discussion

The noctuid moth *Hadena bicruris* strongly responded to some floral scent compounds of *Silene latifolia* in both, the electrophysiological study and in the behavioural tests. The strongest responses of the antennae were elicited by lilac aldehyde isomers, by phenyl acetaldehyde, and by veratrole, when coeluting with the lilac compounds. These compounds were also behaviourally very active. The lilac aldehyde isomers attracted most of the tested *H. bicruris* moths (orientation flight), and were as attractive as the scent of single flowers of *S. latifolia*. Moreover, these oxygenated monoterpenes also attracted some specimens when offering a very low dose of about 0.4ng/2min (data not shown), and it seems that these compounds are very important for attracting *H. bicruris* to flowers of *S. latifolia* from far distances. These compounds are typically found only in a few nocturnal plant species in high amounts (Jürgens et al., 2002a; Knudsen et al., 1993), and in low amounts also in butterfly-pollinated plants (Andersson et al., 2002). They were also the most attractive compounds for another noctuid species, *Autographa gamma* (Plepys et al., 2002b), and are known to elicit responses in the antennae of different butterfly species (Andersson, 2003a). Probably these compounds are generally very important attractants for Lepidoptera species, though more behavioural studies are needed. Lilac aldehydes are oxygenated monoterpenes with three chirality centres, and therefore eight different isomers are possible. They were synthesised (see Figure 1) starting from an isomeric mixture of linalyl acetate resulting in both enantiomeric isomers of the lilac aldehydes A, B, C, D, respectively. Burkhardt and Mosandl (2003), Kreck and Mosandl (2003), and Kreck et al. (2003) studied the lilac aldehydes in detail, and found only four of the eight possible isomers in the nocturnally pollinated *Syringa vulgaris* L. Nothing is known about the exact isomers occurring in the floral scent of *S. latifolia*. The pure isomers were not available, and further research is needed to clarify the role of single isomers for the attraction of *H. bicruris*.

Veratrole and phenyl acetaldehyde also attracted *H. bicruris*, and there were considerable differences between the attractivity of the different doses. High doses of these compounds (comparable to the scent emitted from a small population of *S. latifolia*) attracted significantly more moths than low doses (comparable to the scent of single flowers). This result emphasises the importance of the concentration of compounds for the attraction of moths, such as *H. bicruris* (see also Brantjes, 1976c). These compounds may be of importance for both, for long-distance attraction of *H. bicruris* to a population of flowering plants, and for short-distance orientation of *H. bicruris* in the direct vicinity of *S. latifolia* flowers; in both cases the concentration of scent compounds is high. For *Autographa gamma*, Plepys et al. (2002b) found concentration of compounds an important attraction factor, whereby doses higher or lower than the ideal dose inhibited attraction. Similar conclusions were drawn by Schütz (2001) for the attraction of the colorado potato beetle *Leptinotarsa decemlineata* (Say) to potato plants. It is particularly interesting that phenyl acetaldehyde in high doses elicited significantly more proboscis extensions in *H. bicruris* than the scent of single flowers. Proboscis extension is interpreted as nectar drinking/searching behaviour, and therefore, phenyl acetaldehyde might be an important key compound for orientation of *H. bicruris* closely to or at the flowers, where the concentration of scent compounds is generally highest. Phenyl acetaldehyde is a widespread floral scent compound (Knudsen et al., 1993), and is known as a very attractive compound for butterflies and various moth species (Cantelo and Jacobson, 1979; Cunningham et al., 2004; Haynes et al., 1991; Heath et al., 1992; Honda et al., 1998; Meagher, 2001, 2002; Omura et al., 1999a; Omura et al., 1999b). It is also a potential male sex pheromone of different Lepidoptera species (Bestmann et al., 1977; Honda, 1980). Nothing is known about the attractivity of veratrole, which is typically found in few nocturnal plant species (Knudsen et al., 1993), in any other insect.

Another attractive compound besides lilac aldehyde isomers, phenyl acetaldehyde, and veratrole was 3-methyl-butyl-aldoxime. This compound

is typically found in moth-pollinated species (Kaiser, 1994; Knudsen et al., 1993), and to my knowledge, this nitrogen-bearing volatile alone was (as veratrole) never tested before in behavioural bioassays.

As reported in Chapter 2 the floral scent of *S. latifolia* was proved to be highly variable, and different chemotypes were documented. The most frequent chemotype was dominated by lilac aldehyde isomers. Interestingly, these compounds are also the most important ones for the attraction of the moths, so that *H. bicruris* seems to be well adapted to its most important host plant. Because of the high variability in the floral odour composition of *S. latifolia*, it is advantageous to rely on these marker compounds, and not on a particular scent composition. Moreover, lilac aldehydes are not often found in floral scents (Knudsen et al., 1993), increasing the probability for *H. bicruris* to find *S. latifolia* when using the lilac compounds as key substances for host plant finding together with further typical floral scent compounds of this Caryophyllaceae species such as phenyl acetaldehyde, veratrole or 3-methyl-butyl-aldoxime.

Not all compounds tested in the wind tunnel attracted moths, although they elicited significant signals in the antennae of *H. bicruris*. One of these compounds is benzaldehyde, another one is *cis*-3-hexenyl acetate. Benzaldehyde is typically a minor compound in the flower scent of *S. latifolia* and only in exceptional cases abundant (compare with Chapter 2). This benzenoid is a very widespread plant derived compound often found in floral volatiles (Knudsen et al., 1993). Because benzaldehyde is not very specific for *S. latifolia* its unimportance for the attraction of *H. bicruris* seems reasonable. The probability to find *S. latifolia* when relying on benzaldehyde may be very low for *H. bicruris*. Similarly ineffective in attracting moths is the fatty acid derivative *cis*-3-hexenyl acetate. It is a common green leaf volatile (Pare and Tumlinson, 1999), not typically belonging (exclusively) to floral scents (see also Chapter 5). Therefore it is not surprising that this compound does not attract moths in search for flowers.

Summary and perspectives

In the present study it is shown that the seed-predating pollinator *H. bicruris* is significantly attracted by specific floral volatiles emitted by its main host plant, *S. latifolia*. The most common and abundant floral scent compounds (lilac aldehydes, see Chapter 2) attracted most of the tested moths, indicating a specific adaptation of *H. bicruris* to its host plant. These oxygenated monoterpenoids may be generally very attractive to flower visiting Lepidoptera species, at least to noctuids, though more behavioural tests with other moth taxa are needed to draw general conclusions. Also, more data about the naturally occurring lilac aldehyde isomers are desirable, and the role of the single isomers for the attraction of pollinators needs to be determined.

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5. Spatial fragrance patterns in flowers of *Silene latifolia*: Lilac compounds as olfactory nectar guides?

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Abstract

Floral odour can differ qualitatively and quantitatively between different parts of flowers, and these spatial fragrance patterns within flowers can be used by pollinators for orientation on flowers. However, only few data are available describing distinct volatile compounds or blends emitted by different flower parts. Here we present results of spatial fragrance patterns within flowers of the dioecious *Silene latifolia* (Caryophyllaceae). Volatiles were collected and analysed using a highly sensitive dynamic headspace method, which allows to reduce the sample time dramatically. To determine the parts of female and male flowers responsible for scent emission, volatiles from attached intact flowers were sampled and then single flower parts were progressively removed. After each preparation step, volatiles were collected from the remaining "flower". Especially the petals and the anthophore emitted the typical flower volatiles of *S. latifolia*. However, compounds emitted from the petals differed from the compounds emitted by the anthophore. The anthophore emitted only lilac aldehydes and alcohols, whereas all other compounds of the typical *Silene* scent (e.g. benzenoids, phenylpropanoids) were emitted by the petals.

Lilac aldehydes are known to be very attractive for noctuid moths, and they may serve as nectar guides in *S. latifolia*.

Key words: attraction of moths, Caryophyllaceae, flower parts, lilac compounds, olfactory nectar guides, *Silene latifolia*, solid-phase extraction (SPE), spatial fragrance patterns.

Introduction

Floral volatiles are important cues for the attraction of insect pollinators, and especially nocturnal plant species are known to emit strong scents (Jürgens et al., 2002a; Kaiser, 1993; Knudsen and Tollsten, 1993; Miyake et al., 1998). The emitted compounds may function as long-distance and/or short-distance attractants (reviewed by Dobson, 1994). Moreover, floral odour can differ qualitatively and quantitatively between different parts of the flowers. These spatial fragrance patterns within the flowers are used by pollinators for orientation on flowers (Lex, 1954; Vogel, 1963) and can guide flower visitors to floral rewards, especially nectar (e.g. Bolwig, 1954; Dobson, 1994; Lex, 1954). However, only few data are available describing distinct volatile compounds or blends emitted by different parts of flowers (Bergström et al., 1995; Dobson et al., 1990; Dobson et al., 1996; Flamini et al., 2002; Knudsen and Tollsten, 1991; Pichersky et al., 1994; Raguso and Pichersky, 1999).

