Floral Scent in *Salix* L. and the Role of Olfactory and Visual Cues for Pollinator Attraction of *Salix caprea* L.

Dissertation

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This dissertation is submitted as a "Cumulative Thesis" that includes four (4) publications: two (2) published articles, one (1) submitted article, and one (1) article in preparation for submission. The publications are listed in detail below.

Published:

- **Dötterl S., Füssel U., Jürgens A., and Aas G. (2005):** 1,4-Dimethoxybenzene, a floral scent compound in willows that attracts an oligolectic bee. *Journal of Chemical Ecology* 31:2993-2998 (Part B, Chapter 3).
- **Füssel U., Dötterl S., Jürgens A., and Aas G. (2007):** Inter- and intraspecific variation in floral scent in the genus *Salix* and its implication for pollination. *Journal of Chemical Ecology* 33:749-765 (Part B, Chapter 1).

Submitted:

- **Füssel U., Dötterl S., Jürgens A., Woodring J., and Aas G. (2008)**: Floral reward and advertisement in dioecious *Salix caprea*. Submitted to *Plant Biology* (Part B, Chapter 4).

Prepared for resubmission:

- **Füssel U., Dötterl S., Jürgens A., and Aas G. (2008):** *Salix caprea*: An interaction generalist and multi-specialist with bimodal adaptations of floral scent to bees and moths. Intended for resubmission to *New Phytologist* (Part B, Chapter 2).

Declaration of Self-Contribution of Research

The thesis contains a detailed summary (Part A) and four (4) research articles (Part B), covering various research work on pollination biology and chemical ecology of willows and their pollinators. Most of the research work presented in this thesis was carried out by myself independently at the Ecological-Botanical Garden, University Bayreuth under supervision of PD Dr. Gregor Aas.

Together with my supervisor and all co-authors (Dr. Stefan Dötterl, Dr. Andreas Jürgens, and Prof. Dr. Joseph Woodring) I developed the methods, discussed the results and prepared the manuscripts of all research articles. My practical field and laboratory work was supported by several students and employees of the Ecological-Botanical Garden and the University.

1st article Füssel U., Dötterl S., Jürgens A., and Aas G. (2007): Inter- and intraspecific variation in floral scent in the genus *Salix* and its implication for pollination. *Journal of Chemical Ecology* 33:749-765. (Part B, Chapter 1)

My contribution to this chapter was about 85 %. The experimental design, the main part of the field work as well as the analysis, the presentation, and the interpretation of the results were performed by myself.

2nd article Füssel U., Dötterl S., Jürgens A., and Aas G. (submitted 2007): *Salix caprea*: An interaction generalist and multi-specialist with bimodal adaptations of floral scent to bees and moths. Intended for resubmission to *New Phytologist*. (Part B, Chapter 2)

My contribution to this article was approximately 75 %. Dr. Stefan Dötterl conducted the GC-EAD study and analysed the GC-EAD data. The floral scent samples needed for the electrophysiological measurements were collected and prepared by myself. All the other data were also collected, analysed, presented, and interpreted by myself. Susanne Kern helped to collect and identify flower visitors.

3rd article Dötterl S., Füssel U., Jürgens A., and Aas G. (2005): 1,4-Dimethoxybenzene, a floral scent compound in willows that attracts an oligolectic bee. *Journal of Chemical Ecology* 31:2993-2998. (Part B, Chapter 3)

My contribution to this study was circa 60 %. The data concerning the behavioural experiment with 1,4-dimethoxybenzene were collected, analysed, presented, and interpreted completely by myself. Floral scent samples for the electrophysiological measurements were collected and prepared completely by myself. Data of the electrophysiological measurements were collected, presented, interpreted and discussed by Dr. Stefan Dötterl. He wrote also the first manuscript draft.

4th article Füssel U., Dötterl S., Jürgens A., Woodring J., and Aas G (prepared for submission): Floral reward and advertisement in dioecious *Salix caprea*. Submitted to *Plant Biology*. (Part B, Chapter 4)

My contribution to this manuscript was about 80 %. The experimental design, the main part of the work in field and laboratory, as well as the analysis, presentation, interpretation and discussion of the results were performed by myself. Prof. Dr. Joseph Woodring introduced me in the HPLC method and I performed the nectar analyses myself.

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Particularly I want to thank all the students who helped and supported my work. With their assistance it was possible to perform all the time-consuming experiments during the short flowering time of the willows.

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Content

Part A

Detailed Summary

1 General Introduction and Aims of the Research

Numerous studies have shown that flowers are complex systems in which floral features such as shape, nectar, colour, and odour work together for the benefit of the plants' sexual reproduction. Pollen transfer is either achieved by attraction and manipulation of pollinators (e.g. Stensmyr et al. 2002; Schiestl 2005; Raguso et al. 2007) or by abiotic factors such as wind and water (Ackermann and Kevan 2005). Both diversity and similarity of flowers have been interpreted since Darwin as adaptations to different types of pollinating agents (e.g. Darwin 1862; Delpino 1868-1875; Knuth 1906; Vogel 1954; Baker 1963; Grant and Grant 1965; Stebbins 1970; Fægri and van der Pijl 1979; Johnson and Steiner 2000; Fenster et al. 2004). More or less specialised relationships between abiotic and biotic pollinating agents and plant species are reflected in the widely adopted classification of flowers with different pollination syndromes (Faegri and van der Pijl 1979). Pollination systems of flowers which attract numerous animal species with a broad taxonomic spectrum, or achieve pollination by a mixture of pollination modes and vectors (Robertson 1928; Vroege and Stelleman 1990; Ellis and Ellis-Adam 1993; Ollerton 1996; Waser et al. 1996; Memmott 1999), have long been neglected. But in fact, such generalistic pollination systems seem to be more common than previously thought (Waser et al. 1996). For example, a combination of wind and insect pollination has been found in a number of species from a wide range of taxa and these show a various mixture of traits attributed to wind- and insect pollination (Proctor et al. 1996; de Figueiredo and Sazima 2000; Culley et al. 2002).

Especially, species of the genus *Salix* L. (willows) were often described as pollination generalists (e.g. Karrenberg et al. 2002), because they show traits of insect as well as wind pollination (Stebbins 1970; Faegri and van der Pijl 1979). Depending on species and ecological context, insects (Kevan 1972; Sacchi and Price 1988; Elmqvist et al. 1988; Douglas 1997) as well as wind (Argus 1974; Vroege and Stelleman 1990; Fox 1992) are both important pollen vectors. Besides this mixture of pollination modes, a variety of insects are known as flower visitors and potential pollinators (Vroege and Stelleman 1990; Hilty 2006). Despite their worldwide distribution and great ecological importance, little is known about the specific interaction of *Salix* species with their pollinators and the mechanisms of pollinator attraction, pollination success, and hybridisation. In willows that seemingly combine different pollination modes and a wide array of potential pollinators, nothing is known about the signals that prompt pollinators to visit flowers of both genders repeatedly to ensure pollinators. Taking all its features together, the genus *Salix* seemed to be an interesting case to

be studied within the scope of the graduate college 678 “Ecological significance of natural compounds and other signals in insects – from structure to function”. The present work focuses mainly on plant-insect interactions in the genus *Salix* and the role of floral scent for the attraction of insects. Besides a general survey of floral scent in willow species, I conducted a detailed case study on its role in plant-pollinator interactions of *Salix caprea* L.

1.1 Background

1.1.1 The Genus *Salix*: Distribution and Taxonomy

The genus *Salix* L. comprises 400 to 500 species (Fang 1987; Skvortsov 1999) with a nearly worldwide distribution. *Salix* species occur predominantly in temperate to arctic regions of the northern hemisphere. In Central Europe about 40 species occur, many sympatrically (Lautenschlager-Fleury and Lautenschlager-Fleury 1994; Rothmaler 2002).

From a taxonomical point of view, *Salix* is a problematic genus with difficulties in the delimitation of many species, mainly because of high morphological variability (Argus 1997; Skvortsov 1999), and suggested widespread hybridisation and introgression (Mosseler 1990; Fritz et al. 1998). There are several, different phylogenetic classifications of this genus available, all based on morphological characters (Dorn 1976; Argus 1997: American species, Skvortsov 1999: Eurasian species). The classification used in this study is that of Skvortsov (1999), because it is the most comprehensive for Eurasian species. He divided *Salix* in three subgenera (*Chamaetia*, *Salix*, and *Vetrix*), each with several sections listed in Füssel et al. (2007) (see Part B, Chapter 1).

1.1.2 Pollination System of *Salix*

Salix species are dioecious with often hundreds of flowers arranged in catkins (Kay 1985; Karrenberg et al. 2002) (see Figure 1). The plants show traits of insect as well as of wind pollination. Stiff erect catkins, availability of nectar, and floral scent production fit well with insect pollination, whereas small flower size, absence of a perianth, predominant flowering early in spring before leaf unfolding, and release of large amounts of small pollen are characteristic for wind pollination. Hence, the importance of either mode of pollination in the genus *Salix* is controversial (Karrenberg et al. 2002). Nevertheless, most species are thought

to be mainly entomogamous, though in certain species wind contributes to some degree to pollination (Argus 1974; Sacchi and Price 1988; Vroege and Stelleman 1990; Ohara and Higashi 1994; Peeters and Totland 1999; Totland and Sottocornola 2001; Karrenberg et al. 2002). Reported ratios of insect to wind pollination range from 20-70 % wind pollination in *Salix repens* (Vroege and Stelleman 1990), to 50 % insect pollination in *S. caprea* (Vroege and Stelleman 1990), and almost total insect pollination in *S. arctica* (Kevan 1972). Depending on species and ecological context both, insects (Kevan 1972; Sacchi and Price 1988; Elmqvist et al. 1988; Douglas 1997) and wind (Argus 1974; Vroege and Stelleman 1990; Fox 1992) seem to be important pollen vectors.

With regards to insect pollination it is known that social and solitary bees (Apoidea, Hymenoptera) are the most common flower visitors of many *Salix* species (e.g. van der Werf et al. 1982; Vroege and Stelleman 1990; Hilty 2006). *Salix* is a genus that hosts many different oligolectic bee species (e.g. *Andrena vaga*), probably because of its readily accessible pollen (Michener 2000). Some generalistic bees (e.g. *Apis mellifera*), often visit willow catkins for their pollen and nectar (e.g. van der Werf et al. 1982; Vroege and Stelleman 1990; Hilty 2006).

Some Diptera (van der Werf et al. 1982; Pellmyr and Kärkkäinen 1987; Totland and Sottocornola 2001) and some Lepidoptera and Coleoptera species (Vroege and Stelleman 1990; Urban and Kopelke 2004) have been also observed as flower visitors. However, studies that differentiate the importance of the different insect groups and of diurnal and nocturnal flower visitors, or compare them separately with wind pollination are missing. In most cases it is not clear to what extent particular flower visitors are contributing to pollination (van der Werf et al. 1982).

1.1.3 Floral Signals and Rewards of *Salix*

Floral signals consist in most cases of visual and olfactory cues. Attractants include the visual stimulus of floral shape and colour as well as the production of floral odour (Fraegri and van der Pijl 1979; Passarelli and Bruzzone 2004). The attractivity of floral signals is usually based on the possibility for the animal to find a reward, such as nectar (e.g. Molina-Faeaner et al. 2004), pollen (e.g. Fleming and Nicolson 2002), or other substances (Fraegri and van der Pijl 1979).

Pollen – The process of pollination begins with the exposure and shedding of ripe pollen, which carries the male gametes or their progenitors (Dafni et al. 2005). A pollen "grain" is a haploid microspore that has matured through mitotic divisions. The primary and indispensable function of pollen is to transport the male gametes from staminate flower organs of one flower to pistillate flower organs of another conspecific flower (Lunau 2000). Usage of pollen to reward pollinators most likely evolved from interaction of early seed plants with phytophageous insects that fed on nutrient rich pollen. Assumingly, flowering plants seem to have made the best of it and with occurrence of perfect flowers, they evolved adaptations to exploit pollen-seeking herbivores for pollination (Lunau 2000 and references therein). Pollen thus acts not only as a means for transportation of male gametes, but also as a food reward for potential pollinators (Dafni 2005; Roulston 2005). To solve this problem, plants evolved flowers which either produce a surplus of pollen to satisfy pollinator needs, developed mechanisms to conceal pollen against "unwanted" feeding, or spent resources on the production of alternative rewards such as nectar that are not a direct cost to the plants reproductive system (see Lunau 2000 and references therein).

Nectar – To attract pollinators, plants offer different types of rewards, mainly pollen and nectar. Of these two types, nectar is sought by a wider array of animals than pollen (Simpson and Neff 1981). While pollen grains, essentially the plant's male gametophytes containing male gametes, are essential for the plant's sexual reproduction itself, nectar secretion has usually no other function than attracting and rewarding pollinators. Nectar, basically a sugar solution which satisfies the energetic needs of many insects, is produced in different types of nectaries and offered at different places in the flower, depending on plant species and flower types. Timing of nectar secretion and accessibility of secreted nectar often serve to manipulate potential pollinators to achieve optimal pollen transfer between pollen donor and pollen receptor (Greco et al. 1996).

In *Salix* flowers, nectar is secreted from one or more nectaries projecting from the base of the flower (Figure 2). Nectar is thought to be an important food source for insects, especially for wild bees (e.g. several species of *Andrena*, *Colletes*, and other solitary bees (Proctor and Yeo 1973; Alford 1975)). Early nectar investigations in *Salix* species were done by Percival (1961); she found that nectar of male flowers is sucrose dominated where nectar of female flowers is hexose dominated.

Visual signals – Besides flower shape, floral colour is one of the main visual signals which attracts pollinators (Lunau and Maier 1995; Lunau 1996). The development of different floral

colours in different floral organs and tissues is a result of many factors, e.g. chemical composition of pigments or formation of chelate complexes with metal cationes or carbohydrates (e.g. Lunau 1995, 2000). Many pollinators' spectral perception extends from ultraviolet through the red part of the electromagnetic spectrum, but colour vision of insects is greatly limited by the sensitivity range of photoreceptors (Menzel 1979; Chittka and Kevan 2005). Many flower-visiting insects (e.g. bumblebees, sphingid moths, nymphalid butterflies) are sensitive to ultraviolet, blue and green light, and have three types of photoreceptors each corresponding to a distinct waveband (Hoglund et al. 1973; Steiner et al. 1987; Peitsch et al. 1992). The different flower visitors have different flower colour preference, for example the bumblebees prefer violet (Nakano and Washitani 2003), honeybees prefer yellow (Niggebrügge and de Ibarra 2003), butterflies and moths prefer yellow or blue (Andersson 2003; Kleber et al. 2003). Nocturnal species can discriminate flowers at starlight intensities when humans and honeybees are colour blind (Kleber et al. 2003).

In *Salix*, male catkins are almost always yellowish and female inflorescences are usually greenish (Figure 1), but a coloured perianth as a visual attractant is lacking in *Salix* flowers (Figure 2). In male flowers with long white filaments, the intensive yellow pollen in the anthers is responsible for the colour. In female flowers, ovary, style, and stigma are coloured inconspicuously green. The lack of a colourful perianth, the small size and relatively open exposure of reproductive organs have often been interpreted as adaptations to abiotic pollination by wind (Ackermann and Kevan 2005). Many findings provide compelling evidence that pollen functions not only as a reward but also as a visual signal: Simply because it originally must be released in an exposed position to allow wind pollination, and because of the necessary protective pigments, pollen was predestined to become an attractant signal to visitors (Lunau 2000 and references therein). Since the trichromatic colour vision in insects is phylogenetically older than the habit to visit flowers, Chittka (1996) assumed that early flower visitors were able to detect pollen cues. Thus pollen was recognized as a phylogenetically old signal of flowering plants to attract flower visitors (Osche 1979, 1983, 1986). To attract flower visitors, often mimetic "signal copies" of pollen and/or whole stamens are used, while nectar and nectaries act as less significant signals (Vogel 1998).

Olfactory signals – Quality and quantity of floral scents are assumed to be olfactory cues for attracting pollinators (Wyatt 1983). Floral scents usually consist of a complex mixture of relatively small (five to 20 carbon atoms), volatile organic compounds. They belong to several chemical classes, such as fatty acid derivates, benzenoids, terpenoids, nitrogenous compounds, and sulphur-containing compounds (Dudareva et al. 1999; Knudsen et al. 2006).

Fig. 1: Male (left) and female (right) catkin of *Salix caprea*.