Several attributes make *Silene latifolia* Poir. ssp. *alba* (Mill.) Greut. & Burdet an interesting subject to study spatial fragrance patterns within the flowers. *Silene latifolia* is a nocturnal, dioecious Caryophyllaceae species, and emits a strong scent during the night (Jürgens et al., 2002a). Main pollinators are different nocturnal Lepidoptera species (Altizer et al., 1998; Ellis and Ellis-Adam, 1993; Jürgens et al., 1996; Young, 2002), and the floral scent is known to be responsible for the attraction of at least some flower visitors (Chapters 3 and 4; Brantjes, 1976b, c, d). One of these

flower visitors is the seed-predating pollinator *Hadena bicruris* Hufn. (Lepidoptera: Noctuidae). The female moths lay their eggs in the female flowers of their main host plant, *S. latifolia* (Bopp and Gottsberger, 2004). Further, nectar is consumed by both sexes of the moths from both male and female flowers of *S. latifolia*. Flowers are thereby pollinated and the larvae feed on the developing seeds (Bopp, 2003; Brantjes, 1976b, c). *Hadena bicruris* is attracted to the flowers by floral scent, which also elicits landing on flowers, and even extension of the proboscis (Chapters 3 and 4; Brantjes, 1976c, d). The floral scent of *S. latifolia* is especially dominated by different monoterpenoids, and benzenoids (Jürgens et al., 2002a). However, the qualitative scent variability between different populations is high (Chapter 2). Nevertheless, the most common and abundant compounds in the floral scent of *S. latifolia* are lilac aldehydes (Chapter 2). These monoterpenoids are also the most attractive compounds for *H. bicruris*, and are alone as attractive as the scent of whole flowers (Chapter 4).

In the present work spatial fragrance patterns of five female and seven male flowers of *S. latifolia* were analysed. Furthermore, the scent emitted by male and female flowers was compared in terms of quantity and quality. Because eggs are deposited only in female flowers (Brantjes, 1976b), it is hypothesised that floral scent differences between female and male flowers are used by female *H. bicruris* to distinguish between female and male flowers.

Material and methods

Volatile collection

Floral scent samples were collected and analysed from 5 female flowers, and from 7 male flowers of *Silene latifolia* (see Figure 1) in the laboratory under an extractor hood using dynamic headspace methods. To determine the parts of the flowers responsible for scent emission, volatiles from

attached intact flowers were collected for two minutes and then single flower parts were progressively removed. After each preparation step, volatiles were collected from the remaining "flower" for another two minutes. In male flowers calyx, petals, stamens, anthophore, and the base of the flower were removed separately. In female flowers calyx, petals, styles, gynoecium, and at last the anthophore together with the base of the flower were removed. In female flowers the anthophore is very small, and therefore it was impossible to remove with certainty the anthophore and the flower base separately. The last volatile collection from flowers of both sexes was conducted from the remaining floral stem. The flowers respectively remaining "flowers" were enclosed within a polyethylene oven bag (Toppits®) and the emitted volatiles were trapped in an adsorbent tube through the use of a membrane pump (ASF Thomas, Inc.). The flow rate was adjusted to 200ml/min using a power supply and a flow meter. Samples were collected for 2min in the night, when *S. latifolia* is emitting most of its floral volatiles (S. Dötterl, unpublished data). ChromatoProbe quartz microvials of Varian Inc. (length: 15mm; inner diameter: 2mm) were cut at the closed end, filled with a mixture (1:1) of 3mg Tenax-TA (mesh 60-80) and Carbotrap (mesh 20-40), and used as adsorbent tubes. The adsorbents were fixed in the tubes using glass wool. Simultaneous collections of the surrounding air were used to distinguish between floral compounds and ambient contaminants.

Chemical analysis

The samples were analysed on a Varian Saturn 2000 System using a 1079 injector that had been fitted with the ChromatoProbe kit. This kit allows the thermal desorption of small amounts of solids or liquids contained in quartz microvials (Micro-SPE, Amirav and Dagan, 1997; Wilkinson and Ladd, Varian Application note), or in the present case the thermal desorption of the trapped volatiles. The adsorbent tube was loaded into the probe, which was then inserted into the modified GC injector.

The injector split vent was opened (1/20) and the injector heated to 40°C to flush any air from the system. The split vent was closed after 2 minutes and the injector was heated at 200°C/min, then held at 200°C for 4.2min, after which the split vent was opened (1/10) and the injector cooled down.
A ZB-5 column (5% phenyl polysiloxane) was used for the analyses (60m long, inner diameter 0.25mm, film thickness 0.25µm, Phenomenex). Electronic flow control was used to maintain a constant helium carrier gas flow of 1.8ml min^{-1}. The GC oven temperature was held for 7min at 40°C, then increased by 6°C per min to 250°C and held for 1min. The MS interface was 260°C and the ion trap worked at 175°C. The mass spectra were taken at 70eV (in EI mode) with a scanning speed of 1 scan s^{-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; Davies, 1990). Identification of individual components was confirmed by comparison of both mass spectrum and GC retention data with those of authentic standards.
The samples were quantified by injection of known amounts of lilac aldehydes, *trans*-β-ocimene, *cis*-3-hexenyl acetate, benzaldehyde, phenyl acetaldehyde, veratrole, and the mean response of these compounds was used to determine the amount of scent emitted.

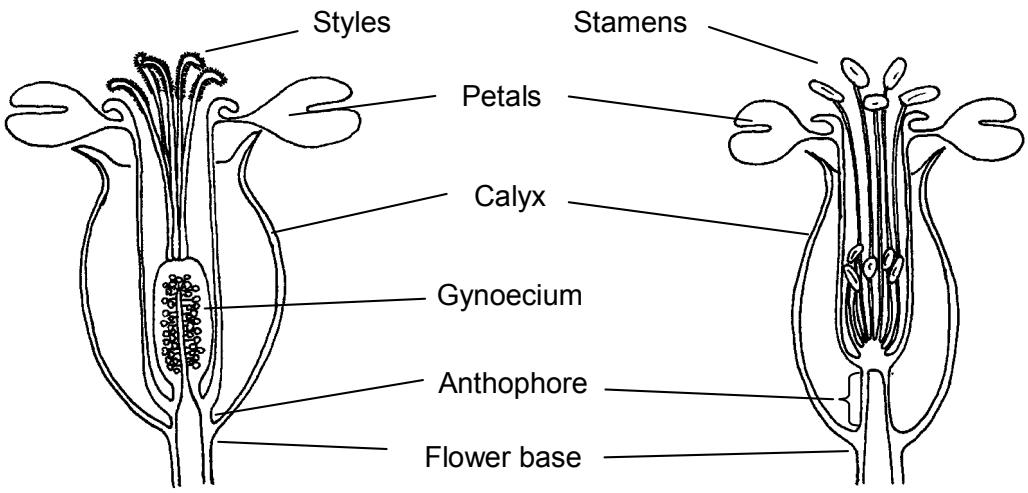

Figure 1: Schematic drawings of a female and a male flower of *Silene latifolia.*

Data analyses

The total amount of emitted volatiles varied greatly between the single flowers of *Silene latifolia*. In order to standardise the results for further data analyses the maximum amount of a particular compound emitted by an individual flower during all sample collections was equated with 100%. For each flower and each flower stage the amount of single compounds is given as percentage of the maximum amount of this particular compound found in the considered flower. These data were used for a correspondence analysis in the STATISTICA package (StatSoft Inc., 2003), to determine compounds showing a similar emission pattern during the preparation process. For compounds found in more than one female or male flower, mean values were calculated, and used in the correspondence analyses. Compounds with similar emission patterns were pooled for further analyses and the differences in the emitted relative amounts of volatiles between different “flower stages” were compared using the Kruskal-Wallis-ANOVA (data not normal-distributed, Kolmogorov-Smirnov test), also in the STATISTICA package (StatSoft

Inc., 2003). The Tukey-Kramer test for non-parametric data was used as post-hoc test (Siegel and Castellan, 1988). The mean amounts of emitted volatiles of female and male flowers were compared using the t-test for independent samples. Kolmogorov-Smirnov test was used as test for normality, and Levene´s test was used as test for homogeneity of variances (STATISTICA, StatSoft Inc., 2003).

Results

The total amount of emitted volatiles varied greatly from 8.3ng/2min to 317.6ng/2min between the single flowers of *Silene latifolia*. The mean emitted amount in males (76.4 ± 108.9; mean ± st.dev.) was higher than in females (42.5 ± 46.6), though this difference is not significant ($t_{df=10}$ = 0.65, p = 0.53).

Twenty-four compounds were detected in the scent of the five female and seven male flowers (see Table 1). The compounds listed in Table 1 are ordered in classes, which to some degree reflect their biosynthetic origin (see Knudsen et al., 1993). Dominant compound classes were monoterpenoids, and benzenoids. Additionally, fatty acid derivatives, phenylpropanoids and nitrogen-bearing compounds were found. The most commonly occurring compounds found in all samples were *cis*-3-hexenyl acetate, benzaldehyde, and *trans*-β-ocimene. Commonly occurring volatiles were also lilac compounds (especially lilac aldehyde isomers), phenyl acetaldehyde, and benzyl benzoate. Seventeen out of the 24 compounds were found in both sexes, four compounds were only found in some of the female flowers (methyl benzoate, benzenepropanal, benzenepropanol, benzenepropyl acetate), and three compounds were only found in one male flower each (veratrole, benzyl acetate, indole).

For both sexes, females and males, separate correspondence analyses of emission-patterns of the compounds during the preparation process produced two groups. The results of females and males were very similar, therefore only data for males are shown in Figure 2.

Table 1: Relative amount of compounds found in five female (F) and seven male (M) flowers of *Silene latifolia*. RI: Kovats retention index.

Compound	RI	F1	F2	F3	F4	F5	M1	M2	M3	M4	M5	M6	M7
Total amount (ng/2min)		124.2	32.6	27.6	8.3	19.7	21.9	317.6	14.2	76.4	22.8	22.5	59.2
Fatty acid derivatives													
cis-3-Hexenol	860	3.1	0.5	1.9	3.8	0.4	-	-	-	0.8	3.8	2.0	0.5
cis-3-Hexenyl acetate*	1016	10.2	3.8	7.3	12.6	2.8	1.4	0.7	0.3	11.0	7.6	7.7	1.3
Benzenoids													
Benzaldehyde*	982	1.7	1.7	3.6	21.5	1.2	0.4	0.5	1.5	4.0	7.7	31.0	15.2
Benzyl alcohol*	1050	5.2	3.7	2.4	2.1	-	-	-	0.3	6.3	2.1	-	0.3
Phenyl acetaldehyde*	1060	-	-	-	3.8	1.3	2.1	4.9	1.3	0.2	9.3	-	35.5
2-Methoxy phenol*	1102	4.7	2.3	-	-	-	-	-	-	-	10.5	0.3	1.3
Methyl benzoate	1107	0.1	0.8	30.9	-	-	-	-	-	-	-	-	-
2-Phenylethanol	1129	0.6	0.2	-	-	-	-	-	-	0.1	-	-	0.3
Veratrole*	1153	-	-	-	-	-	-	-	-	-	0.7	-	-
Benzyl acetate*	1174	-	-	-	-	-	-	-	-	-	13.3	-	-
Methyl salicylate	1208	0.6	-	-	18.8	-	-	-	1.6	8.2	5.1	0.3	0.4
Benzyl benzoate*	1789	2.1	2.9	12.7	1.8	0.7	-	-	14.7	9.6	2.4	-	2.2
Phenylpropanoids													
Benzenepropanal	1178	-	0.1	-	-	-	-	-	-	-	-	-	-
Benzenepropanol	1243	4.0	2.3	-	-	-	-	-	-	-	-	-	-
trans-Cinnamaldehyde	1287	2.7	1.8	-	6.6	7.4	-	0.6	-	-	-	0.8	-
trans-Cinnamyl alcohol	1319	15.4	7.9	-	10.4	22.0	-	2.7	-	-	-	-	-
Benzenepropyl acetate	1380	0.1	-	-	-	-	-	-	-	-	-	-	-
Nitrogen bearing compounds													
3-Methyl-butyl-aldoxime*	858	1.9	5.8	-	-	-	0.5	1.2	-	-	1.0	-	0.8
3-Methyl-butyl-aldoxime*	870	1.3	3.6	-	-	-	-	0.1	-	-	0.2	-	0.7
Indole*	1307	-	-	-	-	-	-	-	-	-	1.9	-	-
Monoterpenoids													
trans-β-Ocimene*	1058	18.4	22.9	10.3	18.8	3.2	3.0	0.2	0.8	4.1	1.7	12.7	1.3
Lilac aldehyde A*	1154	9.3	14.5	9.8	-	15.6	34.9	32.8	26.5	15.2	10.8	13.5	12.4
Lilac aldehyde B+C*	1163	13.6	20.3	15.7	-	40.1	46.1	43.8	45.2	27.1	16.6	22.0	21.1
Lilac aldehyde D*	1178	4.5	3.3	4.9	-	5.1	11.7	11.1	7.3	8.1	4.2	9.4	6.4
Lilac alcohol A*	1211	0.1	0.1	0.1	-	0.2	-	0.3	0.3	0.2	0.3	0.2	0.1
Lilac alcohol B+C*	1219	0.3	0.7	0.2	-	-	-	0.4	0.3	1.3	0.5	0.0	0.1
Lilac alcohol D*	1232	0.2	0.6	0.1	-	-	-	0.6	-	4.0	0.2	0.1	0.1

In the first group all benzenoids, phenylpropanoids, nitrogen-bearing compounds and *trans*-β-ocimene are found, whereas the second group comprises all lilac compounds and the fatty acid derivatives *cis*-3-hexenyl acetate and *cis*-3-hexenol. The compounds of the first group, and the compounds of the second group (excluding the fatty acid derivatives), were pooled for further analyses. The fatty acid derivatives were treated as separate group, because they are mostly known as "green-leaf-volatiles" and are not regarded as typical floral scent compounds (see e.g. Pare and Tumlinson, 1999).

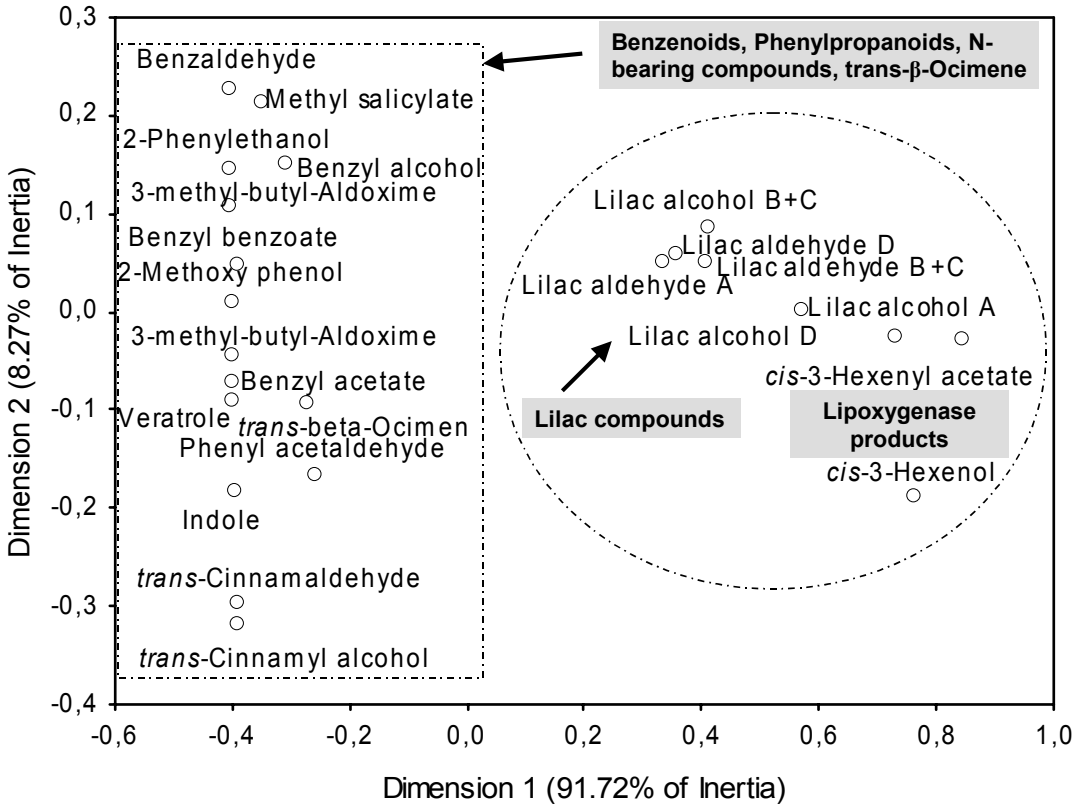

Figure 2: Correspondence analysis of compounds found in male flowers using data of emission patterns of compounds during the preparation process (see Data analyses).