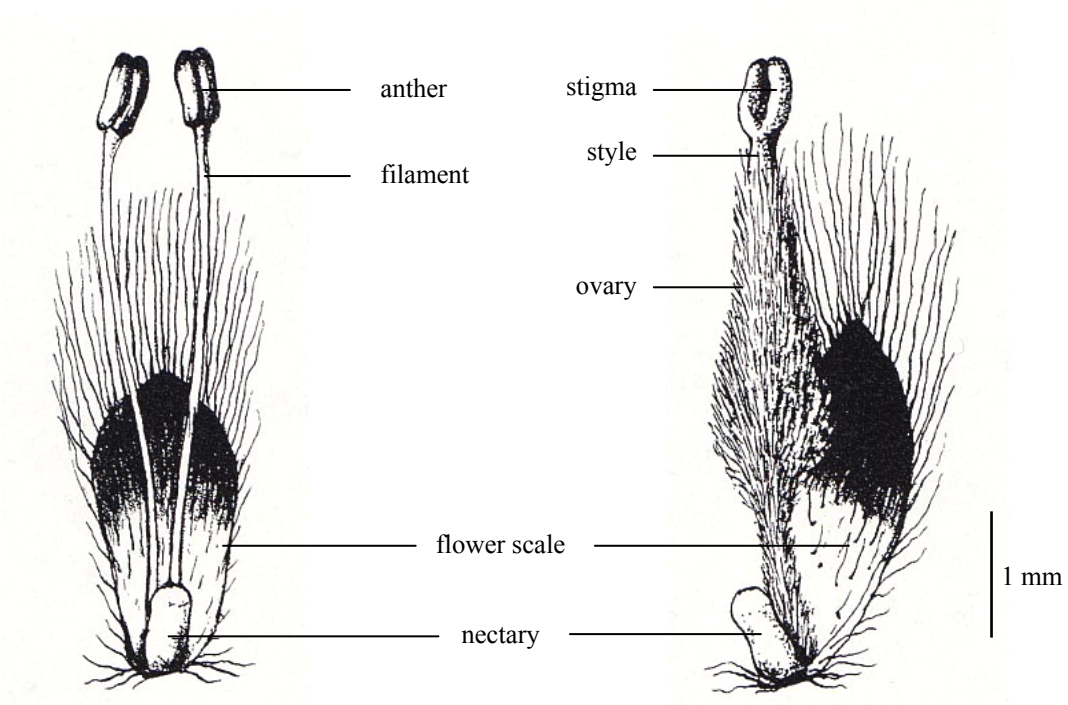

Fig. 2: Single male (left) and single female (right) flower of *Salix caprea*; modified after Lautenschlager-Fleury and Lautenschlager-Fleury (1994).

In contrast to primary plant products, floral scent compounds are typical secondary plant metabolites that are not essential for the plant's growth and development (Schoonhoven 1972). Floral scent compounds can be released continuously, or may be stored in plant tissue and emitted in a defined temporal pattern (Dudareva et al. 1999). Differences in floral scent, scent intensity as well as volatile composition, have been shown to correspond on the one hand to different pollinator assemblages (e.g. Wyatt 1983; Raguso 2001) and on the other hand to taxonomical groups of plants (Jürgens et al. 2003; Jürgens 2004; Jürgens and Dötterl 2004). At the interspecific level the variation of volatiles in floral scents ranges widely. In some groups there is little variation in floral scent composition between closely releated taxa, but in other groups each taxon produces its own specific floral scent blend (Dahl et al. 1990; Tollsten and Bergström 1993; Knudsen and Ståhl 1994; Dobson et al. 1997; Kite et al. 1998; Ervik et al. 1999). At the intraspecific level, scent can vary spatially and temporally within a flower, between plant individuals, between sexes in dioecious plants (Tollsten and Knudsen 1992; Ervik et al. 1999; Ashman et al. 2005; Füssel et al. 2007), and between populations (Tollsten and Bergström 1989; Moya and Ackerman 1993).

Floral scent is an important attractant that plays a key role for chemical communication between plants and animal pollinators (Faegri and van der Pijl 1979; Pellmyr and Thien 1986). Floral scent may be used by insects visiting flowers to feed, mate, and lay eggs, and the species-specific characteristics of floral scents help insects to locate and recognize particular flowers (Dobson 1994; Raguso 2001; Weiss 2001). Detailed knowledge of floral scent coupled with behavioural assays on potential pollinators is needed to understand complex plant-pollinator interaction (Dudareva and Pichersky 2000; Pichersky and Gershenzon 2002; Huber et al. 2005).

Many flowers show a rhythmic scent emission, which is controlled by a circadian clock and/or regulated by light (Jakobsen and Olsen 1994; Helsper et al. 1998). In some species the dynamic nature of scent is not only reflected in quantitative changes in the emission of volatiles but also in qualitative changes in the odour composition (Baldwin et al. 1997; Dötterl et al. 2005a; Hoballah et al. 2005). A rhythmic scent emission is often correlated with the corresponding temporal activity of flower visitors.

The only study that investigated the floral scent of *Salix* species (*Salix caprea*, *S. cinerea*, *S. repens*) was done by Tollsten and Knudsen (1992). The authors found isoprenoids and benzenoids dominating the floral scent. However, the variability of the floral scent in the

genus *Salix* (except the three species) and the importance of the whole floral scent and single compounds for the attraction of potential pollinator remain unknown.

1.2 Aims of the Research

Within the scope of the graduate college 678 "Ecological significance of natural compounds and other signals in insects – from structure to function" I conducted a general survey of floral scent in dioecious willow species, and investigated in a case study the role of olfactory and visual cues for pollinator attraction and pollination success in *Salix caprea* (sallow), a willow with a seemingly generalistic pollination system. I analysed gender specialisation with respect to olfactory signals, visual signals, and nectar reward, and I examined the response of flower visitors to floral signals and their relative importance for reproductive success.

The aim of my research was to answer the following questions:

- What is the chemical composition of *Salix* floral scent and how does it vary with species, gender, and time of the day? (Publications 1, 2, and 4)
- Which are the flower visitors of *Salix caprea*? (Publication 2)
- Which floral scent compounds can be detected by flower visitors of *Salix caprea*? (Publications 2 and 3)
- Do electrophysiological active floral scent compounds act as attractants for potential pollinators in *Salix caprea*? (Publications 2 and 3)
- Which gender of *Salix caprea* is more attractive to *Apis mellifera*? What role do visual and olfactory cues play? (Publication 4)
- Does the nectar reward of male and female flowers of *Salix caprea* differ? (Publication 4)
- What is the contribution of different pollen vectors to reproductive success? (Publication 2)
- Is *Salix caprea* a generalist or a specialist regarding the pollination system? (Publications 1, 2, 3, and 4)

2 Material and Methods

2.1 Plant Material

Nearly all *Salix* plants in this study are growing at the Ecological-Botanical Garden (EBG) Bayreuth, Germany. Ten *Salix* species (*S. alba*, *S. aurita*, *S. babylonica*, *S. caprea*, *S. cinerea*, *S. daphnoides*, *S. fragilis*, *S. purprea*, *S. triandra*, and *S. viminalis*) were sampled additionally at other sites in the vicinity of Bayreuth. After a screening of floral scent emission in the genus *Salix*, *Salix caprea* (sallow) was chosen for a detailed study, because it is a common widely distributed *Salix* species in our region, and further experiments (GC-EAD, bioassays, nectar analyses, pollination experiments) were conducted mainly with this species.

2.2 **Determination of Flower Visitors** (Publication 2)

To analyse the reproductive success of plants it is absolutely essential to understand their pollinator assemblages (Waser et al. 1996; Johnson and Steiner 2000). To determine the spectrum of the flower visitors of *Salix caprea*, visitors of three male and four female trees were recorded in the flowering season 2006. Each *Salix* individual was observed a full day every two hours for 10 min. The total observation time was 60 min (6 x 10 min) during the day and 60 min (6 x 10 min) during the night. All observed flower visitors were caught with an insect net and identifiable species (e.g. honeybees) were recorded (species, number of individuals) and released alive. Others species were stored at -20 °C for further preparation and determination. Nocturnal Lepidoptera were additionally collected with automatic light traps (model Weber, bioform; 12 V, 15 W). The light traps were attached directly in the centre of the trees. Each of the seven *Salix caprea* individuals was investigated from one to four days, depending on the flowering duration of each tree and on weather conditions.

Only flower visitors of Hymenoptera and Lepidoptera were included in further analyses, because some insect groups that are difficult with respect to identification (e.g. Coleoptera and Diptera) are currently with several specialists for determination. A fifth publication containing a complete list of all flower visitors of *Salix caprea* is in preparation.

To determine the abundance of flower visitors on *Salix caprea* in the course of a day the "scan sampling method" according to Sowig (1991) was applied. In intervals of two hours (parallel with floral scent collection from the seven individuals in 2006), one randomly selected branch per individual (length = 30 cm) was observed for 30 s for their flower visitors

and in the following 30 s the result of these observation was recorded. The total observation time was 15 min. This procedure was repeated every two hours 12 times on a selected branch. The mean values of different *Salix* individuals of these observations were determined. Because of the difficult identification of species during foraging, the observed visitors were classified into seven easily distinguishable groups (species) (1 = honeybees; 2 = bumblebees; 3 = medium sized bees [wild bees about honeybee size]; 4 = small bees [wild bees smaller than honeybees]; 5 = butterflies; 6 = moths; 7 = others like flies and beetles).

2.3 **Floral Scent Collection and Analysis** (Publications 1, 2, and 4)

Floral scent was collected using a dynamic headspace MicroSPE method. For this purpose, a certain number of twigs per individual with four to 80 flowering catkins, depending on the experimental design, was enclosed for 10 min in an oven bag (Nalophan), and the floral scent was subsequently trapped for 2.5 min in an adsorbent micro tube (filled with 3 mg of a 1:1 mixture of Tenax-TA 60-80 and Carbotrap 20-40) by using a membrane pump (G12/01 EB, Rietschle Thomas, Puchheim, Germany). After sampling, the glass micro tubes were stored at -20 °C until further analyses.

The samples were analysed on a Varian Saturn 3800 gas chromatograph (GC) fitted with a 1079 injector, and coupled with a Varian Saturn 2000 mass spectrometer (MS). The micro tubes were inserted via Varians Chromatoprobe into the GC injector. The injector vent was opened (1/20) and the injector was heated at 40 °C to flush any air from the system. After 2 min the split vent was closed and the injector heated at 200 °C min^{-1}, then held at 200 °C for 4.2 min, after which the split vent was opened (1/20) and the injector cooled down. For the analyses a ZB-5 column (5 % phenyl polysiloxane, length 60 m, inner diameter 0.25 mm, film thickness 0.25 µm, Phenomenex) was used. Electronic flow control maintained a constant helium carrier gas flow (flow rate of 1.8 ml min^{-1}). The GC oven temperature was held for 7 min at 40 °C, then increased by 6 °C min^{-1} to 260 °C and held for 1 min at this temperature. The mass spectra were taken at 70 eV with a scanning speed of 1 scan s^{-1} from *m/z* 40 to 350.

Anther scent was collected from three different male *S. caprea* individuals in the flowering season 2005. For each sample, 20 anthers from one catkin were put in quartz microvials for direct analysis via thermal desorption and coupled GC-MS (described above). The Chromatoprobe microvial 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 min and the injector was heated at 200 °C/min, then held at 150 °C for 2 min, after which the split vent was opened (1/20) and the injector cooled down. The GC oven temperature was held for 4.6 min at 40 °C, then increased by 6 °C per min to 260 °C and held for 1 min. After each run the column was cleaned by heating at 100 °C/min to 300 °C. The MS interface was 260 °C and the ion trap worked at 175 °C. The mass spectra were taken as described above.

The GC-MS data were analysed by using the Saturn Software package 5.2.1. To identify the floral scent compounds of the GC-MS spectra the data bases NIST 02 and MassFinder 3 were used, and identifications were confirmed by comparison of retention times with published data (Adams 1995). The identification of some compounds was also confirmed by comparison of mass spectra and retention times with those of standards.

The total scent emission is estimated as follows: For quantification of compounds known amounts of lilac aldehydes, *trans*-β-ocimene, *cis*-3-hexenylacetate, benzaldehyde, phenylacetaldehyde, and veratrole were injected, and the mean responses of these compounds were used for quantification.

2.4 **Gas Chromatography Coupled to Electroantennographic Detection (GC-EAD)** (Publications 2 and 3)

To get samples for the electrophysiological analyses (see below) floral scent was collected using a dynamic headspace method. For each sample two or three twigs with 10 to 12 catkins of each *Salix caprea* and *S. atrocinerea* individual were enclosed in a polyethylene oven bag and volatiles were trapped for ca. eight hours between 9 am and 5 pm in large adsorbent tubes filled with 30 mg of a 1:1 mixture of Tenax-TA 60-80 and Carbotrap 20-40. Volatiles were eluted with 70 µl of acetone (SupraSolv, Merck KgaA, Germany) for later use in the GC-EADs.

Electrophysiological analyses were used to identify the compounds in the floral scent of *Salix caprea* eliciting signals in the antennae of abundant flower visitors. The scent samples were tested on the antennae of frequent diurnal (different bee species) and frequent nocturnal flower visitors (different moth species). Bees were caught either at their nesting places or directly from *S. caprea*, and moths were mainly caught by light traps (see 2.2). All measurements were performed with the GC-EAD system described by Dötterl et al. (2005b) (see Figure 3). The GC-EAD system consisted of a gas chromatograph (Vega 6000 Series 2,

Carlo Erba, Rodano, Italy) equipped with a flame ionisation detector (FID), and an EAD setup (heated transfer line, 2-channel USB acquisition controller) provided by Syntech (Hilversum, Netherlands). 1 µl of an acetone sample was injected splitless at 60 °C, followed by opening the split vent after 1 min and heating the oven at a rate of 10 °C min^{-1} to 200 °C. The end temperature was held for 5 min. A ZB-5 column was used for the analyses (length 30 m, inner diameter 0.32 mm, film thickness 0.25 µm, Phenomenex). The column was split at the end by the four arm flow splitter GRAPHPACK 3D/2 (Gerstel, Mülheim, Germany) into two pieces of deactivated capillary (length 50 cm, inner diameter 0.32 mm) leading to the FID and EAD setup. Makeup gas (He; flow rate 16 ml min^{-1}) was introduced through the fourth arm of the splitter. For measurements, an excised antenna was mounted between glass micropipette electrodes filled with insect ringer (8.0 g l^{-1} NaCl, 0.4 g l^{-1} KCl, 4 g l^{-1} $CaCl_2$), and connected to silver wires.

To identify the compounds eliciting signals in the insect antennae, 1 µl of the acetone samples was placed in a quartz vial in the injector port of the GC by means of the ChromatoProbe, and then analysed by GC-MS as described above for samples taken to study floral scent (see 2.3).

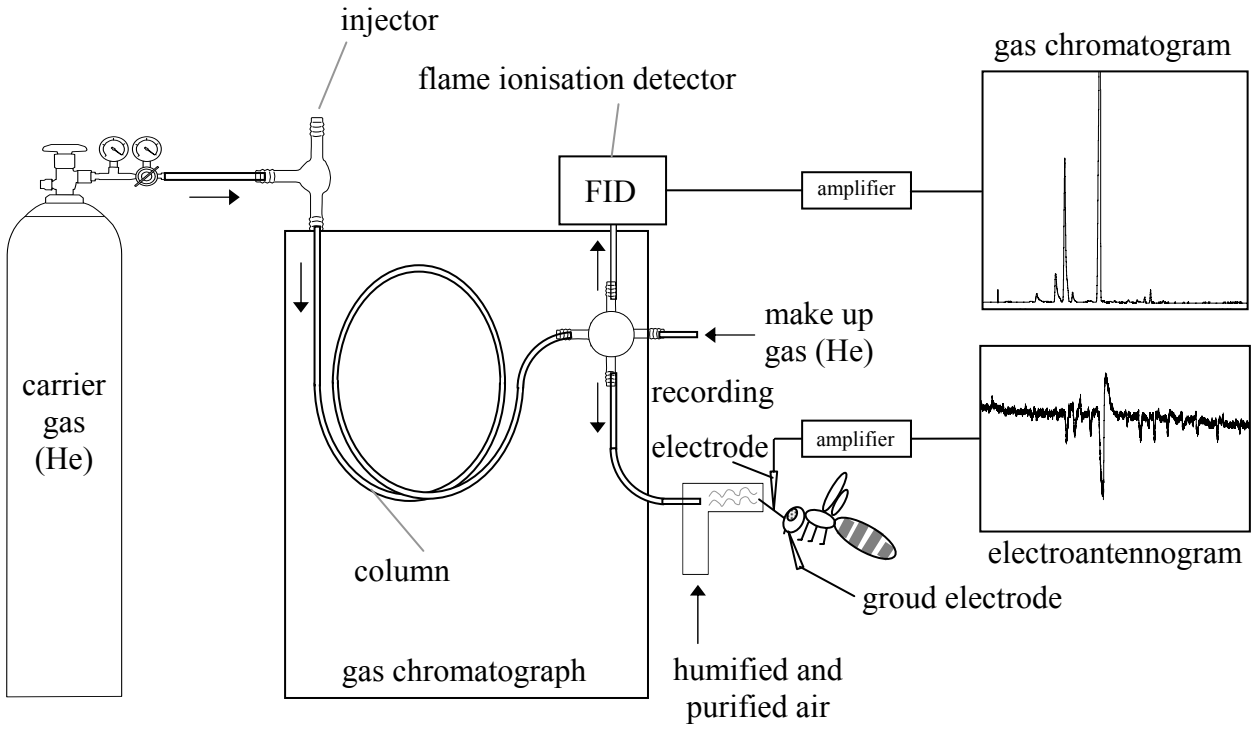

Fig. 3: Scheme of gas chromatography coupled to electroantennography (GC-EAD).