In both, females and males, the intact flower emitted the smallest relative amounts of the green leaf volatiles. The amount of these lipoxygenase products increased in female flowers during flower preparation, but remained relatively constant in male flowers after removal of the calyx (see Figure 3).

A totally different pattern was observed for benzenoids, phenylpropanoids, nitrogen-bearing compounds, and *trans*-β-ocimene. These compounds were only detected in the samples from whole flowers and flowers after removal of the calyx. They disappeared almost completely as soon as the petals were removed. It seems that these compounds are produced in both females and males almost exclusively by the petals (see Figure 3).

The amount of the lilac compounds emitted was relatively constant in the first four samples of males and females. In males, the emitted amount of lilac aldehydes and lilac alcohols decreased dramatically when removing the anthophore. In females it decreased in a first step already when removing the gynoecium, and in a significant second step when removing the anthophore together with the flower base.

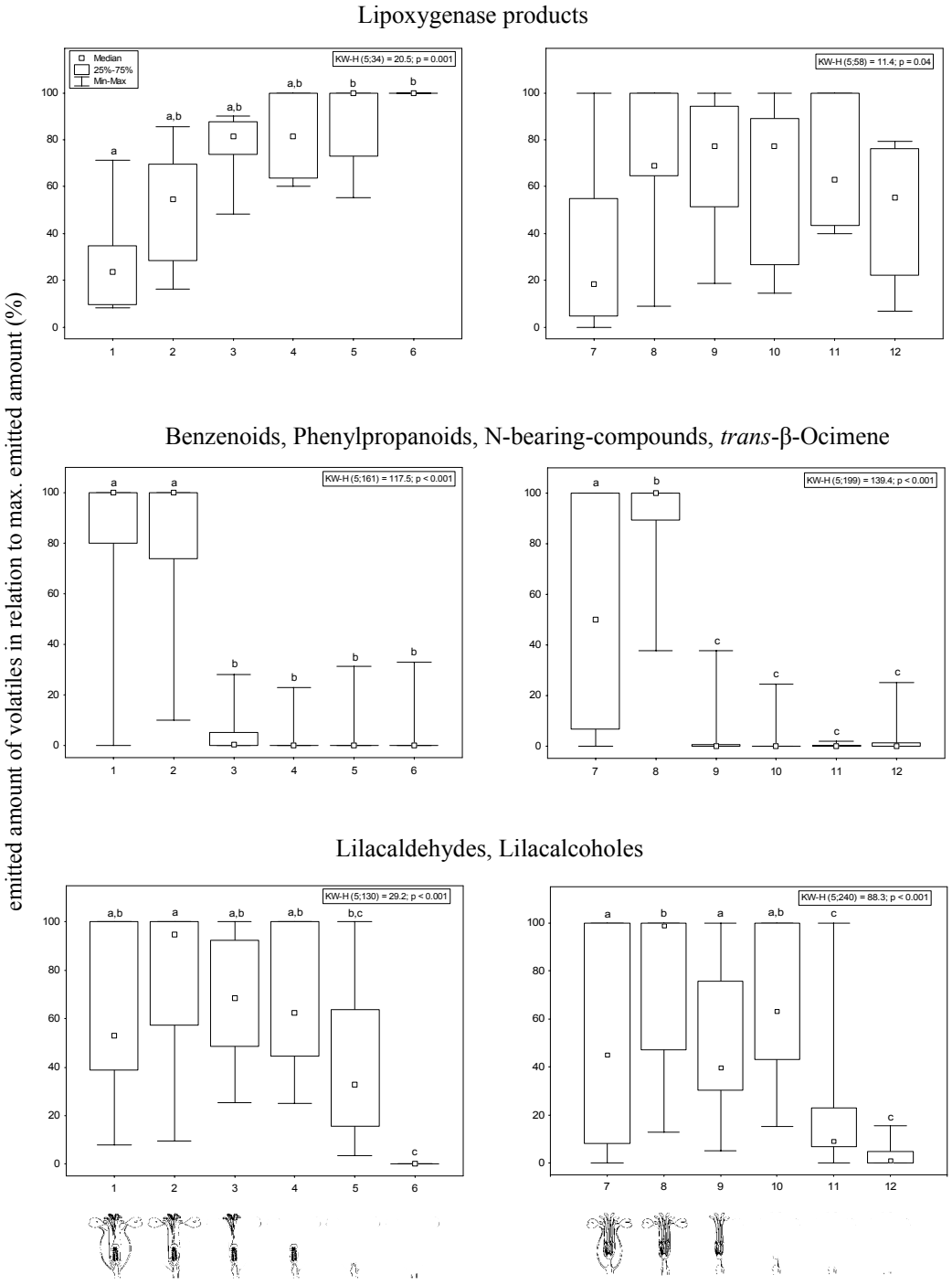

Figure 3: Emission patterns of compound classes in female (left) and male (right) flowers. x-axis: flower and flower preparation stage of females (1-6) and males (7-12) used for collection of volatiles. Volatiles were collected first from whole flowers. Parts of the flowers were then successively removed and volatiles were collected from the remaining "flower" (see Volatile collection).

Discussion

The results of the present work clearly indicate a spatial fragrance pattern within flowers of *Silene latifolia*. The pattern is similar in both female and male flowers. Most compounds are emitted by the petals, but the lilac aldehydes and alcohols are emitted within the flowers, especially by the column like anthophore. In females the anthophore was removed together with the flower base resulting in a decrease of emitted lilac compounds. However, there are indications that also in females the anthophore alone, and not the flower base, is responsible for emission of lilac compounds. When enclosing and collecting scent of pieces of anthophores high amounts of lilac compounds were detected. This was not the case when analysing scent emitted by flower bases (S. Dötterl, unpublished data). All benzenoids, phenylpropanoids, nitrogen-bearing compounds, and *trans*-β-ocimene are emitted by the petals, and staining with neutral red indicates that only the upper white parts (ligulae, lobes) surrounding the flower entrance, and not the green parts (claws) enclosed from the calyx, are responsible for scent emission (Jürgens et al., 1996).

Therefore it seems that lilac compounds are marking the flower opening, and they may guide flower visitors to the entrance of the floral tube and finally to the nectar, which is secreted at the base of the filaments (which are also present in female flowers in a very reduced form, see Witt, 2003), and stored at the calyx base (Vogel, 1998) near the anthophore emitting lilac compounds.

In *S. latifolia* lilac aldehydes are much more abundant compared to the lilac alcohols, and it was proved that they effectively attract *Hadena bicruris* (Chapter 4), and *Autographa gamma* (Plepys et al., 2002b). These two noctuids are prominent flower visitors of *S. latifolia* (Jürgens et al., 1996), and the *Hadena* species is furthermore a highly specific fruit predator (Brantjes, 1976b). Maybe these moths use lilac aldehydes for both, attraction to the flower from far distances, and for orientation at the flowers to find the nectar. Also, petal odours may contribute to the attraction of flower visitors and to short-distance orientation near or at the

flowers, because some of these odours are reported to be behaviourally active in potential *S. latifolia* pollinators, especially in noctuid Lepidoptera species (Chapter 3; Bruce and Cork, 2001; Fraser et al., 2003; Haynes et al., 1991; Heath et al., 1992; Meagher, 2001, 2002; Plepys et al., 2002b). More detailed behavioural studies are now needed to test these hypotheses. Nevertheless, it is known especially from bees that insects can use distinct scenting parts of flowers for location of food rewards (Lex, 1954; von Aufsess, 1960). However, it was not possible for the latter authors to identify the compounds emitted by different parts of the flowers. Floral scent "nectar guides" were also found or at least suggested in *Ranunculus acris* L. (Bergström et al., 1995), *Clarkia breweri* (Gray) Greene (Raguso and Pichersky, 1999), and *Laurus nobilis* L. (Flamini et al., 2002), but with the exception of *R. acris* only chemical data point towards nectar guides, and no information is available about the attractivity of the nectar guiding compounds to particular flower visitors. The nectary portions of the petals in *R. acris* emit more volatiles, and different relative amounts of some compounds than the non-nectary portions of the petals (Bergström et al., 1995). Such differences are perceptible to bees (von Aufsess, 1960), and may be helpful in locating the nectaries, thus increasing the foraging efficiency of bees (Bergström et al., 1995).

The styles and stamens did not significantly contribute to the floral odour of *Silene latifolia*. This result is in contrast to the findings from other plant species (Bergström et al., 1995; Dobson et al., 1990; Dobson et al., 1996; Flamini et al., 2002; Knudsen and Tollsten, 1991; Pichersky et al., 1994; Raguso and Pichersky, 1999), where at least one of these sexual organs was characterised by a particular scent profile differing from other flower parts. Knudsen and Tollsten (1991) e.g. found in *Bombus*-pollinated *Pyrola* species, and in *Moneses uniflora* (L.) A. Gray characteristic scent profiles of petals and stamens. Pollen is collected from these species by vibration of the poricidal anthers, and pollination is dependent on how

effectively the bees can orientate themselves towards the anthers. The authors assume that scent can obviously function as a close range orientation cue for the bumblebees in locating food rewards, or can bring pollinators in position for effective pollination. Bergström et al. (1995) drew a similar conclusion for *Ranunculus acris*. Pollen was characterised in this study by a scent profile distinct from all other studied floral parts.
However, the plants studied by Knudsen and Tollsten (1991), and Bergström et al. (1995) are pollinated by pollen collecting bees. This is not the case in *S. latifolia*, where especially noctuids, sphingids, and geometrids are important pollinators (Altizer et al., 1998; Ellis and Ellis-Adam, 1993; Jürgens et al., 1996; Young, 2002). These Lepidoptera species visit the flowers of *S. latifolia* only for nectar drinking (and oviposition in case of *Hadena bicruris*), but not for pollen reward. As Brantjes (1976c) could show for *H. bicruris* moths, they thereby automatically pollinate the flowers, because styles and stamens are protruding from the entrance of the flowers, and flower visitors have to pass these sexual organs to reach the nectar (see also Figure 1). For *S. latifolia* it is therefore not necessary to have “attractive” anthers and styles, as it is sufficient for successful pollination to guide the flower visitors to the nectar in the depth of the floral tube.

In *S. latifolia* the lipoxygenase products *cis*-3-hexenol and *cis*-3- hexenyl acetate were not typical floral scent compounds as they were also emitted from the floral stems, when the flowers were already totally removed. The amount of these fatty acid derivatives increased as soon as the flowers were injured, and parts of the flowers were removed (see Figure 3). It is known that these “green leaf odours” are produced by the autolytic oxidative breakdown of membrane lipids, and are released when parts of the plant are mechanically damaged (Pare and Tumlinson, 1999).

When comparing female to male flowers, emission rates of volatiles in *S. latifolia* did not differ significantly, but at least some qualitative differences

were found (see Table 1). Female *H. bicruris* moths drink nectar from both flowers, but eggs are deposited only in female flowers (Bopp, 2003; Brantjes, 1976b, c). It was assumed that volatiles could be important cues for female moths to distinguish between female and male flowers (Bopp, 2003; Brantjes, 1976c). However, this would only make sense if some compounds were exclusively emitted from all females, and not from males (or vice versa). In the present study some compounds were detected only in female flowers, but they were found only in some, and not in all females. Therefore it is unlikely that the floral volatiles reported here serve as cues for *H. bicruris* to distinguish between female and male flowers. This assumption was confirmed comparing the scent of 118 male flowers/specimens with 40 female flowers/specimens, because no gender specific scent compounds were found at all (S. Dötterl, unpublished data).

Summary and perspectives

The present study showed that especially the petals and the anthophore emitted the flower volatiles of *S. latifolia*. In females also the gynoecium seems to play a role in emitting floral volatiles. The compounds emitted from the petals were qualitatively quite different to the volatiles emitted by the anthophore and the gynoecium. The latter parts of the flowers solely emitted the lilac aldehydes and alcohols. The lilac aldehydes are known to be very attractive for noctuid moths, and these monoterpenoids may serve as nectar guides in *S. latifolia*. However, biotests are necessary to prove, if (naïve or experienced) moths use the observed spatial floral scent patterns for food location.

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6. Synopsis

This thesis gives a detailed description of the importance of flower volatiles in a nursery pollination system, in which an insect reproduces within the flowers it pollinates. In detail, the variability and spatial patterns of the floral scent of *Silene latifolia* Poir. ssp. *alba* (Mill.) Greut. & Burdet, the main nectar and larval host plant of the nursery pollinator *Hadena bicruris* Hufn., were studied. Furthermore, electrophysiological and behavioural responses of *H. bicruris* to the scent of whole flowers as well as to single scent compounds of *S. latifolia* were examined. This is the first study combining data about floral scent variability with electrophysiological and behavioural data of a pollinator, which in the present case also is a highly specific predator. In general, little data about how pollinators respond to single floral scent compounds are available (Dudareva and Pichersky, 2000).

Floral scents are important signals for chemical communication between flowering plants and animal pollinators (Pellmyr and Thien, 1986). Especially nocturnally pollinated plant species are known to have strong scents, and to rely on floral scents for attraction of pollinators (Jürgens et al., 2002a; Knudsen and Tollsten, 1993; Miyake et al., 1998; Raguso et al., 2003; Raguso and Pichersky, 1995). In general, the attracted insects pollinate the flowers, and are rewarded with food, like pollen, nectar, or oil (Vogel, 1983). However, there are also examples in which the floral volatiles themselves are the reward (e.g. Eltz et al., 2003), or in which volatiles mimic female sex pheromones of hymenopteran insects to attract males, which “copulate” with the flower, and thereby pollinate it (pseudocopulatory pollination). In latter systems, the plants exploit the male pollinators, because they do not provide any reward (e.g. Schiestl et al., 2000). There are also systems known, in which pollinators reproduce within the flowers they pollinate (nursery pollination, see review by Dufay

and Anstett, 2003). One of these systems is the interaction between the noctuid moth *Hadena bicruris* and the Caryophyllaceae *Silene latifolia*.

Silene latifolia (Caryophyllaceae) is a dioecious plant and relies especially on different nocturnal Lepidoptera species for pollination (Jürgens et al., 1996; Young, 2002), though also day-active insects were observed on its flowers (Altizer et al., 1998; Jürgens et al., 1996; Shykoff and Bucheli, 1995; Young, 2002). Seed set resulting from moth pollination is more than twice as seed set resulting from visitation by diurnal insects (Young, 2002). The pollinators are attracted in the night by a strong and complex floral scent blend (Chapters 1 and 4; Jürgens et al., 2002a). Like most plant taxa (Faegri and van der Pijl, 1979), *S. latifolia* is under selective pressure to increase pollination efficiency. Therefore it seems reasonable that selection is modifying (adapting) the floral scent of *S. latifolia* in order to keep attraction of pollinators and thus pollination optimal to the plants needs. One of the various pollinators of *S. latifolia* is *H. bicruris*, which is also attracted to the flowers by their scent (Brantjes, 1976b, c). Female and male moths of *H. bicruris* use flowers of both sexes to drink nectar, and female moths additionally lay their eggs in female flowers, and the larvae feed on the growing seeds (Bopp and Gottsberger, 2004). *Hadena bicruris* is therefore pollinator and fruit predator at the same time. To ensure high reproductive success, *H. bicruris* has to find its most important host plant effectively, because seeds of *S. latifolia* are most profitable for nutrition of *H. bicruris* larvae compared to seeds of other Caryophyllaceae (Bopp and Gottsberger, 2004; P. Oberpaul, personal communication). Moreover, it is important for females to effectively pollinate the flowers chosen for egg deposition to guarantee a food supply for the larvae. Data about pollen loads on museum specimens of *H. bicruris* indicate that this moth indeed effectively finds its host plant (S. Dötterl, unpublished data). On 26 of 34 studied specimens exclusively pollen typical for *S. latifolia* was found. Pollination efficiency of *H. bicruris* (Brantjes, 1976c) showed that females transfer up to 276 pollen grains to the stigmas of female *S.*

latifolia flowers before egg deposition, indicating a highly effective pollination mechanism. Flowers of *S. latifolia* have in the mean 540 ± 63 ovules (Jürgens et al., 2002b).

The interaction between *S. latifolia* and *H. bicruris* can be interpreted as parasitism rather than as mutualism (see Bopp, 2003; Brantjes, 1976c; Dufay and Anstett, 2003). It was therefore hypothesised that *S. latifolia* and its floral scent are generally adapted to moth pollination, and not specifically to *H. bicruris*, though *H. bicruris* is specifically adapted to the scent of *S. latifolia*, its main host plant, ensuring effective host plant detection. Furthermore, the question arose whether *S. latifolia* might be able to avoid or at least to reduce parasitism by *H. bicruris* by means of floral scent.