2.5 **Behavioural Tests** (Publications 2, 3, and 4)

Behavioural tests are essential to assess the effect of floral scent compounds. Electrophysiological activity does not tell how potential pollinators react towards a compound. They may be attracted or repelled, or they may even behave indifferent to electrophysiologically active compounds (Omura et al. 2000). Three different behavioural tests were conducted in this study. First, I compared the responsiveness of the honeybee (*Apis mellifera*) and the moth species *Orthosia gothica* to the benzenoid 1,4-dimethoxybenzene and the isoprenoid lilac aldehyde (Publication 2). Second, I tested the attraction of a solitary bee that visits *S. caprea* flowers, *Andrena vaga,* to 1,4-dimethoxybenzene (Publication 3). Finally, the attractiveness of olfactory and visual signals of male and female *Salix* individuals to *Apis mellifera* was investigated in two-choice bioassays (Publication 4).

1) To test the attractiveness of 1,4-dimethoxybenzene and lilac aldehyde two-choice bioassays were conducted in a flight cage with *Apis mellifera* and in a wind tunnel with *Orthosia gothica* in spring 2007. The two floral scent compounds of *Salix caprea* were chosen, because 1,4-dimethoxybenzene elicited the main signal in the antennae of bees and lilac aldehyde elicited a stronger signal in the antennae of moths than in the antennae of bees.

Two-choice bioassay with *Apis mellifera*. A flight cage (7.20 m × 3.60 m × 2.20 m) was placed in a greenhouse to create a closed system. Before flowering of *S. caprea* one bee hive with nine honeycombs of naïve honeybees was placed in the flight cage. One rubber GC septum impregnated with 10 µl of a 1,4-dimethoxybenzene solution (99 %, Aldrich; 10 µl 1,4-dimethoxybenzene dissolved in 90 µl paraffin) and one rubber GC septum with 10 µl of a lilac aldehyde solution (synthesised as described in Dötterl et al. (2006); 10 µl lilac aldehyde dissolved in 90 µl paraffin) were presented in the flight cage (distance of the septa: 1 m) around noon for 40 min, when the activity of bees was highest. Every 10 minutes the order of the rubber GC septum was changed. The reaction of bees was classified as "zigzagging" when the honeybees flew upwind toward one of the septa up to 10 cm.

Two-choice bioassay with *Orthosia gothica*. A wind tunnel (160 cm × 75 cm × 75 cm) was used for bioassays (Figure 4). A Fischbach speed controller fan (D340/E1, FDR32, Neunkirchen, Germany) continuously circulated the necessary air through the tunnel with an airspeed of 0.35 m s^{-1}. The incoming air was passed through four charcoal filters (145 mm × 457 mm), with a carbon thickness of 16 mm (Camfil Farr, Laval, Quebec, Canada). The temperature and humidity were adjusted to 22-24 °C and 30-32 %, respectively.

Experiments were carried out during the beginning of the dark period, under dim red light. One rubber GC septum was impregnated with 10 µl of a 1,4-dimethoxybenzene solution (10 µl 1,4-dimethoxybenzene dissolved in 90 µl paraffin) and the second rubber GC septum with 10 µl of a lilac aldehyde solution (synthesised as described in Dötterl et al. (2006); 10 µl lilac aldehyde dissolved in 90 µl paraffin). The two rubber GC septa were alternatively offered from both left and right sides. The septa were offered at the upwind end of the tunnel behind polyester gauze and metal grid, so that they were invisible to the moths. For the tests, individual moths were used singly. Moths, which had been caught with a light trap (see 2.2) the night before were kept over day dark and cool. Five hours before the bioassay started, they were adjusted to room temperature. During dusk (ca. 9 pm), moths were released from a holding chamber at the downwind end of the tunnel, and their behaviour was observed for 5 min. In this experiment, 22 male and 24 female moths were tested. Only 20 male and 22 female moths were active and of these 11 male and 12 female moths flew to the ceiling of the wind tunnel. Ten males and eight females flew in the wind tunnel to the GC septa. The behaviour of a single moth was counted as attraction (response) to the odour when moths zigzagged within a radius of 10 cm on the gauze in front of the odour source.

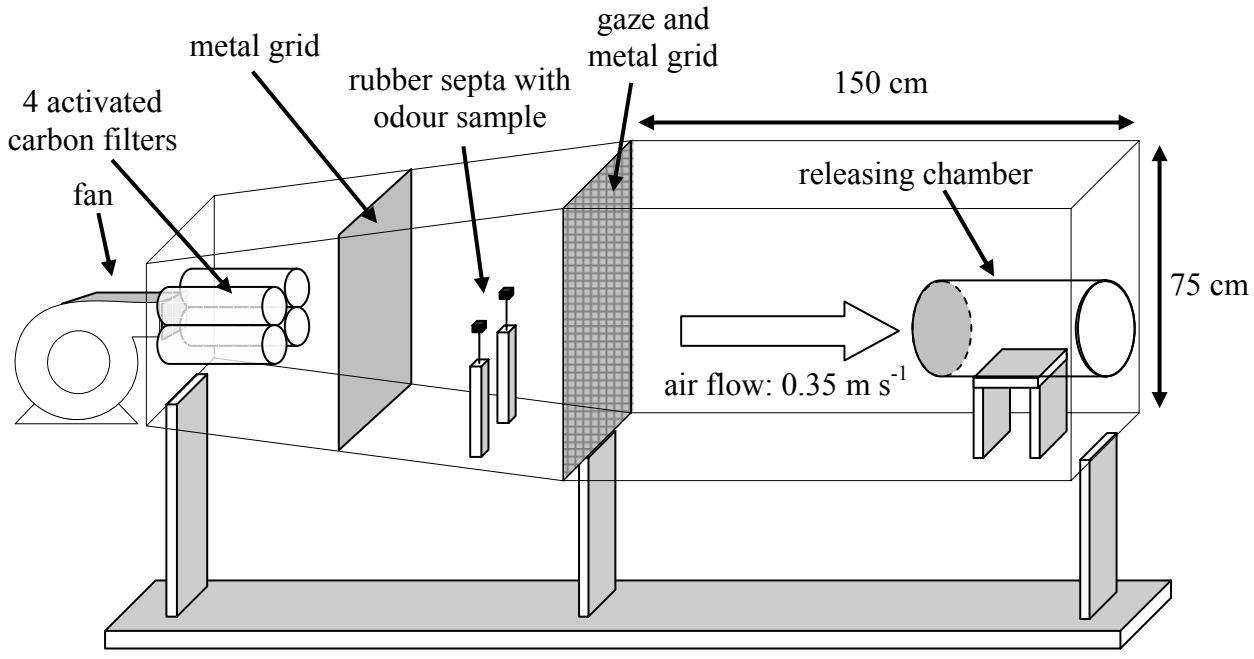

Fig. 4: Design of the wind tunnel used for the two-choice bioassay with *Orthosia gothica.*

2) To test the attractiveness of 1,4-dimethoxybenzene to *Andrena vaga* a two-choice bioassay was conducted in spring 2005 in the Ecological-Botanical Garden near a nesting site of

A. vaga. One rubber GC septum impregnated with 10 µl of 1,4-dimethoxybenzene (99 %, Aldrich) and one blank rubber GC septum were presented on a stand around noon for 20 min, when activity of bees was high. The positive reaction of bees was classified as "zigzagging" when the bees flew upwind towards one of the septa up to within 10 cm, and as "landing" when the bees had contact with a septum.

3) To test the attractiveness of male and female *Salix caprea* to *Apis mellifera* a two-choice bioassay was performed. The experimental design (Figure 5) consisted of three different test series (see points 1 to 3 below); each test series was conducted with three different arrangements (see Figure 5-1, 5-2, 5-3):

1. Comparison of the attractiveness of different floral traits against a control: The attractiveness of olfactory and visual cues as well as both cues combined was tested separately against a control (Figure 5-1).
2. Comparison of the attractiveness of floral traits against each other: The attractiveness of floral scent vs. visual cues, floral scent and visual cues combined vs. floral scent, floral scent and visual cues combined vs. visual cues (Figure 5-2).
3. Comparison of the attractiveness of sexes: The two genders of *Salix caprea* were compared regarding attractiveness of floral scent, visual cues, and olfactory and visual cues combined (Figure 5-3).

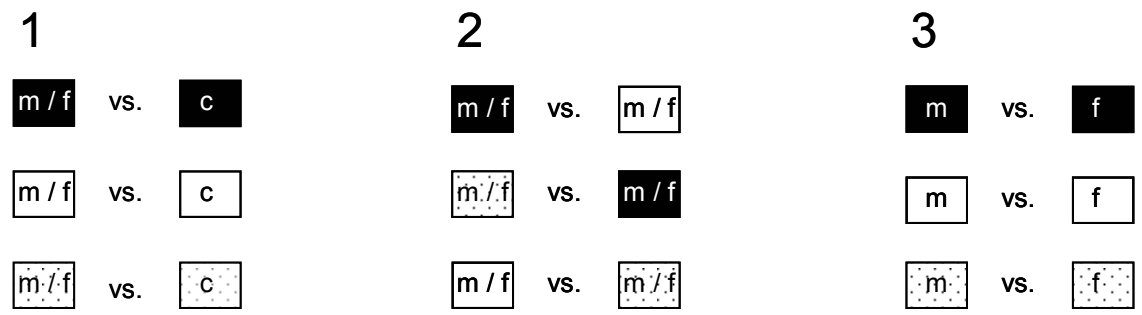

Fig. 5: The cylinder arrangement of the three test series: attractiveness of different floral traits against control (1), attractiveness of the different floral traits against each other (2), attractiveness of males against females (3). Filled squares = olfactory traits; open squares = visual traits, dotted squares = olfactory and visual traits combined; black squares with c (control) = empty cylinders; m = male branches, f = female branches used for the different tests.

Quartz glass cylinders were used to set-up the bioassays (Figure 6). One cylinder consisted of two pieces of quartz glass (cap and body, thickness of glass: 0.3 cm) and a sleeve composed of macrolon® (thickness 0.8 cm), which connected and sealed cap and body hermetically. The

macrolon® sleeve had 60 holes (diameter 0.2 cm), arranged in three horizontal lines to allow diffusion of floral scent. The cylinders were mounted with their bottoms on a PVC disc (diameter 11 cm) which was painted with a black, semi matte varnish. The disc was attached to a quadratic wooden table. A connecting element coupled the cylinder with a membrane pump (G12/01 EB, Rietschle Thomas, Puchheim, Germany).

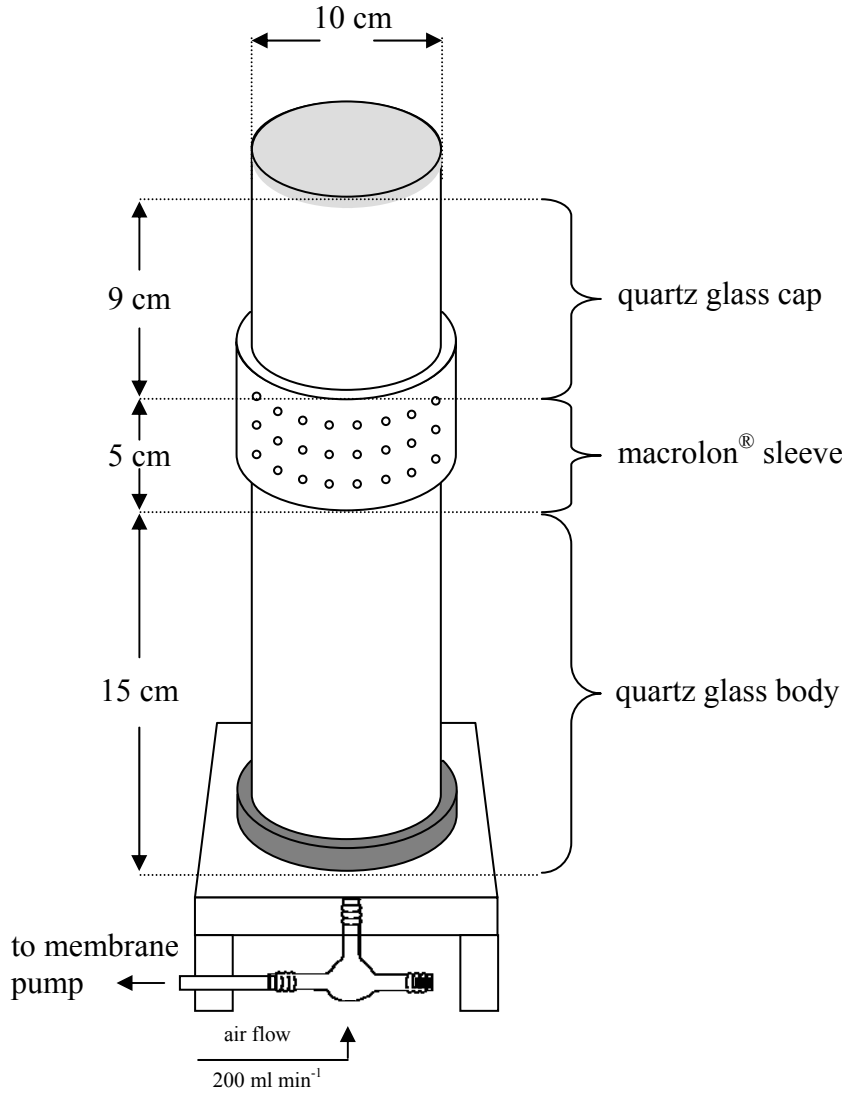

Fig. 6: Basic appearance of quartz glass cylinders used in the behavioural experiments to test the attractiveness of both genders of *Salix caprea* to *Apis mellifera*.

The design of this standard cylinder construction was modified according to the requirements of the particular test series, as described below:

- A standard cylinder as described above was used for testing attraction to olfactory and visual stimuli in combination.
- A cylinder without holes was used for testing visual attraction only.

- A cylinder with holes, but totally painted black with semi matte varnish was used for testing olfactory attraction only.
- For the empty control cylinders of test series 1, we used for each arrangement the cylinder type corresponding to the cylinder loaded with willow branches.

For all three cylinder types all varnished surfaces were dried for one week at 50 °C in a drying oven to eliminate scent emission of the varnish.

Bioassays were performed during the flowering season in 2007 (from March 12th to March 30th). Flowering branches of seven male and four female plants were cut in the field and placed in the cylinders. Cut ends were wrapped in moist tissue paper and placed in polyacetate oven bags to prevent scent emission from damp tissues. In all arrangements of the tests series 1 and 2, four female and four male flowering branches of one plant individual (eight branches had altogether approximately 80 catkins) were enclosed together in one cylinder. In all arrangements of test series 3, either eight male or eight female branches with approximately 80 catkins, respectively, were enclosed in different cylinders. If possible, for each arrangement and replicate of the tests, branches from different plant individuals were used.

The two-choice bioassay was performed in a flight cage (see above, behavioural test 1). Until the beginning of the experiment on March 12th, the bees had been fed with sugar solution. For each experimental arrangement both test cylinders were built up 3 m apart from the bee hive and 1 m apart from each other. All experiments were performed only on days with comparable weather conditions (sunny, at least 10 °C air temperature) between 12 pm and 3 pm, when the activity of bees was highest according to previous field observations (Füssel et al. submitted). According to these field observations, bee activity was higher on male sallows than on females around 12 pm, but at 2 pm honeybees usually visited both male and female catkins with comparable frequencies. Therefore, this time of the day seemed to be appropriate for bioassays testing different cues and sexes separately in order to eliminate as much as possible the effect of preferences of the honeybees for pollen collection or nectar foraging and different sexes at different times of the day. Each test was conducted for 20 min, then, it took 10 min to exchange the arrangement of the cylinders for the next test. For all three test series each arrangement was repeated once 20 min after the first trial. Usually, about 50 bees or more were active at a time during the bioassays. All active bees that flew to within 10 cm of a cylinder and started “zigzagging”, or contacted after “zigzagging” either the macrolon® sleeve (positive “landing” response to floral scent), or the cylinder where the

catkins where visible (positive "landing" response to visual stimuli) were counted and classified into two behavioural groups: bees that zigzagged only = Z, and those that landed after zigzagging = ZL. For later comparison we also summarised both groups (Z+ZL).