To test these hypotheses and to get answers to the question, different approaches were made. In a first step, the variability of the floral scent was analysed, characterising the scent of 98 *S. latifolia* specimens of different European and North American populations, thus determining the most typical floral scent compounds emitted by this species. These data allow conclusions about the adaptation of the floral scent to pollinators in general. The specific adaptation of *H. bicruris* to the floral scent of *S. latifolia* was studied testing the reaction of the moths to different natural scent blends as well as to single scent compounds of *S. latifolia* in electrophysiological and behavioural studies.

Adaptation of floral scent of Silene latifolia to nocturnal pollinators

The flowers of *S. latifolia* open in the evening and close by midmorning. During the day the petals are closing the entrance to the calyx tube (Jürgens et al., 1996). Floral scent is emitted during the night, when the flowers are open (Jürgens et al., 1996, 2002a). The scent is dominated especially by monoterpenoids and benzenoids with different lilac aldehyde isomers, *trans*-β-ocimene, benzaldehyde, phenyl acetaldehyde, benzyl acetate, and veratrole as typical compounds (Chapter 2; Jürgens et al., 2002a). Most of these compounds are frequently found in species

pollinated by noctuid moths or hawkmoths (Jürgens et al., 2002a; Kaiser, 1993; Knudsen and Tollsten, 1993; Miyake et al., 1998), and at least for some of these components it is shown that they attract nocturnal Lepidoptera, among them *H. bicruris* (Chapter 4; Bruce and Cork, 2001; Fraser et al., 2003; Haynes et al., 1991; Heath et al., 1992; Meagher, 2001, 2002; Plepys et al., 2002b).

When analysing the scent of specimens originating from different European and North American populations, a high variability, and several different chemotypes were found (see Chapter 2), comparable to other studies, where the variability of floral scent was determined (summarised by Knudsen, 2002). Compounds dominating a particular chemotype were only minor compounds or even absent in other chemotypes (e.g. lilac aldehydes, *trans*-β-ocimene, phenyl acetaldehyde, veratrole). Nevertheless, the most common chemotype was characterised by high amounts of lilac aldehydes, followed by samples dominated by phenyl acetaldehyde. These compounds are known to be most attractive for nocturnal Lepidoptera (Chapter 4; Cantelo and Jacobson, 1979; Cunningham et al., 2004; Haynes et al., 1991; Heath et al., 1992; Meagher, 2001, 2002; Plepys et al., 2002b). The lilac aldehydes furthermore seem to be nectar guides in *S. latifolia* (Chapter 5). Considering the morphology of the *S. latifolia* flowers (see Chapter 5), the flower visitors automatically get in contact with pollen (anthers) or styles, when drinking nectar, and thereby pollinate the flowers. The reproductive success of *S. latifolia* may therefore increase when guiding the insects to the nectar with attractive compounds.

Consolidating the data of chemical composition of floral scent (Chapter 2), and of spatial fragrance patterns within the flowers (Chapter 5), the scent of *S. latifolia* seems quite well adapted to nocturnal pollinators. Besides other floral traits, such as phenology or morphology, chemical composition and spatial fragrance patterns may contribute to a high reproductive success of *S. latifolia*.

Adaptation of Hadena bicruris to the flower scent of Silene latifolia

Since Brantjes (1976b, c) investigated the relationship between *H. bicruris* and *S. latifolia*, it was known that the moth is attracted by floral scent. However, nothing was known about the floral scent compounds important for the attraction of *H. bicruris*. In general, little is known about how pollinators respond to single floral scent compounds (Dudareva and Pichersky, 2000; Pichersky and Gershenzon, 2002).

When testing different floral scent chemotypes of *S. latifolia* in a GC-FID/EAD or GC-MS/EAD study to *H. bicruris*, all tested extracts elicited significant signals in the antennae of the moths (see Chapter 3). Moreover, the moths could clearly distinguish electrophysiologically between most chemotypes tested (Chapter 3). Interestingly, when testing the most common chemotypes (Chapters 2, and 3) that are dominated either by lilac aldehydes or by phenyl acetaldehyde, these main compounds elicited main signals in the antennae. Furthermore, wind tunnel bioassays with single flowers have shown that flowers emitting high amounts of lilac aldehydes or phenyl acetaldehyde were very attractive to *H. bicruris* (Chapter 3). When testing uncommon *S. latifolia* chemotypes, dominated e.g. by β-myrcene or methyl benzoate, these main compounds did not elicit main signals in the antennae of the moths.

The most common and abundant compounds in the scent of *S. latifolia* (lilac aldehydes) proved to be the most attractive ones, when testing them alone in the wind tunnel. Surprisingly, they were as attractive as the scent of single flowers (Chapter 4). Furthermore, the spatial emission pattern of these oxygenated monoterpenoids in flowers suggests that they serve as nectar guides (see above). In conclusion, *H. bicruris* relies on compounds for attraction which are the most common and abundant ones in floral scent of *S. latifolia*, and which may guide the moths to the food reward (nectar). The moths pollinate the female flowers by drinking nectar ensuring food reward for the larvae (Brantjes, 1976c). These results indicate an adaptation of the moth to the scent of its host plant.

Floral scent variation - Options for reducing seed predation by Hadena bicruris in Silene latifolia?

The interaction between *H. bicruris* and *S. latifolia* was studied in detail in the 70ies, and in a recent work, yet (Bopp, 2003; Brantjes, 1976b, c). These studies dealt with the question whether the interaction should be regarded as mutualism or as parasitism. Both authors concluded that this relationship has more tendencies being a form of predation by the moth (parasitism) than a mutualism, because the costs for the plant in regard to the activities of the moth (seed predation) are higher than the benefit (pollination) for the plant. However, parasitism of larvae by hymenopteran species as well as abiotic factors can dramatically reduce the number of larvae, so that more seeds are “produced” by pollination than fed by the larvae (Bopp, 2003). Also Dufay and Anstett (2003) regarded these relationship more as parasitism than as mutualism. They described conflicts of interests occurring in 13 known nursery pollination systems, where pollinators reproduce within the flowers/inflorescences they pollinate, among them also the popular mutualistic interactions between yucca and yucca moths, and fig and fig wasps (Riley, 1892; Wiebes, 1979). In contrast to these popular systems, and in general in contrast to most other nursery pollination mutualisms, *S. latifolia* is not dependent on the nursery pollinator (*H. bicruris*) for pollination, because of the occurrence of several co-pollinators at least in its original distribution area Europe. This observation is underlined by the fact that *H. bicruris* is absent from North America (Hacker, 1996; Wolfe, 2002), where *S. latifolia* is regarded as a pest, and has spread since its introduction in the early part of the 19th century (McNeill, 1977). *Hadena bicruris* can be found in about 90% of European populations of *S. latifolia*, and dramatically affects the fitness of the plant, because the larvae feed on about 25% fruits/seeds produced (Wolfe, 2002). These suggest control mechanisms for *S. latifolia* to avoid or at least control predation by *H. bicruris*. Such control mechanisms are known from other nursery pollination systems, e.g. between figs and fig wasps, and yucca and yucca moths (Bao and

Addicott, 1998; Kjellberg et al., 1987; Pellmyr and Huth, 1994). Syconia on “male” and female trees of “dioecious” *Ficus carica* for example are not receptive at the same time, and fig wasps breeding at this species can only reproduce in syconia of the “male” trees. The short-lived wasps emerge from “male” syconia, and most (but not all) of the wasps are not able to reproduce because there are no receptive syconia on “male” trees. They pollinate flowers in syconia on female trees and die, because they cannot leave syconium once they have entered (Kjellberg et al., 1987). Also *Yucca baccata* is known to control its nursery pollinator by “cheating” in its obligate nursery pollination mutualism with yucca moths, which oviposit at the apex of ovaries containing only inviable ovules (Bao and Addicott, 1998). *Yucca baccata* is characterised by two distinct flower types. Only 30% of individuals have flowers with many potentially viable ovules, and 70% of individuals have only few potentially viable ovules. Yucca moths cannot distinguish between the two flower types for oviposition, and larvae living in the flower type with few potentially viable ovules die of starvation.

In *S. latifolia*, no such mechanisms for controlling the infestation of *H. bicruris* are known. However, is it possible that the floral scent is used to reduce the rate of parasitism by *H. bicruris*? After hand-pollination of female *S. latifolia* flowers it could be observed that in the following night floral scent production has stopped (S. Dötterl, unpublished data). Furthermore, Meagher and Delph (2001) have shown in pollination experiments that longevity of pollinated flowers (3 days) is significantly shorter than longevity of unpollinated flowers (6 days). Interestingly, the life-span of flowers pollinated to 50% was the same compared to flowers pollinated to 100% (Meagher and Delph, 2001). These data indicate that female flowers are withering even if the seed set after pollination reaches only 50%. For *H. bicruris* it was shown that the moths transfers up to 276 pollen grains to the stigmas (see above), and also other nocturnal Lepidoptera effectively transfer pollen (see Young, 2002). Additionally, Jürgens et al. (1996) and Young (2002) found 250 to 300 seeds per fruit in

S. latifolia. Therefore, may be concluded that a single visit of a pollinator resulting in up to 50% seed set may be sufficient for initialising fruit set combined with an immediate stop of floral scent emission. Because risk for parasitism of a female flower by *H. bicruris* increases with flower life-span, a shortening of flower anthesis might be one mechanism to lower predation risk.

Silene latifolia clearly relies on nocturnal moths for pollination, and *H. bicruris* is not necessary to maintain reproductive success (see above). Main pollinators or at least flower visitors in Europe, where *S. latifolia* is native, but also in North America, are noctuids and sphingids (Altizer et al., 1998; Jürgens et al., 1996; Young, 2002). Scent plays a major role for attraction of all these species. Nevertheless, is it possible that *S. latifolia* emits specific volatiles that are attractive to these pollinators, but not to *H. bicruris*? Compounds found in the floral scent of *S. latifolia* (Chapter 2; Jürgens et al., 2002a) are common constituents in floral scents of moth-pollinated species (e.g. Kaiser, 1993; Knudsen and Tollsten, 1993; Levin et al., 2001; Miyake et al., 1998). Some of them contribute to the so-called “white-floral” odour described by Kaiser (1993). It is suggested that nocturnal moths are attracted largely by the same compounds. Meagher (2002), e.g., has shown that several noctuid moths of different genera were attracted by benzenoids, especially phenyl acetaldehyde and benzyl acetate. Therefore, it should be difficult for *S. latifolia* to change the floral scent profile resulting in lower attractiveness to *H. bicruris*, without loosing attractiveness for other pollinating moths. Nevertheless, as reported in Chapter 4 the most attractive floral scent compounds to *H. bicruris* are lilac aldehydes. In contrast to other common floral scent compounds of *S. latifolia,* these oxygenated monoterpenoids are not very widespread (Knudsen et al., 1993). Furthermore, they are also not common as “white-floral” odours (Kaiser, 1993). *Silene latifolia* flowers not emitting lilac aldehydes, but other compounds typically attractive to noctuids, may reduce the attractivity to *H. bicruris*, though still ensuring pollination by

other moths. The observed variability in floral scent (Chapter 2) may indicate such a trend. Most samples collected were dominated by lilac aldehydes, but in some extracts, the amount of lilac aldehydes was decreased, whereas the amount of other compounds, also often found in moth pollinated plants, was increased (e.g. benzyl acetate, *trans*-β-ocimene, benzaldehyde, phenyl acetaldehyde). However, it is not known how fast local populations of *H. bicruris* moths could adapt to new chemotypes, or if there are already *H. bicruris* populations existing that are adapted to different chemotypes.

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7. Summary

In the present study, the role of floral volatiles for the interaction between the nocturnal Caryophyllaceae *Silene latifolia*, and the noctuid moth *Hadena bicruris* was determined. This insect-plant relationship is one of the known nursery pollination systems, where pollinators reproduce within the flowers they pollinate. *Silene latifolia* is a dioecious weed, native to Europe and formerly introduced to North America. It is the main larval host plant of *H. bicruris*, which is distributed in Europe and North Africa. Especially night-active moths, among them *H. bicruris*, which are attracted by the flower scent, pollinate *S. latifolia*. However, until now, nothing was known about the role of single flower scent compounds for the attraction of the moths. This thesis describes the chemical composition and the geographical variability in the flower scent of *S. latifolia.* Furthermore, electrophysiological and behavioural tests with floral scent extracts and single authentic standard compounds were carried out in *H. bicruris* to identify the attractive compounds of the complex floral scent. To get an insight into the role of floral scent in guiding potential pollinators on flowers, the spatial fragrance pattern within the flowers of *S. latifolia* was determined, additionally.

Chemical composition and variability in flower scent of Silene latifolia

To determine the chemical composition and variability in floral scent of *S. latifolia*, the scent from single flowers of 98 different specimens from 15 European and 19 North American populations was analysed using a highly sensitive method. The variability in floral scent was very high, especially between different populations, and different chemotypes were characterised. The differences in floral scent composition between European and North American populations were small. Typical

compounds in floral scent of *S. latifolia* were lilac aldehyde isomers, *trans*-β-ocimene, benzaldehyde, phenyl acetaldehyde, or veratrole. Some of these compounds are known to attract nocturnal Lepidoptera species.

Antennal and behavioural responses of Hadena bicruris to chemotypes of Silene latifolia, and to Silene vulgaris

To characterise antennal and behavioural responses of *H. bicruris* to various floral scent chemotypes of *S. latifolia*, and to *S. vulgaris* (which is rarely also used as host plant), different *S. latifolia* extracts, and a *S. vulgaris* extract were analysed using GC-MS methods. These extracts were further used in GC-FID/EAG and GC-MS/EAG detections, respectively. Main compounds in the tested extracts often elicited main signals in the antennae (e.g. lilac aldehydes, phenyl acetaldehyde). Some compounds elicited main signals in the antennae, though they were only minor components in the extracts (e.g. 3-methyl-butyl-aldoxime, benzaldehyde). Other compounds elicited only weak signals in the antennae, though they were abundant in the extracts (e.g. β-myrcene, methyl benzoate). The compounds of the most common chemotypes of *S. latifolia* were very sensitively detected by *Hadena bicruris*, whereas compounds of less abundant chemotypes were less sensitively detected. Floral scent blends that were dominated by lilac aldehydes or phenyl acetaldehyde effectively attracted moths. *Hadena bicruris* can electrophysiologically and behaviourally distinguish between its main host plant, *S. latifolia,* and the similarly scented *S. vulgaris,* another rarely used larval host plant, only by their floral scent.

Antennal and behavioural responses of Hadena bicruris to floral volatiles of Silene latifolia

To identify floral scent compounds of *S. latifolia* that are important for the attraction of *H. bicruris*, the GC-FID/EAD or the GC-MS/EAD method was

used in a first step to identify compounds that elicit signals in the antennae of the moth. Electrophysiologically very active compounds were tested in wind tunnel bioassays, and the attractivity of these compounds was compared to the attractivity of the natural scent of whole flowers of *S. latifolia*.

The antennae of *H. bicruris* detected substances of several compound classes such as monoterpenoids, benzenoids, fatty acid derivatives, and nitrogen-bearing compounds. Lilac aldehydes were the most attractive compounds in wind tunnel bioassays, and attracted 90% of the tested moths, as did the scent of single flowers. Some compounds did not attract any moth, though they elicited significant signals in the antennae.

Spatial fragrance patterns in flowers of Silene latifolia

To determine the parts of the female and male flowers responsible for scent emission, volatiles from attached intact flowers were sampled and then single flower parts were progressively removed. After each preparation step, volatiles were collected from the remaining “flower”. Especially the petals and the anthophore emitted the typical flower volatiles of *S. latifolia*; and compounds emitted from the petals differed from the compounds emitted by the anthophore. The anthophore emitted only lilac aldehydes and alcohols. Lilac aldehydes are known to be behaviourally very attractive for noctuid Lepidoptera such as *Autographa gamma* and *Hadena bicruris*, and they may serve as nectar guides in *S. latifolia*.

8. Zusammenfassung

Die Rolle von Blütendüften für die Interaktion zwischen der diözischen, nachtblütigen Weißen Lichtnelke (*Silene latifolia*, Caryophyllaceae) und der nacht-aktiven Nelkeneule *Hadena bicruris* (Lepidoptera: Noctuidae) wurde erstmals detailliert untersucht. Bei diesem System parasitieren die Larven der Falter an den Samen der Pflanzen, die die Falter beim Nektartrinken und bei der Eiablage in den weiblichen Blüten bestäuben. Für *H. bicruris* ist *S. latifolia* zwar die wichtigste Wirtspflanze, umgekehrt ist *H. bicruris* aber nur einer von vielen anderen bestäubenden Nachtfaltern. Sowohl *Hadena* als auch die anderen Falter werden vom Blütenduft angelockt, wobei bisher aber nicht bekannt war, welche Duftstoffkomponenten für die Anlockung der Falter von Bedeutung sind bzw. welche Rolle sie im einzelnen spielen.