2.6 Sugar Composition and Concentration of Nectar in Flowers of *Salix caprea* (Publication 4)

Nectar volume, nectar sugar concentration and composition were analysed to determine differences in the floral reward common to male and female flowers.

In 2006, 25 nectar samples were collected from flowers of fully abloom inflorescences of 11 female and 14 male individuals of *Salix caprea*. Sampling took place between 11 am and 2 pm on sunny days with at least 10 °C air temperature. Nectar samples were taken with 0.5 µl capillaries ("Minicaps" from Hirschmann Laborgeräte). From each individual plant, one nectar sample, containing nectar from five to 15 flowers of a single catkin was taken. Nectar volume was determined and nectar was transferred into an Eppendorf reaction tube filled with 200 µl Milli-Q-Water. All samples were immediately frozen at -80 °C until further analysis.

The samples were analysed by using high performance liquid chromatography (HPLC – Jas.co PU-1580) equipped with a CarboPac PA 100, 4 x 250 mm column. Frozen nectar samples were thawed and diluted appropriately 1:10 to 1:100 with Milli-Q-Water, and a 2 µl subsample was injected for analysis. Elution took place in Milli-Q-Water with a 0.5 M NaOH gradient from 3 to 70 % at a flow rate of 1 ml min^{-1}. An electrochemical detector (Dionex ED 40) was used for sugar detection. Borwin Chromatogram software created the respective chromatograms. Nectar sugar composition of *Salix caprea* was determined by comparison with standards (glucose, fructose, and sucrose). Sugar amount per single flower (µg), nectar sugar concentration (mol l^{-1}), and nectar sugar composition (proportion % of single sugars in relation to total sugar content) were calculated.

2.7 Pollination Experiment (Publication 2)

In 2006, five female *Salix caprea* individuals of similar size and age (same subset as for pollinator observations described in 2.2) were chosen for pollination experiments. Before

stigmas became receptive, I selected per plant four twigs each with five to 25 catkins for the following four pollination treatments:

(1) day- and night pollination (control): no exclusion of insects;

(2) day pollination: exclusion of insects during night (8 pm until 6 am);

(3) night pollination: exclusion of insects during day (6 am until 8 pm);

(4) wind pollination: exclusion of insects during day and night.

To exclude insects, twigs were enclosed with a nylon net (unifilar fabric of gossamer). To guarantee natural progress of fruit and seed development, all nylon nets were removed after the twigs had ceased flowering. Shortly before seed maturity, single fruit catkins were enclosed in dialysis tubing (cellulose, Visking, Type 1-7/8, diameter 79 mm). When fruits opened inside the dialysis tubing the catkins were harvested. The number of seeds and capsules per catkin were counted and the number of seeds per capsule was calculated. Since the calculated numbers of seeds per catkin and seeds per capsule varied greatly within pollination treatments among the different plant individuals, the data were standardised for further analyses. The maximum seed set of open day- and night pollination (control) of an individual was equated with 100 %. For the other pollination treatments (2-4) the amount of seeds per catkin and seeds per capsule is given as percentage of the maximum seed set found in the corresponding control.

3 Results and Discussion

3.1 What Is the Chemical Composition of *Salix* Floral Scent? How Does it Vary with Species, Gender, and Time of the Day? (Publications 1, 2, and 4)

Floral scent composition of various *Salix* species, the variability of floral scent among species (Publication 1), within species (Publication 1), and between genders (Publications 1 and 4) as well as temporal variation of floral scent emission (Publication 2) were examined.

In 32 European and two Asian *Salix* species a total of 48 compounds was detected, most of them being isoprenoids and benzenoids. Commonly occurring compounds included *trans*-β-ocimene, *cis*-β-ocimene, benzaldehyde, D-limonene, α-pinene, *cis*-3-hexenylacetate, linalool, 1,4-dimethoxybenzene, and β-pinene. Many floral scent compounds identified in

Salix species are known as typical floral odour compounds from other plant species (compare e.g. Knudsen et al. 2006).

Interspecific variation

Analyses of floral scent composition of species of the two subgenera *Salix* ($N = 5$) and *Vetrix* ($N = 28$) revealed no differences between these subgenera (CNESS, ANOSIM: $R = -0.035$; $p = 0.66$). However, within the subgenus *Vetrix*, significant differences between species of the section *Arbuscella* ($N = 4$) and *Vetrix* ($N = 8$) were found (CNESS, ANOSIM: $R = 0.274$; $p < 0.005$). *cis*-3-Hexenylacetate and 1,4-dimethoxybenzene were the main variable compounds between these two sections. A relatively high amount of *cis*-3-hexenylacetate was found in the section *Arbuscella* and of 1,4-dimethoxybenzene in the section *Vetrix*.

Differences of floral scent composition (relative amounts) among 34 *Salix* species, based on the CNESSm = 1 index are visualised in Figure 7, using nonmetric multidimensional scaling (stress: 0.19).

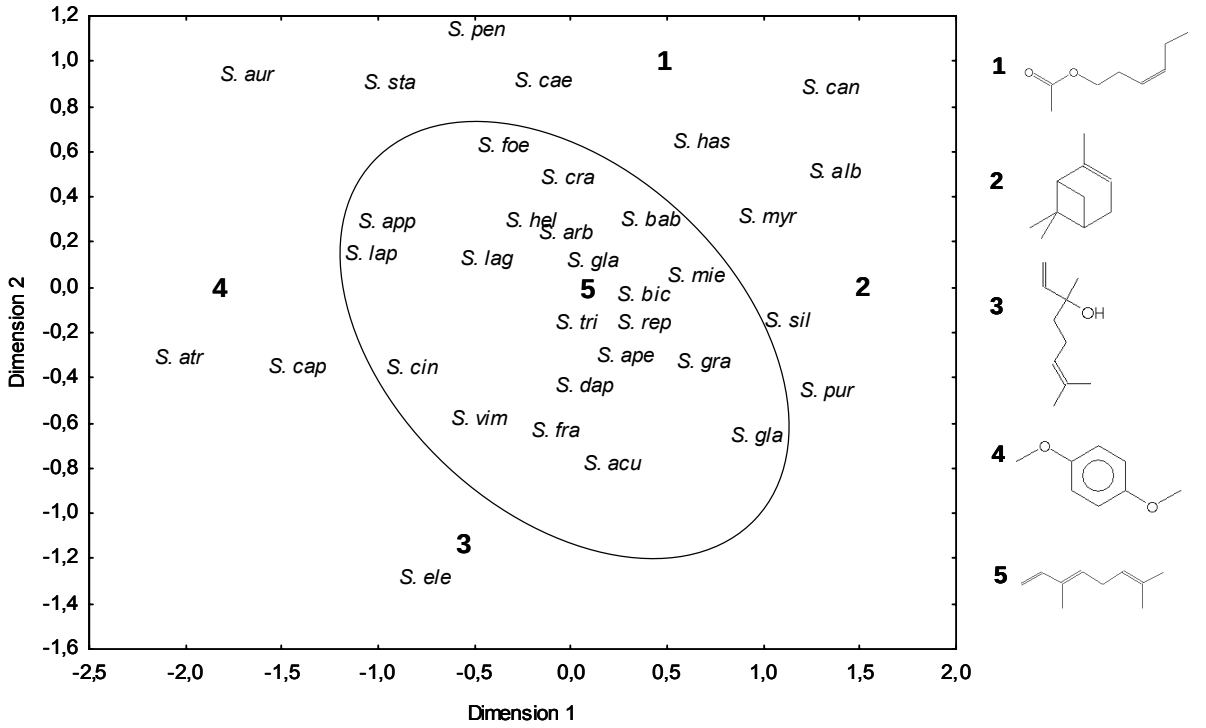

Fig. 7: Nonmetric multidimensional scaling (NMDS) of floral scent profiles of 34 *Salix* species based on the CNESSm = 1 index (stress: 0.19). The structures and names of the five main compounds: (1) *cis*-3-hexenylacetate, (2) α-pinene, (3) linalool, (4) 1,4-dimethoxybenzene, (5) *trans*-β-ocimene dominating the scent of different species are presented in the figure. The circle comprises species with more than 30 % relative amount of *trans*-β ocimene. The abbreviations of the *Salix* species are listed in Part B, Chapter 1, Table 1.

In general, no clear separation of species groups was found. Most species were more or less evenly distributed, and clear separation of species subgroups was hardly possible. However, species in the centre of the scatter plot were characterised by the emission of high relative amounts of *trans*-β-ocimene (more than 30 %), while the proportion of this monoterpene was lower in species at the margins. In *Salix caprea*, *S. atrocinerea*, *S. aurita*, and *S. cinerea*, 1,4-dimethoxybenzene was a dominant compound (more than 50 %). In other species (*S. mielichhoferi*, *S. myrsinifolia*, and *S. silesiaca*), high amounts of α-pinene (25-35 %) were detected. High amounts of the green leaf volatile *cis*-3-hexenylacetate (50-65 %) were emitted by *S. starkeana* and *S. pentandra*, and the isoprenoid linalool occurred in large amounts (32 %) in *S. eleagnos*.

In a subset of eight extensively sampled species (*S. bicolor*, *S. caprea*, *S. cinerea*, *S. fragilis*, *S. myrsinifolia*, *S. repens*, *S. triandra*, and *S. viminalis*), except of *S. bicolor* and *S. repens* all others had a characteristic floral scent composition; half of the pairwise species comparisons confirmed significant differences. The results show that variation in floral scent in *Salix* may provide specific signals which may guide pollinators and thus contribute to the reproductive isolation of compatible and co-occurring species.

Intraspecific variation

The variability within species could be explained by sex differences at least in three (*Salix fragilis*, *S. myrsinifolia*, and *S. triandra*) out of a subset of eight species (Publication 1). The significant gender differences (ANOSIM: $R = 0.623$; $p < 0.001$) in floral scent of *Salix caprea* (Figure 8) found in Publication 4 are contradicting the data published in our first study on intra- and interspecific variability of floral scents in the genus *Salix* (Füssel et al. 2007; Publication 1). But also in Publication 4, most substances were found in scent samples of both genders of *S. caprea*, and differences were often only semiquantitative. Tollsten and Knudsen (1992) found also high resemblances in floral scent of male and female inflorescences, but they also demonstrated at least small differences in the floral scent profile between sexes for *S. caprea*. These authors found dissimilarities of male and female scent of only 10.6 %, while we found 32.2 %. Different methods were used in the two studies (e.g. different adsorbents, thermodesorption vs. extraction of volatiles from filter using solvent), and perhaps these methodical differences were responsible for the differing results (see Füssel et al. 2007). Both studies found that male flowers produced relatively more 1,4-dimethoxybenzene than other substances, but Tollsten and Knudsen (1992) detected

methylsalicylate only in low relative amounts, whereas in our study methylsalicylate is one of the four main compounds (1,4-dimethoxybenzene, *trans*-β-ocimene, methylsalicylate, linalool) explaining altogether more than 60 % of the observed variability between male and female floral scent composition.

Anther and pollen volatiles differed significantly from male and female inflorescence scent emission (ANOSIM: $R = 0.48$; $p < 0.001$). Direct comparison of absolute emission between anthers and inflorescences is hardly possibly because of the different methods used, however, as the strong dominance of 1,4-dimethoxybenzene in male headspace is not reflected in the composition of anther volatiles (dominated by *trans*-β-ocimene), it can be concluded that other floral organs than anthers and pollen alone are responsible for the male-specific scent emission which is characterised by relatively and absolutely high amounts of 1,4-dimethoxybenzene.

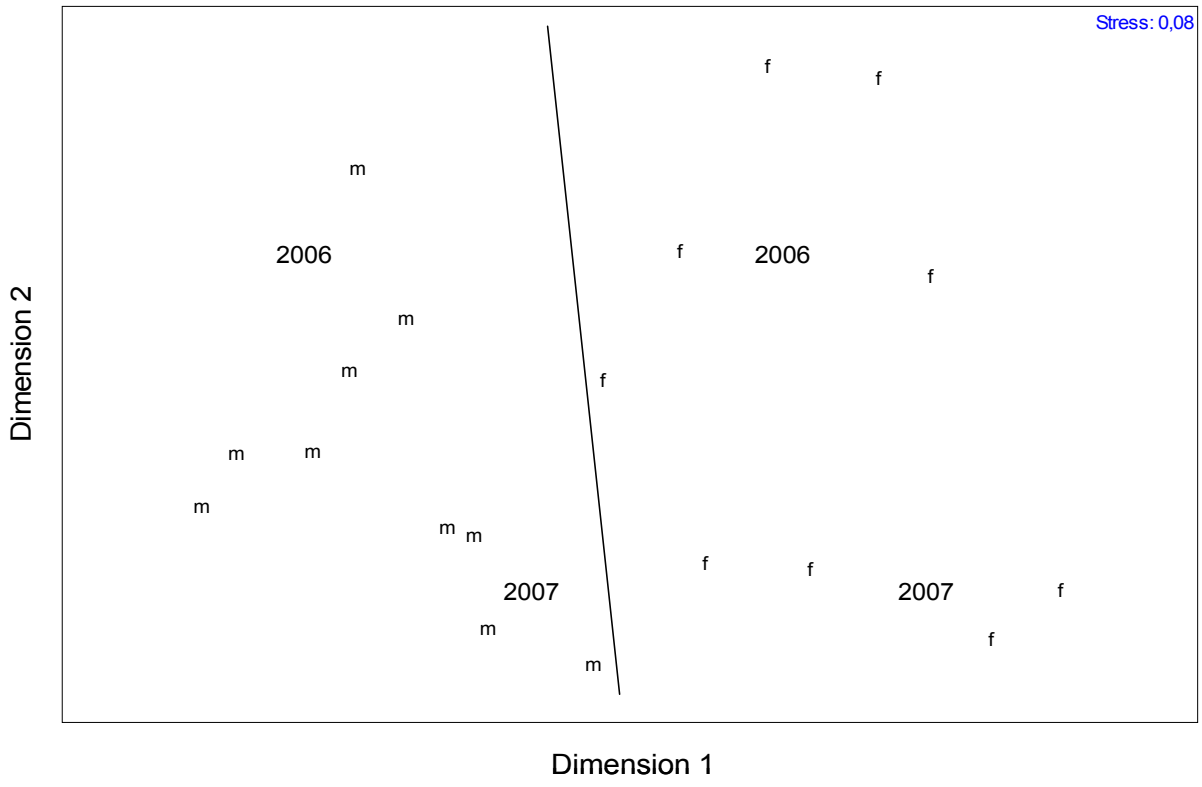

Fig. 8: Nonmetric multidimensional scaling (NMDS) of floral scent composition of different sets of male (m) and female (f) individuals of *Salix caprea* sampled in 2006 and 2007 (stress: 0.08).

Circadian rhythmicity of floral scent emission

In *Salix caprea,* during the day a significantly higher total amount of floral scent was emitted compared to the night. Furthermore, a strong correlation between floral scent emission and temperature (Figure 9) was found. Most likely, temperature influences floral scent emission of

S. caprea over a day. Similar circadian rhythms were reported in other plant species (see e.g. Matile and Altenburger 1988; Picone et al. 2004), and some authors explained differences of the quantity of fragrance emission by temperature effects (Jakobsen and Olsen 1994; Wang and Pichersky 1998; Dudareva and Pichersky 2000). However, in our study, contrary to total scent emission, some single floral scent compounds (e.g. lilac aldehyde isomers) were emitted in higher relative amounts as well as total amounts during night when the temperature was much lower compared to day-time. The increased emission of lilac aldehydes at night may be the result of an upregulation of genes, which are involved in the biosynthesis of these monoterpenes, in the evening. Such an upregulation of genes in the late day was demonstrated for example in *Petunia hybrida* line W115 (Mitchel) (Solanaceae), a plant emitting the highest relative amount of benzenoids at dusk (Verdonk et al. 2003). The emission of high amounts of volatiles at night is typically found in plants that are pollinated by nocturnal insects (Dobson 2006). In case of *Nicotiana attenuata* (Solanaceae), night-pollinating insects such as *Manduca sexta* hawkmoths could be attracted by the high relative nocturnal emission of the compound benzylacetone (Kessler and Baldwin 2006). Huber et al. (2005) showed that phenylacetaldehyde in *Gymnadenia odoratissima* (Orchidaceae) was emitted in higher relative amounts during night and attracted effectively nocturnal moths. Our data likewise suggest that the isomers of lilac aldehyde, which were emitted during night in higher relative as well as total amounts than during day, represent an adaptation for attraction of nocturnal moths, particularly *Orthosia* species which visit *S. caprea* flowers in highest numbers at the time of relatively highest lilac aldehyde emission.

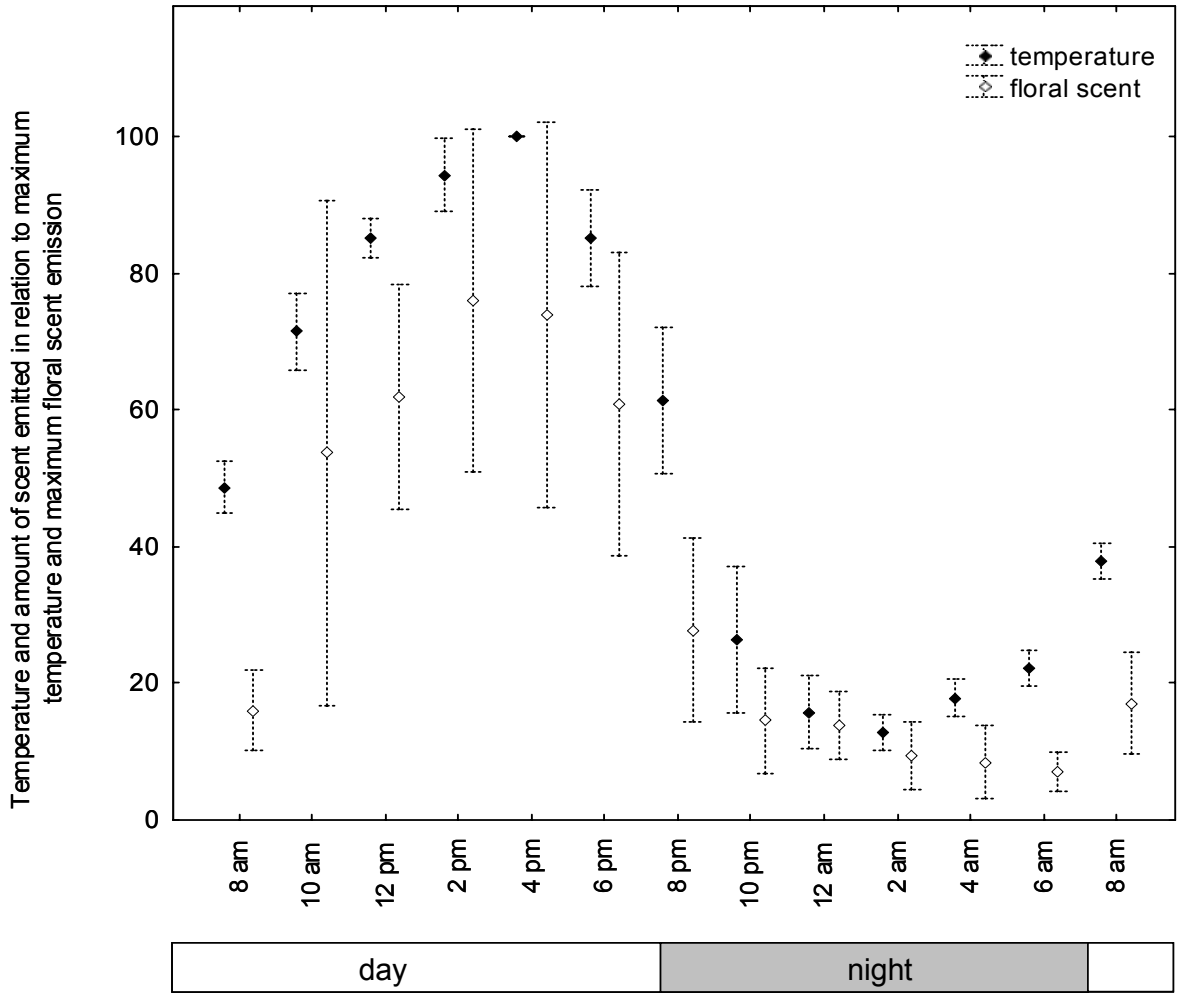

Fig. 9: Circadian emission of relative floral scent amounts of seven *Salix caprea* specimens (mean ± SE) and relative average air temperature during scent collection (mean ± SE, *n* = two days).

3.2 Which Are the Flower Visitors of *Salix caprea*? (Publication 2)

The spectrum of flower visitors of *Salix caprea* comprised a high number of different species: About 150 species of Diptera (unpublished data, determination is still in progress), 25 species of Lepidoptera (predominantly night-active moths), 20 species of Hymenoptera, 20 species of Coleoptera, and 10 species of Hemiptera were recorded. Until identification of all other visitor groups (e.g. Coleoptera and Diptera) is accomplished, data analyses focuses on the orders Lepidoptera and Hymenoptera, because they were the most frequently observed and usually pollen carrying flower visitors. It is known that flies are considered as flower visitors of *Salix*, but the frequency is depending on the *Salix* species (Totland and Sottocornola 2001). In this work I found different species of Diptera, but the total numbers which are detected on the catkins of seven *S. caprea* during the course of day was ten. Surprisingly, flies were more

often detected on male *S. caprea* individuals. Hence their role as potential pollinators may decrease.

The abundance of different flower visitor groups (honeybees, bumblebees, medium sized bees, small bees, butterflies, moths, other insects) during the course of the day is shown in Figure 10. Activity was highest between 10 am and 4 pm. The most frequently observed insects during day were bees, butterflies, and other insects (e.g. 2 pm: 38 bees, four butterflies, ten other insects per 15 min). From dusk onwards (8 pm) the total number of flower visitors declined, and moths (six moths per 15 min) were the most common flower visitors. With the beginning of dawn (6 to 8 am) first active bumblebees were recorded and the assemblage changed again to day-active bees and other insects.

In this study, many nocturnal moth species were observed as visitors of willow catkins. Several of these species, e.g. *Orthosia gothica*, visited *Salix* frequently; these moths use willow flowers as an important source of nectar in the early spring. Potential pollinators may be both bees as well as diurnal and nocturnal Lepidoptera, which were frequently seen to contact the anthers, carry pollen and transfer the pollen from male flowers to female flowers. Further investigations will give information about the role of the flower visitors of the orders Diptera, Coleoptera, and Hemiptera.

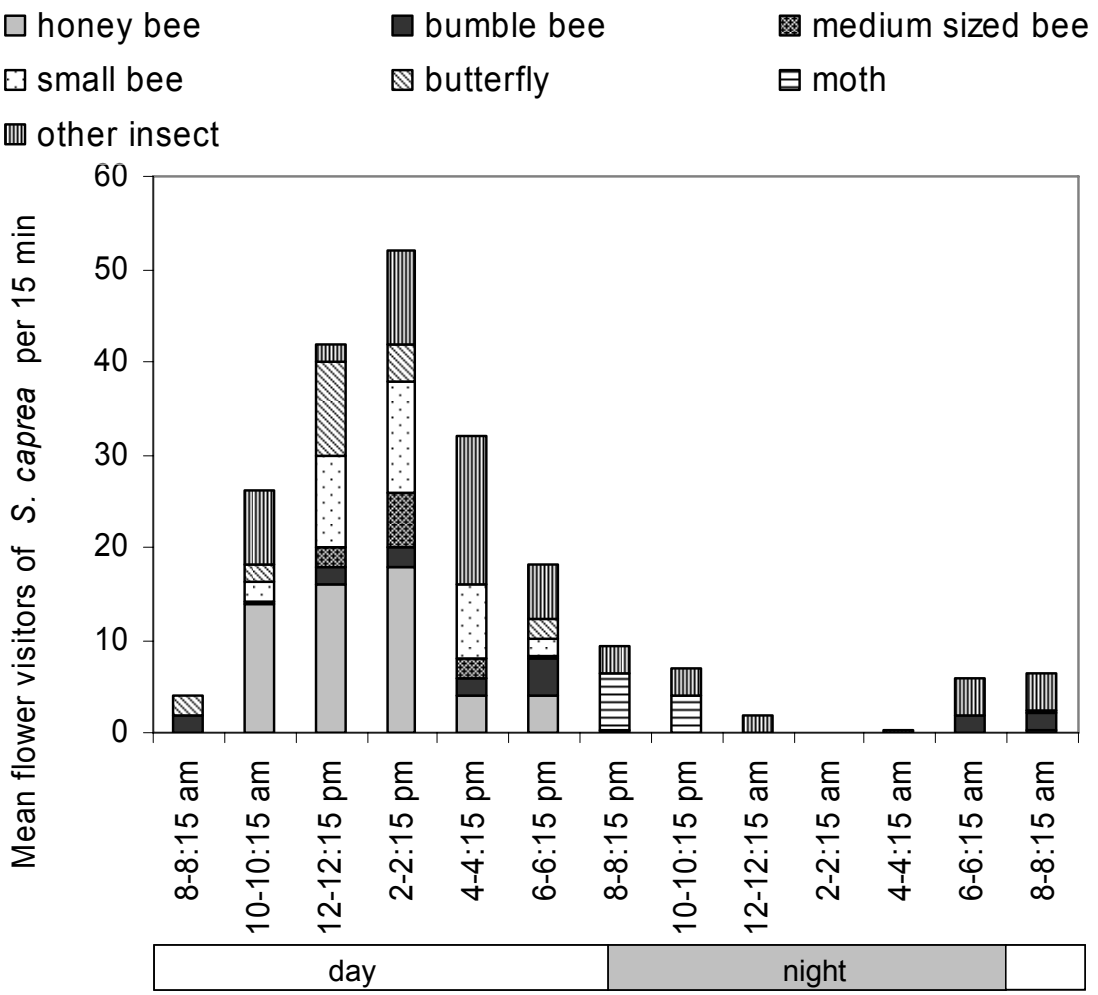

Fig. 10: Mean number of flower visits (type and number of observed flower visitor individuals per time) of *Salix caprea* (n = 7) per 15 minutes in the course of a day (n = 6).

3.3 Which Floral Scent Compounds Can Be Detected by Flower Visitors of *Salix*? (Publications 2 and 3)

To evaluate the role of floral scent compounds for attraction of flower visitors of *Salix,* electroantennographic studies were performed. In the electroantennographic (GC-EAD) study, 25 out of 38 floral scent compounds of *Salix caprea* elicited signals in the antennae of potential pollinators (oligolectic and generalistic bees as well as moths). Interestingly, bees and moths responded nearly to the same subset of compounds, however, the strength of the response to certain components differed between both groups. Interestingly, the moths strongly responded to the co-eluting compounds lilac aldehyde A, benzylnitrile, and 4-oxoisophorone, while the response of the bees was less pronounced. It is unclear, which of the three co-eluting compounds were responsible for the observed differences between moths

and bees. Actually, only antennal responses of moths to different lilac aldehyde isomers (including lilac aldehyde A) were shown (Plepys et al. 2002b; Dötterl et al. 2006), and it is unknown, whether moths also respond to benzylnitrile and 4-oxoisophorone. Lilac aldehyde is often found in plants pollinated by moths (Dobson 2006; Knudsen et al. 2006), and it was proven in the present study as well as in previous studies to be highly attractive for moths (Plepys et al. 2002a; Dötterl et al. 2006).

In all measurements with bee antennae, 1,4-dimethoxybenzene, which was found to be a major component of male inflorescence scent in relative and absolute terms (contrary to female floral scent) elicited the highest signals, whereas the responses to the other compounds were comparatively small.

3.4 Do Electrophysiological Active Compounds Act as Attractants for Potential Pollinators of *Salix caprea*? (Publications 2 and 3)

Electrophysiologically active compounds were tested in field bioassays to identify possible attractants for potential pollinators of *Salix caprea.* Bioassays (two-choice experiments) were conducted with 1,4-dimethoxybenzene and lilac aldehyde, two components which elicited the strongest antennae signals in the most frequent diurnal and nocturnal flower visitor species of *Salix caprea*. Honeybees responded most strongly to 1,4-dimethoxybenzene, which was emitted at a higher relative amount as well as total amount during day-time, whereas most moths responded besides 1,4-dimethoxybenzene also to the isomers of lilac aldehyde (Figure 11) which are emitted in higher percentage as well as total amount at night. It seems that *S. caprea,* although an interaction generalist, evolved temporally fine tuned scent emission with quantitative and qualitative changes in the scent composition in adaptation to the preferences of different types of potential pollinators.

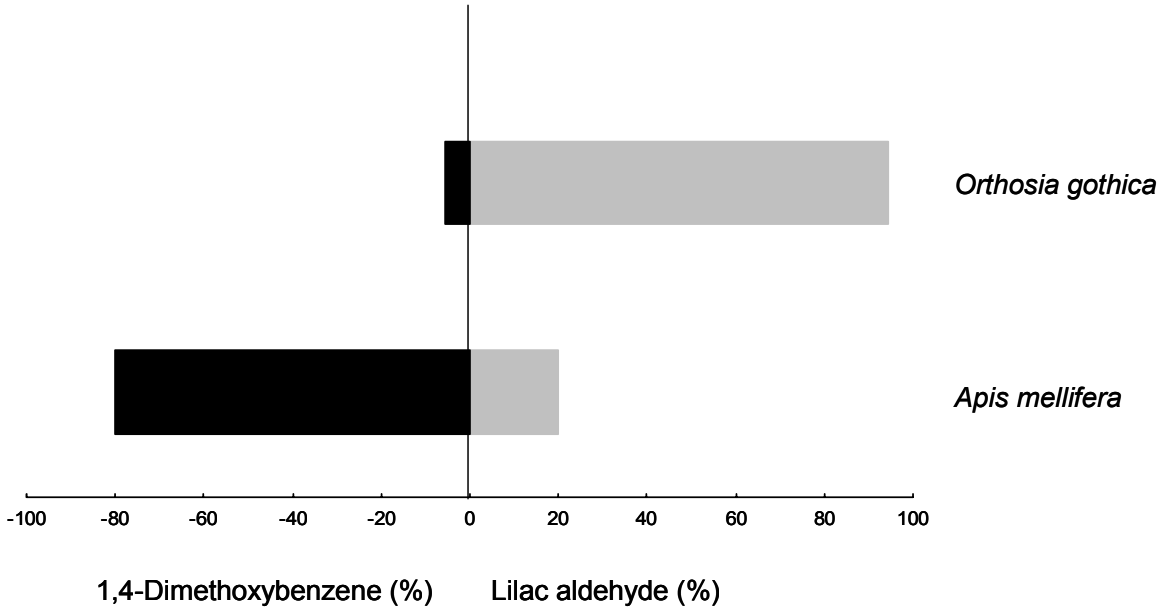

Fig. 11: Attraction of *Apis mellifera* (*n* = 101) and *Orthosia gothica* (*n* = 18) by 1,4-dimethoxybenzene (black) and lilac aldehyde (grey).

3.5 Which Gender of *Salix caprea* Is More Attractive to *Apis mellifera*? What Role Do Visual and Olfactory Cues Play? (Publication 4)

For successful pollination in dioecious plant species like *Salix caprea* it is necessary that pollinators visit both genders repeatedly, but gender separation is often linked to gender specialisation and divergence in floral traits, such as reward and advertisement. As this is clearly the case in *S. caprea,* where male flowers offer pollen and nectar whereas females offer only nectar, the attractiveness of both genders of *Salix caprea* to *Apis mellifera* was examined.

In *Salix caprea* honeybees respond to both olfactory and visual cues. However, we found that floral scent is more attractive than visual cues alone. Nevertheless, the combination of floral scent and visual signals attracts more bees than either cue alone.

Interestingly, floral scent of male and female *Salix caprea* catkins was similarly attractive to its main flower visitor *Apis mellifera*, despite the differing total scent emission (male floral scent = 350.61 ng; female floral scent = 79.88 ng) and significant sex-specific differences of relative scent composition. Thus, although scent of *S. caprea* is used by honeybees as a cue to find flowers and is advertising different sets of rewards in the genders (pollen and nectar in male, only nectar in female flowers), scent alone had no effect on flower choice of honeybees. Altogether, floral scent alone is a relatively uncertain cue to discriminate male and female

flowers of *S. caprea*: Total scent intensity is depending on other factors such as wind or distance, and composition is different but not consistently distinct enough across time and space. Reason for this might be that anther and pollen volatiles are not determining male plants' scent. Although male willows may have billions of anthers open at a time, and anthers contain an extremely specific and distinct spectrum of volatiles, the emitted scent spectra of male plants are not corresponding with anthers volatile composition. In the bioassay a combination of olfactory and visual signals of male flowers attracted more honeybees than olfactory and visual cues from female flowers. Accordingly, differing visitation rates to male and female sallows were reported from field observations (Füssel et al., unpublished data). Female individuals of *S. caprea* were visited by honeybees at a lower intensity than males, possibly due to the yellow signalling colour of anthers. Different visitation rates of the two genders might be advantageous, because successful pollination requires a prior visit of one or several male willow flowers to load the pollinator with sufficient pollen for subsequent pollination of female flowers. If visitation frequency to male willows is higher, the probability of successful pollination of a female willow might increase. Moreover, with increasing visit frequency to males, the higher probability of pollen transfer from a diverse array of male individuals to females might increase the genetic diversity of the progeny.