Im Rahmen der vorliegenden Dissertation wurde neben der Zusammensetzung des Blütenduftes auch dessen geographische Variabilität mit Hinblick auf die unterschiedlichen Verbreitungsgebiete der beiden Interaktions-Partner ausführlich analysiert. Die Bedeutung der Blütendüfte der Weißen Lichtnelke für die Anlockung von *H. bicruris* wurde mittels Elektroantennogrammen und Biotests im Windkanal bestimmt. Zusätzlich wurde das räumliche Duftmuster an bzw. in den Blüten von *S. latifolia* mit Hinblick auf das Verhalten der bestäubenden Blütenbesucher analysiert.

Chemische Zusammensetzung und Variabilität des Blütenduftes von Silene latifolia

Zur Bestimmung der Zusammensetzung und der Variabilität des Blütenduftes von *S. latifolia* wurde der Duft von 98 verschiedenen Individuen aus insgesamt 15 europäischen und 19 nordamerikanischen Populationen untersucht. Dazu wurde der Duft von Einzelblüten mittels

„dynamic-headspace“ -Methoden gesammelt und mit Hilfe der Gaschromatographie, gekoppelt mit der Massenspektrometrie (GC-MS), analysiert. Der Blütenduft der Weißen Lichtnelke ist sehr variabel, und es fanden sich unterschiedliche Chemotypen, welche durch bestimmte Duftkomponenten charakterisiert waren. Die Variabilität innerhalb der Populationen war signifikant niedriger als die Variabilität zwischen den Populationen. Die Unterschiede in der Blütenduftzusammensetzung zwischen europäischen und nordamerikanischen Populationen waren klein. In Europa waren die Duftunterschiede zwischen Pflanzen nah gelegener Populationen tendenziell größer als zwischen Individuen von weit entfernten Populationen. In Nordamerika hatte die räumliche Verteilung der Populationen keinen Einfluss auf die Zusammensetzung des Blütenduftes. Typische Duftkomponenten waren unterschiedliche Monoterpene wie Lilakaldehyde oder (*E*)-Ocimen sowie unterschiedliche benzenoide Verbindungen (Benzaldehyd, Phenylacetaldehyd, Veratrol). Einige dieser Stoffe sind bekannt dafür, dass sie Nachtfalter anlocken.

Elektrophysiologische Signale der Antennen und Verhaltensreaktionen von Hadena bicruris auf verschiedene Chemotypen von Silene latifolia und auf S. vulgaris

Die Extrakte unterschiedlicher Chemotypen von *S. latifolia* wurden mittels GC-MS analysiert, und mit Hilfe von der Gaschromatographie bzw. Gaschromatographie und Massenspektrometrie gekoppelt mit der Elektroantennographie (GC-FID/EAD, GC-MS/EAD) an *H. bicruris* getestet. Alle getesteten Extrakte riefen deutliche Signale in den Antennen von *H. bicruris* hervor, und die Falter konnten elektrophysiologisch zwischen den meisten Chemotypen unterscheiden. Hauptkomponenten in den untersuchten Extrakten führten oft zu den größten Signalen in den Antennen (z.B. Lilakaldehyde, Phenylacetaldehyd). Einige Komponenten lösten große relative elektrophysiologische Reaktionen aus, obwohl sie nur in kleineren Mengen in den Extrakten zu finden waren (z.B. 3-methyl-

butyl-Aldoxim, Benzaldehyd), während andere Komponenten nur schwache Signale in den Antennen auslösten, obwohl sie in relativ großen Mengen in den Extrakten auftraten (β-Myrcen, Methylbenzoat).
Im Biotest lockten Blüten, welche v.a. Lilakaldehyde bzw. Phenylacetaldehyd abgaben, viele Falter an. *Hadena bicruris* konnte bei den Elektroantennogrammen sowie bei den Windkanal-Biotests trotz relativ ähnlicher Blütenduftzusammensetzung zwischen *S. latifolia* und *S. vulgaris* unterscheiden.

Elektrophysiologische Signale der Antennen und Verhaltensreaktionen von Hadena bicruris auf einzelne Blütenduftkomponenten von Silene latifolia

Zur Identifizierung der wichtigsten Blütenduftstoffe von *S. latifolia* für die Anlockung von *H. bicruris* wurde die GC-FID/EAD- bzw. GC-MS/EAD-Methode eingesetzt, um in einem ersten Schritt die Komponenten von *S. latifolia* zu identifizieren, die von *H. bicruris* wahrgenommen werden können. Elektrophysiologisch bedeutsame Komponenten wurden in weiterführenden Analysen im Windkanal getestet, und deren Attraktivität mit der Attraktivität des Duftes ganzer Blüten von *S. latifolia* verglichen.
Die Antennen von weiblichen und männlichen Faltern detektieren gleichermaßen Substanzen aus verschiedenen Stoffklassen (Monoterpene, benzenoide Verbindungen, Fettsäurederivate, stickstoffhaltige Verbindungen). Die stärksten Antennensignale wurden von Lilakaldehyd-Isomeren und von Phenylacetaldehyd hervorgerufen. Die Lilakaldehyde waren auch die attraktivsten Komponenten in den Winkanalbiotests. 90% der getesteten Falter reagierten auf sie – genauso viele wie auf den Duft von Einzelblüten. Alle anderen getesteten Substanzen lockten weniger Falter an. Einige Komponenten lockten keine Falter an, obwohl sie deutliche Signale in den Antennen auslösten. Wurden Stoffe in unterschiedlichen Konzentrationen getestet, reagierten die Falter verstärkt auf die höhere Dosis.

Räumliche Duftmuster bei Blüten von Silene latifolia

Zur Bestimmung der für die Duftemission verantwortlichen Blütenteile wurde zuerst der Duft ganzer weiblicher und männlicher Blüten analysiert. Anschließend wurden von den Blüten einzelne Teile entfernt und jeweils die Duftstoffe von der „Restblüte“ bestimmt.

Die Blütenduftstoffe von *S. latifolia* wurden v. a. von den Blütenblättern und dem Anthophor emittiert, wobei sich die Stoffe je nach Emissionsort unterschieden. Der Anthophor gab ausschließlich Lilakaldehyde und Lilakalkohole ab, während die Blütenblätter sämtliche benzenoiden, phenylpropanoiden und stickstoffhaltigen Verbindungen sowie (*E*)-Ocimen abgaben. Die Lilakaldehyde sind bekannt für ihre große Attraktivität auf Nachtfalter und sind möglicherweise zum Nektar weisende „Saftmale“ von *S. latifolia*.

Darstellung des Eigenanteils

Kapitel 1, 2, 5-8: Diese Kapitel wurden ausschließlich von mir verfasst. Die Ergebnisse der Kapitel 2 und 5 wurden ausschließlich von mir gewonnen. Das für die Durchführung von Kapitel 2 benötige Saatgut von unterschiedlichen Europäischen und Nordamerikanischen Populationen habe ich von Dr. Lorne M. Wolfe aus den USA erhalten.

Kapitel 3: Bis auf die Beschreibung der elektrophysiologischen Methode (see Material and methods) wurde dieses Kapitel von mir verfasst. Die elektrophysiologische Methode beschrieb Prof. Dr. Stefan Schütz aus Göttingen. Die elektrophysiologischen Messungen wurden zusammen mit Prof. Dr. Stefan Schütz und Dr. Bernhard Weißbecker auf deren System in Göttingen durchgeführt. Die dazu benötigten Tiere wurden von mir gezüchtet und Duftextrakte wurden von mir hergestellt. Die erhaltenen elektrophysiologischen Daten wurden ausschließlich von mir ausgewertet. Die Windkanal Biotests wurden ausschließlich von mir durchgeführt und ausgewertet.

Kapitel 4: Bis auf die Beschreibung der elektrophysiologischen Methode und der Lilakaldehyd Synthese (see Material and methods) wurde alles von mir verfasst. Prof. Dr. Karlheinz Seifert und Thorsten Laube beschrieben die Duftstoff-Synthese und führten sie durch. Die elektrophysiologische Methode beschrieb Prof. Dr. Stefan Schütz aus Göttingen. Die Lilakaldehyde sowie weitere kommerziell erworbene Duftstoffe wurden ausschließlich von mir im Windkanal getestet. Die erhaltenen Ergebnisse wurden ausschließlich von mir ausgewertet. Wie in Kapitel 2 wurden die elektrophysiologischen Messungen zusammen mit Prof. Dr. Stefan Schütz und Dr. Bernhard Weißbecker auf deren System in Göttingen durchgeführt. Die dabei erhaltene Daten wurden wieder ausschließlich von mir ausgewertet.

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst und dabei keine anderen als die angegebenen 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 27. Mai