3.6 Does the Nectar Reward of Male and Female Flowers of *Salix caprea* Differ? (Publication 4)

The different attractiveness of the sexes is due to the different rewards, but as our results show information about the different reward offers is better mitigated by visual than by olfactory cues. Besides pollen that is only offered by males, we found also differences in nectar. Female *Salix caprea* flowers produce tendencially more nectar sugar per flower than male flowers. However, flower number per catkin is higher in males than in females (Kay 1985; Karrenberg et al. 2002). We found that females offer significantly higher concentrated nectar thus confirming the results of Elmqvist et al. (1988), and Katoh et al. (1985). Nectar composition also differs significantly between sexes. Similar results were reported from Percival (1961), Goukon et al. (1976), Katoh et al. (1985), and Elmqvist et al. (1988) from different willow species. According to the classification of Baker and Baker (1983), females have hexose-rich nectar ($S/(F+G) = 0.52$) in contrast to sucrose-dominated nectar ($S/(F+G) = 5.22$) in males (Mann-Whitney-U-Test: $Z = 4.22$; $p < 0.001$) (S, F, and G: amount of sucrose, fructose, and glucose, respectively). With respect to the single three

sugars, nectar composition of females is relatively well balanced, a phenomenon that according to Percival (1961) is relatively rare in plants. It is known that honeybees prefer balanced nectars with more or less equal amounts of all three sugars (therefore usually hexose-rich nectars according to the classification of Baker and Baker (1983) over sucrose-dominated nectars) (Wykes 1952). It may be hypothesised that female flowers compensate for the lack of pollen with higher concentrated nectar which matches the preferences of bees better than nectar from male catkins. Further behavioural tests in the field are necessary to determine if flower visitors, such as honeybees, link sex-specific visual cues to nectar quantity and quality of the genders. Greco et al. (1996) stated that the activity or rather the visitation rate of honeybees is associated with the circadian availability of resources. According to our own field observations, the visitation rate by honeybees on male *Salix* inflorescences is high in the late morning when activity in general is high, whereas female plants have a higher visitation rate in the afternoon when activity in general is decreasing. Most likely, a combination of changing reward presentation and changing pollinator preferences in the course of the day account for this visitation pattern.

3.7 What Is the Contribution of Different Pollen Vectors to Reproductive Success? (Publication 2)

Floral scent analyses and behavioural tests point towards a temporally fine tuned scent emission of *Salix caprea* with specific adaptation to the preferences of different types of potential pollinators, such as bees during the day, and moths at night. To verify the importance of different functional groups of flower visitors and wind for the reproductive success of *S. caprea* pollination experiments were performed. They revealed that day-active visitors contributed most to the reproductive success in terms of seed set, whereas wind and nocturnal flower visitors played a minor role, the latter possibly due to low activity in response to the low temperature at night (see Figure 12). These results correspond to other studies where both nocturnal and diurnal potential pollinators were found visiting flowers of the same plant species and where diurnal pollinators were usually found to be more abundant than nocturnal ones, resulting in higher visitation rates and greater seed yields (Jennersten 1988; Jennersten and Morse 1991; Altizer et al. 1998; Miyake et al. 1998; Balmford et al. 2006). However, neither diurnal, nor nocturnal pollinators, nor wind alone, achieved maximal reproductive success. Even a combination of all pollen vectors in the open pollination experiment did not result in maximum seed set of all flowers and ovules. It seems that

S. caprea is still pollen-limited and therefore any additional pollinating agent is advantageous. However, the contribution of the different pollinator types and wind pollination to the reproductive success of the plant may vary between years, and future studies are needed to consider possible resource limitation that might prevent maximum seed.

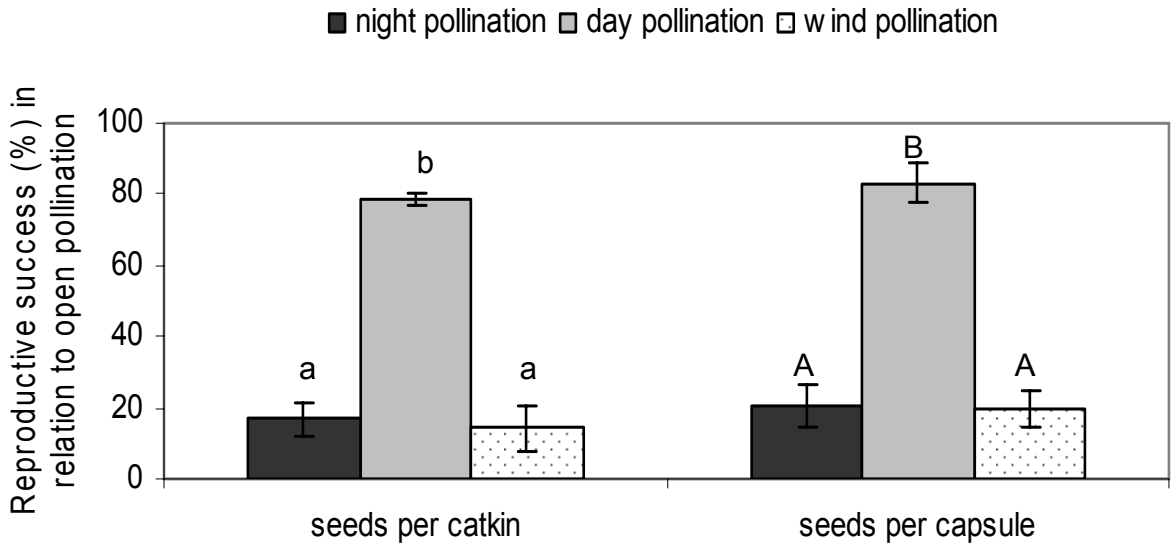

Fig. 12: Reproductive success, represented as percentages of seeds per catkin and seeds per capsule of *Salix caprea* ($n = 5$) resulting from different pollination treatments (night- , day- and wind pollination; means ± SE) in relation to open pollination (control). Significant differences of seed set between pollination regimes (LSD test: $p < 0.001$): Capital letters = per capsule, small letters = per catkin.

3.8 Is *Salix caprea* a Generalist or a Specialist Regarding the Pollination System? (Publications 1, 2, 3, and 4)

The pollination system of *Salix* is generally regarded as a generalistic pollination system, with both insects of different systematic and functional groups and wind as pollen vectors (e.g Vroege and Stelleman 1990; Karrenberg et al. 2002). However, it is generally assumed that a generalistic pollination system evolves little adaptations to specific pollen vectors. Contrary, my data give evidence that the interaction generalist *S. caprea* shows not only specific adaptations to wind- and insect pollination, but has furthermore evolved a specific pattern of floral scent emission as adaptation to its two main functional pollinator groups (diurnal pollen- and nectar-seeking bees, nocturnal nectar-seeking moths), which both contribute effectively to total reproductive success: Thus *S. caprea* is an interesting example supporting Aigner's (2006) hypothesis that floral characteristics may represent adaptations to pollinators that are neither most numerous nor most effective, but provide nevertheless a marginal fitness

gain. This view differs from Stebbins' (1970) "most effective pollinator principle" which states that "the characteristics of flowers will be moulded by those pollinators that visit it most frequently and effectively". Altogether, this case study is challenging the existing concepts of specialisation/generalisation of plant-pollinator interactions. Regarding the aspect of interactions, *S. caprea* is a generalist, but looking at the aspect of adaptations, *S. caprea* can be regarded as a multi-specialist with respect to its floral scent emission. Considering the third aspect of specialisation, the importance of different pollinator types (bees versus moths versus wind), *S. caprea* takes an intermediate position, with bees seeming the most important but not too dominant pollinating agent.

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5 Short Summary

The present work studied the role of floral scent in plant-insect interactions of the dioecious genus *Salix*. Besides a general survey of floral scent in willow species, I conducted a detailed case study on its role for pollinator attraction in *Salix caprea* (see Figure 13).

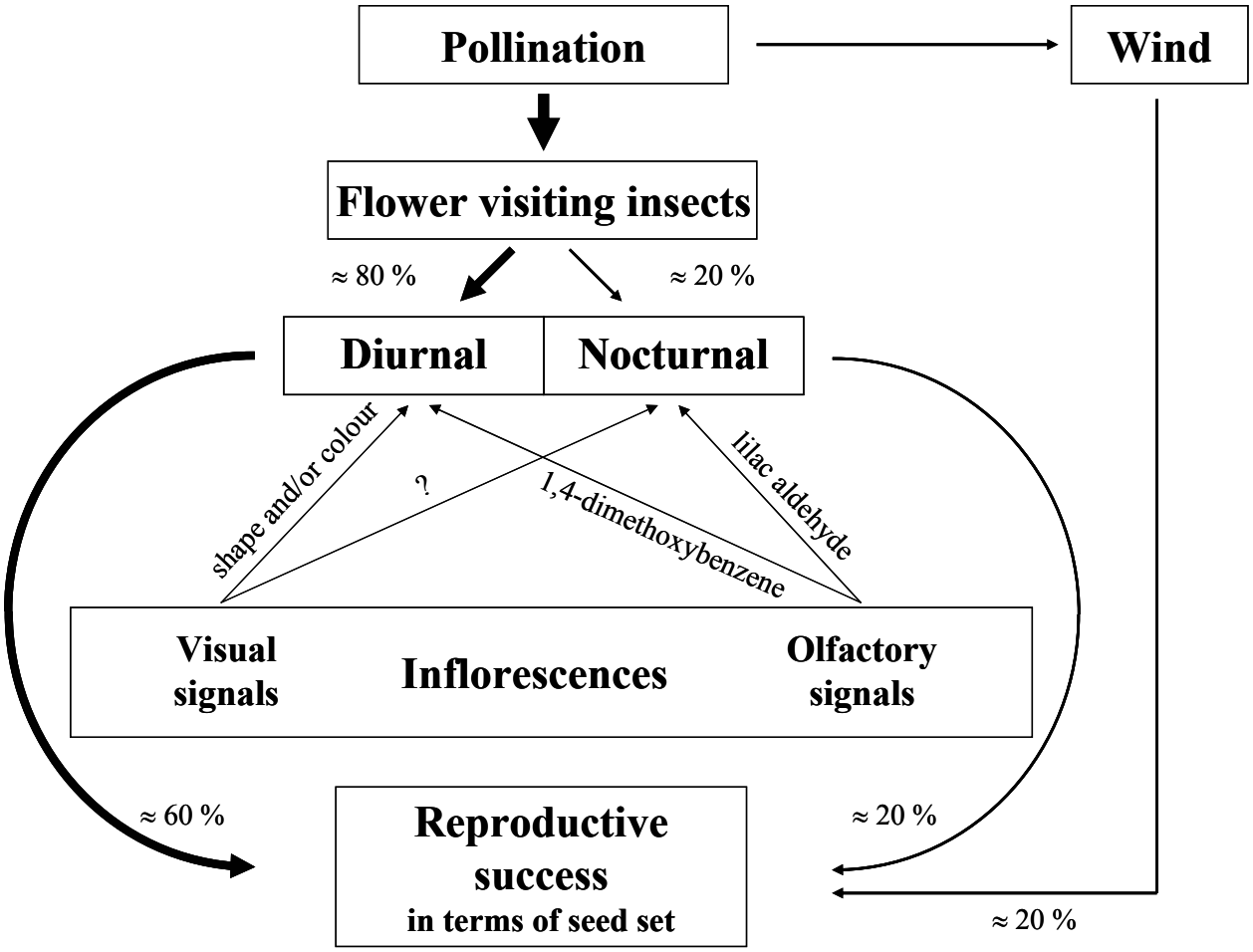

Fig. 13: Pollination system of *Salix caprea* (schematic) according to the results presented in this thesis.

Besides adaptations to insect pollination, *Salix caprea* shows also traits of wind pollination, but according to my results wind played only a minor role for reproductive success in terms of seed set. Flower observations show that the catkins of *Salix caprea* are visited by numerous insect species with a broad taxonomic spectrum. During day, flowers were mainly visited by diurnal bees (e.g. *Apis mellifera*, *Bombus terrestris*, *Andrena praecox*, *A. clarkella*), after sunset, nocturnal moths (e.g. *Orthosia cerasi*, *O. gothica*, *O. gracilis*) were the nearly exclusive flower visitors.

Insect pollinated flowers advertise themselves by olfactory and visual cues. Olfactory cues in terms of volatile composition as well as scent intensity correspond to different pollinator

assemblages. Totally 48 floral scent compounds were detected in 34 *Salix* species, most of them isoprenoids (e.g. *trans*-β-ocimene, D-limonene, and lilac aldehyde) and benzenoids (e.g. 1,4-dimethoxybenzene, benzaldehyde). Only two of these components, the benzenoid 1,4-dimethoxybenzene and the isoprenoid *trans*-β-ocimene, were responsible for most of the interspecific variation between genders, with males emitting relatively and absolutely higher amounts of 1,4-dimethoxybenzene than females.

Salix caprea flowers show a rhythmic scent emission. The floral scent emission was higher in the day than during the night, which is most likely due temperature effects. However, in our study, contrary to total scent emission, some single floral scent compounds (e.g. lilac aldehyde isomers) were emitted in higher relative amounts during night. The increased emission of lilac aldehydes at night may be the result of an upregulation of genes, which are involved in the biosynthesis of these monoterpenoids in the evening.

EAD studies and bioassays with diurnal *Apis mellifera* (Hymenoptera) and nocturnal *Orthosia gothica* (Lepidoptera) showed that the responses of these insect species correspond well to the circadian patterns of emitted compounds: Honeybees responded most strongly to 1,4-dimethoxybenzene, while moth species responded besides 1,4-dimethoxybenzene also to a group of co-eluting compounds including lilac aldehyde A, benzylnitrile, and 4-oxoisophorone.

Attracting pollinators is especially crucial in dioecious plants like *Salix* species, where sexual reproduction depends on pollen transfer from male to female individuals and it can be assumed that strong selective pressures are working on the odour composition to optimize repeated visitation of both genders and thus pollination. However, the floral scents of male and female *Salix* individuals are very similar in some species, whereas in other *Salix* species like *S. caprea*, *S. fragilis*, *S. myrsinifolia*, and *S. triandra* the genders emitted significantly different floral scent spectra. Such sex differences are often related to different attractiveness of the flowers for pollinators and differing pollinator behaviour. But in case of *Salix caprea* the divergence in floral scent between male and female individuals, and a clearly distinct anther and pollen volatile composition had no significant effect on the attractiveness of the two genders. Visual cues of *S. caprea* seem to play a major role for flower finding and gender differentiation by its pollinator. Male flowers of *Salix* may often be more attractive to pollinators because they offer both nectar and pollen, and especially the latter advertises itself by its obvious visual stimulus whereas female *Salix* flowers offer only nectar and present inconspicuously greenish stigmata. In *Salix caprea* the visitation frequency of female flowers

is lower than of males. It was often hypothesised that this is of no disadvantage, because the male function (pollen dispersal) needs higher visitation rates than the female function (pollen receipt) to be accomplished. By the higher visitation frequency to male willows, the probability of successful pollination of a female individual may increase. Additionally, the higher probability of pollen transfer from diverse male individuals to one female individual might enhance the genetic diversity of the progeny.

Altogether, this case study is challenging the existing concepts of specialisation/ generalisation of plant-pollinator interactions. Regarding the aspect of interactions, *S. caprea* is a generalist, but looking at the aspect of adaptations, *S. caprea* can be regarded as a multi-specialist with respect to its floral scent emission. Considering the aspect of specialisation, the importance of different pollinating agents (diurnal insects versus nocturnal insects versus wind), *S. caprea* takes an intermediate position, with diurnal insects seeming the most important but not exclusive pollen vectors.

Part B

Publications

Four publications resulted from the different working packages. They are listed in this part (Part B) of the present work, as chapters 1 to 4.

Chapter 1 *Inter- and intraspecific variation in floral scent in the genus* Salix *and its implication for pollination*

The inter- and intraspecific variation in floral scent in the genus *Salix* was determined. The scent of 32 European and two Asian *Salix* species was collected using a dynamic headspace MicroSPE method and analysed using gas chromatography coupled with mass spectrometry. Of special interest was the variability within the genus and between male and female individual within certain species. The variability in floral scent was calculated using the dissimilarity index CNESS and visualised using the nonmetric multidimensional scaling (NMDS).

Chapter 2 Salix caprea*: An interaction generalist and multi-specialist with bimodal adaptations of floral scent to bees and moths*

Salix caprea (Sallow) is a pollination generalist that is pollinated besides wind by diverse pollinators, e.g. bees and moths. I tested the general hypothesis that plant species, which are pollinated by diverse groups of pollinators are unlikely to develop specific adaptations for a single group of pollinators. Therefore, the diurnal and nocturnal flower visitors of *Salix caprea* and the circadian rhythmicity of floral scent emission were determined. Electrophysiological and behavioural responses of different flower visitors/pollinators to the scent of whole flowers or single scent compounds were tested. It seems that in sallow, the circadian change of the quality and quantity of floral scent, is a possible adaptation to the differing preferences of different co-pollinating flower visitors at the same time.

Chapter 3 *1,4-Dimethoxybenzene, a floral scent compound in willows that attracts an oligolectic bee*

Gas chromatography coupled to electroantennography (GC-EAD) elucidated the floral scent compounds of *Salix caprea* and *S. atrocinerea* that elicit signals in the antennae of female and male *Andrena vaga*, an oligolectic bee to *Salix*. The compound that elicited the main signal in the antennae of bees (1,4-dimethoxybenzene) was further tested for attraction in a field bioassay.

Chapter 4 *Floral reward and advertisement in dioecious* Salix caprea

Behavioural tests as well as chemical analyses of floral scent and nectar were conducted to investigate the interaction between *Salix caprea* (sallow) and *Apis mellifera* (honeybee). The role of olfactory and visual signals for the attraction of honeybees to male and female individuals of *S. caprea* was analysed. I tested if male flowers of *Salix caprea* are more attractive to honeybees than female flowers.

1 Inter- and Intraspecific Variation in Floral Scent in the Genus *Salix* and its Implication for Pollination

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Abstract

The floral scent composition of 32 European and two Asian *Salix* L. species (Salicaceae) was analyzed. Intra- and interspecific variation was compared for a subset of 8 species. All *Salix* species are dioecious and floral scent was collected from both male and female individuals by using a dynamic headspace MicroSPE method, and analyzed by GC-MS. A total of 48 compounds were detected, most of them being isoprenoids and benzenoids. Commonly occurring compounds included *trans*-β-ocimene, *cis*-β-ocimene, benzaldehyde, D-limonene, α-pinene, *cis*-3-hexenyl aceatate, linalool, 1,4-dimethoxybenzene, and β-pinene. Two compounds, 1,4-dimethoxybenzene and *trans*-β-ocimene, were responsible for most of the interspecific variation. In a subset of eight extensively sampled species, six had a characteristic floral scent composition; half of the pairwise species comparisons confirmed significant differences. In three of these eight species, intraspecific variability could be explained by sex differences. Variation in *Salix* floral scent may provide specific signals that guide pollinators and thus contribute to the reproductive isolation of compatible and cooccurring species.

Keywords Dioecy · Floral scent · GC-MS · Intraspecific variation · Interspecific variation · *Salix* · Salicaceae

Introduction

The genus *Salix* L., composed of approximately 400 to 500 species (Skvortsov, 1999), has an almost worldwide distribution, but occurs predominantly in temperate to arctic regions of the northern hemisphere. In Central Europe, about 40 species occur and many are sympatric.

From the taxonomic point of view, *Salix* is a problematic genus with difficulties delimiting many species because of high morphological variability (Argus, 1997; Skvortsov, 1999) and supposed widespread hybridization and introgression (Mosseler, 1990). There are several, in some parts dissentient, phylogenetic classifications of the genus available, all based on morphological characters (Dorn, 1976; Argus, 1997: American species; Skvortsov, 1999: Eurasian species). Because it is the most comprehensive for Eurasian species, the classification of Skvortsov (1999) is used here. Skvortsov (1999) divided *Salix* into three subgenera (Chamaetia, Salix, and Vetrix), each with several sections.

Normally, willow species are dioecious with flowers arranged in catkins. The plants show traits of insect as well as wind pollination. Stiff erect catkins and the availability of nectar fit with insect pollination, whereas small flower size, the absence of a perianth, and the predominant flowering early in spring before leaf unfolding, match with the wind pollination syndrome. Hence, the importance of either mode of pollination in *Salix* is controversial (Karrenberg et al., 2002). Nevertheless, most species are thought to be mainly entomogamous, though in certain species wind contributes to some degree to pollination (Argus, 1974; Sacchi and Price, 1988; Vroege and Stelleman, 1990; Totland and Sottocornola, 2001; Karrenberg et al., 2002). Flowers of both sexes are visited by a wide variety of insects, including Diptera (Vroege and Stelleman, 1990; Tollsten and Knudsen, 1992; Totland and Sottocornola, 2001), Hymenoptera (van der Werf et al., 1982; Vroege and Stelleman, 1990; Tollsten and Knudsen, 1992; Totland and Sottocornola, 2001), Lepidoptera (Vroege and Stelleman, 1990; Totland and Sottocornola, 2001), Coleoptera (Vroege and Stelleman, 1990), and occasionally birds (Kay, 1985). Flower-visiting animals are rewarded with easily accessible pollen and nectar (male flowers) or solely with nectar (female flowers). In most cases, it is not clear to what extent particular flower visitors contribute to effective pollination (van der Werf et al., 1982).

From hybridization experiments (Argus, 1974; Salick and Pfeffer, 1999; Palme et al., 2003) and analyses of natural populations (Mosseler and Papadopol, 1989; Mosseler and Zsuffa, 1989; Rechinger, 1992; Triest et al., 1999), it is clear that many willow species are able to hybridize. For example, more than 50 different hybrid combinations are known from the

approximately 30 species that occur in Germany (see Rothmaler, 2002). However, how often hybridization occurs under natural conditions and what role introgressive hybridization plays (Dorn, 1976; Triest et al., 1997; Salick and Pfeffer, 1999; Totland and Sottocornola, 2001) is still a matter of discussion. Our understanding of the nature and efficiency of isolating mechanisms in sympatric compatible willow species, e.g., phenological differentiation (Argus, 1974; Dorn, 1976; Mosseler and Papadopol, 1989) or incongruity (Argus, 1974; Mosseler, 1989; Adler, 2000) is still incomplete. Floral scent is one trait that might function as a reproductive isolating mechanism in entomogamous species by guiding pollinating insects to specific species. However, there are few studies available that compare floral scent across several species within a genus to test this hypothesis. The only study in *Salix* that investigates floral scent variability within and among species was done by Tollsten and Knudsen (1992). They studied two sympatrically occurring, insect-pollinated species, *Salix caprea* and *Salix cinerea*, and both displayed relatively similar floral scent profiles. They concluded that floral scent does not promote reproductive isolation between these two species, resulting in the frequently observed hybridization.

In dioecious plants, such as *Salix* species, it is essential that pollen is transported from male to female flowers and that pollinators fly among them. Tollsten and Knudsen (1992) hypothesized that the floral scent of males and females should not differ within a species; otherwise, pollinators could learn to associate the scent of either gender with its rewards, resulting in preference for one sex. Indeed, they found no difference in scent between male and female flowers within either *S. caprea* or *S. cinerea*, suggesting that pollinating insects cannot discriminate among the sexes of these species.

In the present study, the floral scent of 34 willow species was analyzed by using a dynamic headspace MircoSPE method. The main objectives were to provide an overview of scent production in this interesting genus, with respect to its pollination biology, and to determine intrageneric, interspecific, and intraspecific variation. Based on our results, we discuss the potential of floral scent patterns as reproductive isolation barriers, and as cues for pollen collecting bees to discriminate between male and female individuals.

Methods and Materials

Plant Material Among the 34 species of *Salix* studied, 23 had been planted in the Ecological–Botanical Garden Bayreuth, Germany (EBG). Details on the geographic origin of these plants are listed in Table 1. All other species studied either grew wild in the EBG and/or at sites near Bayreuth. Thirty-two of the studied species are native to Europe; two occur naturally in Asia only (*Salix babylonica*, *Salix gracistyla*).

For 26 species, only a few individuals were available (Table 1), and floral scent could be collected only from one or two male and/or female specimens. For 8 species, several plants of both sexes were available and at least two male and three female specimens were sampled for variability among sexes within these eight species, and to compare intraspecific with interspecific variability. Five out of these eight extensively sampled species (*S. caprea*, *S. cinerea*, *Salix fragilis*, *Salix triandra*, and *Salix viminalis*) grow wild at sites near Bayreuth. Specimens of the other three species—*Salix bicolor*, *Salix myrsinifolia*, and *Salix repens*—have been planted at the EBG and have different geographical origins each (Table 1).

Volatile Collection Floral scent samples were collected from individuals in full bloom in the field from March to May 2005. Scent samples were taken during the day (10:00–17.00) by using a dynamic headspace method. For each individual plant, one twig with four to ten flowering catkins, depending on catkin size, was enclosed for 10 min in an oven bag (Nalophan). The emitted floral scent was subsequently trapped for 2.5 min in a microtube filled with absorbent (3 mg of a 1:1 mixture of Tenax-TA 60–80 and Carbotrap 20–40) by using a membrane pump (G12/01 EB, Rietschle Thomas, Puchheim, Germany). Airflow rate during volatile collection was 200 ml min^{-1}. After sampling, the microtubes were stored in a freezer (at -20°C) until analysis.

Gas Chromatography and Mass Spectrometry (GC–MS) The samples were analyzed on a Varian Saturn 3800 gas chromatograph (GC) fitted with a 1079 injector, and a Varian Saturn 2000 mass spectrometer (MS). A ZB-5 column (5% phenyl polysiloxane, length 60 m, inner diameter 0.25 µm, film thickness 0.25 µm, Phenomenex) was used for the analyses. Microtubes were inserted via Varians' Chromatoprobe into the GC injector. The injector vent was opened (1/20) and the injector heated at 40°C to flush any air from the system. After 2 min, the split vent was closed and the injector heated at 200°C min^{-1}, then held at 200°C for 4.2 min, after which the split vent was opened (1/20) and the injector cooled down.

Table 1 Species, systematic position (according to Skvortsov, 1999), number of samples from males (M) and females (F), location of sampled plants, and geographic origin (data as far as available) of willow plants studied

Species	Abbreviation	Section	M	F	Location	Geographic origin[a]
Subgenus *Chamaetia*						
S. glauca L.	*S. gla*	Glaucae		1	EBG[b]	N (west), Grotli/Geiranger, 1,250 m
Subgenus *Salix*						
S. triandra L.	*S. tri*	Amygdalinae	2	3	Wild[c]	D, Bavaria, Bayreuth, 365 m
S. pentandra L.	*S. pen*	Pentandrae		1	EBG	D, Saxony-Anhalt, Quedlinburg, 455 m
S. alba L.	*S. alb*	Salix	2		Wild	D, Bavaria, Bayreuth, 340 m
S. fragilis L.	*S. fra*	Salix	3	3	Wild	D, Bavaria, Bayreuth, 340 m
S. babylonica L.	*S. bab*	L.Subalbae		1	Wild	D, Bavaria, Bayreuth, 365 m
Subgenus Vetrix						
S. arbuscula L.	*S. arb*	Arbuscella	1		EBG	N (south), Kongsvoll, 1,000 m
S. arbuscula L.		Arbuscella	1		EBG	CH, St. Gallen, Gamperfin, 1,320 m
S. bicolor Willd.	*S. bic*	Arbuscella	2		EBG	F (east), Vogesen, Hohneck, 1,200 m
S. bicolor Willd.		Arbuscella		1	EBG	No data
S. bicolor Willd.		Arbuscella	1		EBG	N (west), Gjevil see, Oppdal, 600 m
S. bicolor Willd.		Arbuscella		1	EBG	CZ (nord), Tatra, 1,800 m
S. cantabrica Rech.F	*S. can*	Arbuscella		1	EBG	E (north), Kantabrien, Sia Pass, 1,050 m
S. foetida DC.	*S. foe*	Arbuscella	1		EBG	I, Aosta, Gr. St. Bernhard, 2,020 m
S. foetida DC.		Arbuscella		1	EBG	No data
S. eleagnos Scop	*S. ele*	Canae		1	EBG	CH, St. Gallen, Neckertal, 580 m
S. acutifolia Willd.	*S. acu*	Daphnella	1		EBG	No data
S. daphnoides Vill.	*S. dap*	Daphnella	1		Wild	D, Bavaria, Bayreuth 365 m
S. daphnoides Vill.		Daphnella	1		EBG	A, Steiermark, Graz, 440 m
S. daphnoides Vill.		Daphnella		1	EBG	CH, St. Gallen, Sitterufer, 570 m
S. crataegifolia Bertol.	*S. cra*	Glabrella	1		EBG	I, Tuscany, Orto di Donna, 1,450 m
S. glabra Scop.	*S. gla*	Glabrella	1		EBG	CH, Tessin, Val Colla, Fojorina-Nord, 1,650 m
S. hastata L.	*S.has*	Hastatae		2	EBG	CZ (north), Sudeten Mountains, 1,300 m
S. hastata L.		Hastatae	1		EBG	No data
S. caesia Vill.	*S. cae*	Helix	1		EBG	F (southeast), Col de Larche, 1900 m
S. caesia Vill.		Helix		1	EBG	CH, Grisons, Bevers, Ebene, 1,700m
S. purpurea L.	*S. pur*	Helix	2	1	Wild	D, Bavaria, Bayreuth, 365 m
S. repens L.	*S. rep*	Incubaceae	1		EBG	No data
S. repens L.		Incubaceae	2		EBG	No data
S. repens L.		Incubaceae	1		EBG	PL (east), Brzezno, 200 m
S. repens L.		Incubaceae		2	EBG	DK (south), Bornholm, 30 m
S. repens L.		Incubaceae		1	EBG	N (south), Bergen, 43 m
S. apennina Skv.	*S. ape*	Nigricantes		1	EBG	I, Tuscany, Cisa-Pass, 450 m
S. apennina Skv.		Nigricantes		1	EBG	I, Verona, Apua, Mte Altissimo, 1,300 m
S. mielichhoferi Sauter.	*S. mie*	Nigricantes	1		EBG	A, Salzburgerland, Radstätter Tauern, 1,700m
S. mielichhoferi Sauter.		Nigricantes	1		EBG	I, Südtirol, Seiseralp, 1,200 m
S. mielichhoferi Sauter.		Nigricantes		1	EBG	A, Steiermark, Tauern, 1,750 m
S. myrsinifolia Salisb.	*S. myr*	Nigricantes	1		EBG	CH, St. Gallen, Wattwil, 620 m
S. myrsinifolia Salisb.		Nigricantes	1		EBG	N (west), Gjevil See, 700 m
S. myrsinifolia Salisb.		Nigricantes	1	1	EBG	CH, Grisons, Vorderrhein, 1,500 m
S. myrsinifolia Salisb.		Nigricantes		2	EBG	CH, St. Gallen, Wattwil, 620 m
S. gracilistyla Miq.	*S. gra*	Subviminales	1		EBG	J (cultivated)
S. appendiculata Vill.	*S. app*	Vetrix	1		EBG	CH, Tessin, Airolo, 1,200 m
S. atrocinerea Brot.	*S. atr*	Vetrix		1	EBG	IR (east), Wicklow, Glendalaugh, 600 m
S. atrocinerea Brot.		Vetrix	1		EBG	CH, St. Gallen, Rohrspitz, 400 m
S. aurita L.	*S. aur*	Vetrix	1		Wild	D, Bavaria, Bayreuth, 365 m
S. caprea L.	*S. cap*	Vetrix	3	2	Wild	D, Bavaria, Bayreuth, 365 m
S. cinerea L.	*S. cin*	Vetrix	2	3	Wild	D, Bavaria, Bayreuth, 365 m
S. cinerea L.		Vetrix	1		EBG	CH, St. Gallen, Wattwil, 670 m
S. laggeri Wimm.	*S. lag*	Vetrix	1		EBG	CH, Wallis, Gletschboden, 1,780 m
S. laggeri Wimm.		Vetrix		1	EBG	A, Tirol, Stubei, 1,600 m

Table 1 (continued)

Species	Abbreviation	Section	M	F	Location	Geographic origin[a]
S. silesiaca Willd.	*S. sil*	Vetrix	1		EBG	CR (north), Sudeten Mountains, 1,400 m
S. silesiaca Willd.		Vetrix	1	1	EBG	PL (east), W-Tatra, 1,300 m
S. silesiaca Willd.		Vetrix	1		EBG	CR (north), Sudeten Mountains, 1,300 m
S. starkeana Willd.	*S. sta*	Vetrix		1	EBG	S, (east), Jämtland, Tännäs, 20 m
S. helvetica Vill.	*S. hel*	Villosae	2		EBG	CH, Wallis, Grimselpass, 2,040 m
S. helvetica Vill.		Villosae		1	EBG	CH, Wallis, Gletschboden, 1,780 m
S. lapponum L.	*S. lap*	Villosae	1		EBG	CR (north), Sudeten Mountains, 1,400 m
S. lapponum L.		Villosae	1		EBG	CR (north), Sudeten Mountains, 1,300 m
S. viminalis L.	*S. vim*	Vimen	3	3	Wild	D, Bavaria, Bayreuth, 365 m

[a] The geographic origin is described with the shortcut of European countries and m declared the level about sea.

[b] EBG = individuals cultivated in the Ecological-Botanical Garden Bayreuth.

[c] Wild = growing wild in natural habitats.

Electronic flow control was used to maintain a constant helium carrier gas flow of 1.8 ml min^{-1}. The GC oven temperature was held for 7 min at 40°C, then increased by 6°C min^{-1} to 260°C, and held for 1 min at this temperature. Mass spectra were taken at 70 eV with a scanning speed of 1 scan/sec from *m/z* 30 to 350.

The GC-MS data were processed with the Saturn Software package 5.2.1. To identify floral scent components, GC-MS spectra were compared to, the NIST 02 and MassFinder 3 databases. Identifications were confirmed by comparison of retention times with published data (Adams, 1995). Identification of some compounds was also confirmed by comparison of mass spectra and retention times with those of authentic standards.

Statistics To determine (semi)-quantitative differences among single samples, we used chord-normalized expected species shared (CNESS) dissimilarity index, ranging between 0 and square root of 2. These semiquantitative comparisons were based on the percentage amount of components. Comparison of the absolute peak areas and the amounts were impractical because emission rate varied extensively both within and among species across individuals and flowering period. In cases where more individuals per species had been sampled, mean relative amounts per species were calculated. The CNESS index was calculated by using the updated version of the Combinatorial Polythetic Agglomeration Hierarchical Clustering (COMPAH) program (Boesch, 1977), provided by Gallagher at UMASS/Boston (http://www.es.umb.edu/edgwebp.htm).

Qualitative differences in floral scent (presence or absence of compounds) among samples were determined by using Sørensen's index of similarity (Sørensen, 1948). RELATE was used (program package Primer, version 5.2.9) to correlate and compare the CNESS with the Sørensen matrix (Kendall correlation coefficient).

We utilized nonmetric multidimensional scaling (NMDS) in the STATISTICA 7 package to identify meaningful dimensions and to visualize both similarities and dissimilarities among individual samples or different species (see Borg and Lingoes, 1987). A stress value is given to calculate how well the particular configuration produces the observed distance matrix. The smaller this value, the better is the fit of the configuration to the reproduced distance matrix (Clarke, 1993).

Analysis of similarities (ANOSIM, one-way design) in the program package Primer (version 5.2.9) was used to test for differences in floral scent among species of subgenera *Salix* and *Vetrix*, and within subgenus *Vetrix* among species of sections *Arbuscella* and *Vetrix*. We used these combinations because too few species were sampled in subgenus *Chamaetia* and the other sections making a statistical test less powerful.

Analysis of similarities (two-way crossed design; factors: species and sex) was further used to test for differences in floral scent among eight species (with five or six individuals sampled), and within these species between male and female individuals.

CNESS dissimilarity matrices were used for all ANOSIM analyses. This test calculates the test statistic *R* as well as a level of significance. *R* value ranges between 0 and 1 (-1) and can be interpreted as follows: 1 indicates complete separation of the sample groups (e. g., subgenera), and small values (close to zero) imply no segregation (Clarke and Warwick, 2001).

We used ANOVA as a global test and subsequently the Tukey–Kramer test as a post hoc test to compare the mean relative amount of the two most variable scent compounds between species. Normality was tested by using the Kolmogorov–Smirnov test and homogeneity of variances was tested with the Hartley test.

A variance component analysis in the STATISTICA 7 package was utilized to estimate the contribution of single floral scent compounds to the total observed variation (relative amount) between species.

Results

The compounds found in the floral scent samples of 93 willow plants from 34 species, are listed in Table 2. A total of 48 compounds were detected and 43 were identified. Dominant compound classes included isoprenoids and benzenoids, but fatty acid derivates and N-containing compounds were also present. The most commonly occurring compounds were *cis*-

Table 2 Chemical composition of floral scents: occurrence and relative amount of each compound detected in the flower scent of 93 individuals (52 male and 41 female) of 34 *Salix* species

Compound[a]	R_i[b]	Occurence[c]	Relative Amount[d]					
			Male			Female		
			Median	Quartiles	Min-Max	Median	Quartiles	Min-Max
Isoprenoids								
α-Phellandrene[e]	934	11	0	0-0.25	0-1.41	0	0	0-0.11
α-Pinene[e]	957	29	1.01	0.11-7.37	0-42.34	1.01	0.2-3.57	0-84.46
Camphene[e]	958	10	0	0-0.08	0-3.33	0.23	0-1.61	0-4.79
Sabinene[e]	987	18	0	0-1,18	0-6,67	0	0-0,51	0-4,88
β-Pinene[e]	995	20	0.67	0-3.72	0-26.45	0.16	0-1.6	0-15.54
β-Phellandrene[e]	1,026	9	0	0-0.03	0-1.52	0	0	0-3.33
D-Limonene[e]	1,045	31	1.04	0.29-4.15	0-18.32	0.74	0.12-3.14	0-33.32
cis-β-Ocimene[e]	1,048	33	6.48	3.32-9.59	0-32.50	5.21	3.04-11.94	0-19.78
trans-β-Ocimene[e]	1,058	34	26.76	12.13-45.78	0.59-93.94	21.18	9.21-48.03	0-87.98
γ-Terpinene[e]	1,071	13	0	0-0.53	0-10.28	0	0	0-4.14
trans-Linalool oxide[e]	1,099	2	0	0-1.84	0	-	-	-
Linalool[e]	1,104	25	0.61	0-7.25	0-32.84	3.39	0.1-6.74	0-64.19
Lilac aldehyde A[e]	1,153	17	0.03	0-0.96	0-9.40	0	0-0.11	0-4.26
Camphor	1,153	4	0	0	0-0.76	0	0	0-0.49
Lilac aldehyde B + C[e]	1,163	23	0.19	0-1.24	0-13.91	0.08	0-1.74	0-17.03
Lilac aldehyde D[e]	1,178	13	0	0-0.22	0-7.23	0	0	0-3.56
4-Terpineol	1,191	1	0	0	0-0.59	-	-	-
α-Terpineol	1,202	5	0	0	0-3.45	-	-	-
Lilac alcohol A[e]	1,211	4	0	0	0-3.33	0	0	0-0.17
Lilac alcohol B + C[e]	1,219	5	0	0	0-1.70	0	0	0-0.41
Lilac alcohol D[e]	1,232	3	0	0	0-0.85	0	0	0-0.14
D-Verbenone[e]	1,228	14	0	0-0.76	0-21.01	0	0-0.27	0-32.33
α-Copaene	1,397	5	0	0	0-0.18	0	0	0-3.46
β-Bourbonene	1,407	11	0	0-0.01	0-0.62	0	0	0-9.89
(*E*)-Caryophyllene[e]	1,447	9	0	0	0-5.33	0	0-0.02	0-10.43
E-Geranylacetone[e]	1,336	19	0	0-0.3	0-5.33	0.02	0-0.5	0-5.03
Cubebene	1,334	3	-	-	-	0	0	0-2.47
(*E*,*E*)-α-Farnesene[e]	1,508	15	0.01	0-0.19	0-1.69	0	0	0-0.39
Benzenoids								
Benzaldehyde[e]	982	31	0.78	0.15-1.81	0-10.22	0.23	0-1.61	0-4.79
Benzyl alcohol[e]	1,050	4	0	0	0-3.36	0	0	0-0.96
Phenylacetaldehyde[e]	1,060	3	0	0	0-22.19	0	0	0-0.95
Salicylaldehyde	1,063	4	0	0	0-8.66	0	0	0-0.01
4-Methylbenzyl-alcohol	1,077	4	0	0	0-0.21	0	0	0-0.5
2-Phenylethanol[e]	1,123	7	0	0	0-1.83	0	0	0-6.31
Veratrole[e]	1,153	11	0	0	0-1.82	0	0	0-18.34
1,4-Dimethoxy-benzene[e]	1,175	21	0.5	0-5.78	0-77.36	0	0-2.79	0-74.58

Table 2 (continued)

Compound[a]	R_i[b]	Occurence[c]	Relative Amount[d]					
			Male			Female		
			Median	Quartiles	Min-Max	Median	Quartiles	Min-Max
Methyl salicylate[e]	1,208	24	0.54	0-4.39	0-27.94	0.35	0-2.03	0-16.53
N-bearing compounds								
Benzyl nitrile[e]	1,144	5	0	0	0-1.12	-	-	-
Indole[e]	1,254	6	0	0	0-0.08	0	0	0-1.44
Fatty acid derivates								
cis-3-hexen-1-ol[e]	860	21	0.08	0-1.81	0-8.4	0.19	0-1.73	0-9.61
cis-3-hexenyl acetate[e]	1,016	28	6.16	0.1-17.05	0-36.79	3.75	0.31-13.99	0-64.69
(*E*)-4,8-Dimethyl-1,3,7-nonatriene[e]	1,118	24	0.34	0-0.82	0-3.37	0.07	0-0.46	0-10.00
4-Oxoisophorone[e]	1,159	16	0	0-0.66	0-7.89	0	0-0.6	0-18.67
Unknown substance								
65, 77, 93, 105, 121, 136	1,033	8	0	0-0.08	0-0.71	0	0	0-0.21
39, 77, 93, 105, 121, 136	1,100	13	0	0-0.33	0-4.58	0	0	0-0.38
39, 65, 79, 91, 107, 122	1,130	22	0,1	0-0.74	0-2.27	0.26	0-0.57	0-1.59
41, 67, 82, 105, 122, 138	1,172	5	0	0	0-4.91	0	0	0-1.61
41, 57, 67, 82, 103, 120	1,204	22	0	0-0.31	0-4.14	0.14	0-0.45	0-13.29

[a] Compounds within classes are listed according to Kovat's index.

[b] Kovat's retention index.

[c] Number of species where a compound was detected.

[d] Relative proportion (%) of the compounds in the floral scent bouquets of 52 male and 41 female samples.

[e] Identity confirmed by comparison of MS and retention time with those of authentic standards.

and *trans*-β-ocimene (found in 33 and 34 species, respectively), D-limonene (31 species), benzaldehyde (31species), *cis*-3-hexenyl aceatate (28 species), linalool (25 species), 1,4-dimethoxybenzene (21 species), and α- and β-pinene (29 and 20 species, respectively). The number of compounds detected in each species ranged from a low of four in *Salix acutifolia*, and five in *S. silesiaca*, and *S. glauca* to a high of 29 in *S. myrsinifolia*. The scent profiles in all species were dominated by few components only. Dominant compounds reaching on average at least 50% of the total scent mixture within a species were *trans*-β-ocimene (in *S. viminalis*, *S. daphnoides*, *S. repens*, *S. triandra*, *S. apennina*, *S. bicolor*, *S. glabra*, *S. acutifolia*, *S. babylonica*, and *S. gracilistyla*) and 1,4-dimethoxybenzene (in *S. caprea*, *S. atrocinerea*, *S. aurita,* and *S. cinerea*).

Interspecific Variation Comparing the relative amounts of floral scent compounds among all species (by using a variance component analysis), seven compounds explained 94.7% of the total observed variation among the species. Two compounds, 1,4-dimethoxybenzene (35.9%) and *trans*-β-ocimene (32.5%), were responsible for most of the interspecific variation, followed by α-pinene (11.1%), *cis*-3-hexenyl acetate (9.2%), linalool (3.0%), D-limonene (1.8%) and D-verbenone (1.2%).

Differences in floral scent composition (relative amounts) among 34 *Salix* species based on the CNESSm=1 index are shown in Fig. 1, using nonmetric multidimensional scaling (stress=0.19). In general, no clear separation of species groups was found. Most species

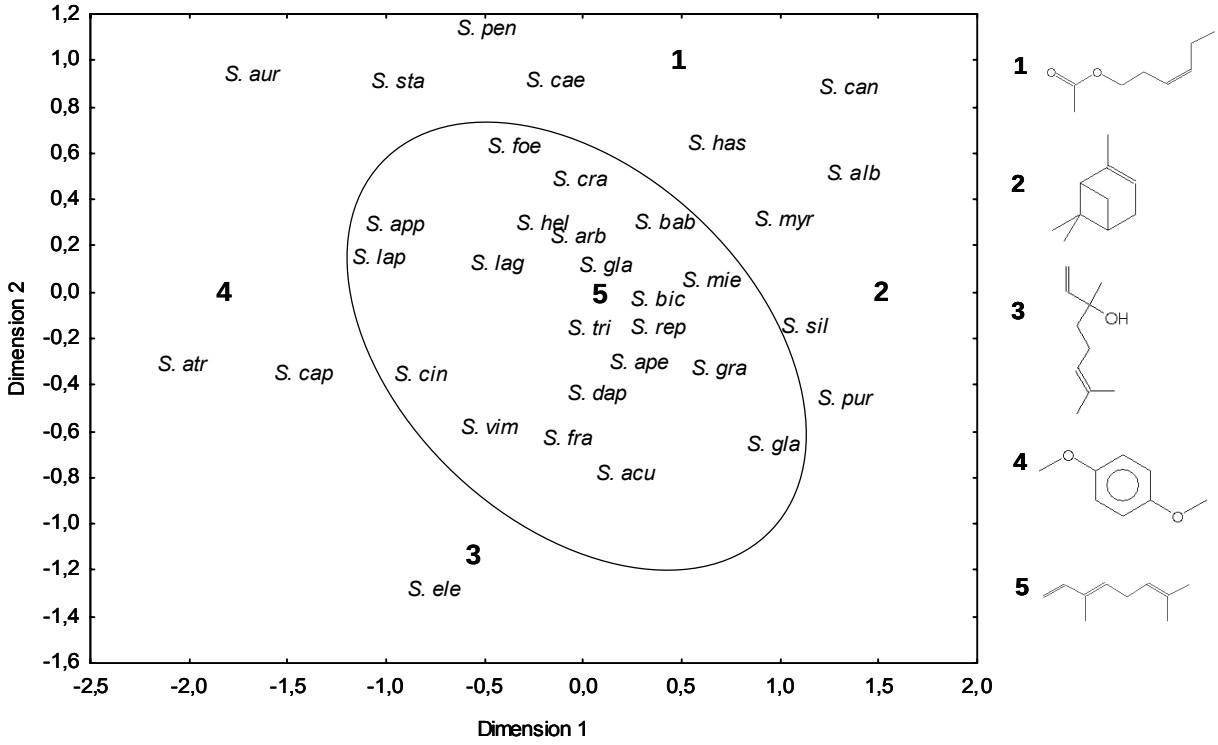

Fig. 1 Nonmetric multidimensional scaling (NMDS) of floral scent profiles of 34 *Salix* species based on the CNESSm=1 index (stress: 0.19). The structures and names of the five main compounds: *1 cis*-3-hexenyl acetate, *2* α-pinene, *3* linalool, *4* 1,4-dimethoxybenzene, *5 trans*-β-ocimene dominating the scent of different species are presented in the figure. The *circle* comprises species with more than 30% relative amount of *trans*-β-ocimene

were more or less evenly distributed, and clear separation of subgroups was hardly possible. Species in the centre of the scatter plot were characterized by the emission of high relative amounts of *trans*-β-ocimene, while the amount of this monoterpene was lower in species at the margins. In *S. caprea*, *S. atrocinerea*, *S. aurita*, and *S. cinerea*, high amounts of 1,4-dimethoxybenzene were found. In other species (*S. mielichhoferi*, *S. myrsinifolia*, and *S. silesiaca*), high amounts of α-pinene (25-35%) were detected. High amounts of the green

leaf volatile *cis*-3-hexenyl acetate (50-65%) were emitted by *S. starkeana* and *S. pentandra*, and the isoprenoid linalool occurred in large amounts (32%) in *S. eleagnos.*

When analyzing the data qualitatively by using the Sørensen index, which considers similarity based on the presence or absence of single compounds for comparison and not their relative amount, the results were similar with most species being evenly distributed according to nonmetric multidimensional scaling (stress=0.17), indicating that categorization of species based on scent composition is hardly possible. The CNESS and Sørensen matrices were strongly correlated (RELATE Kendall: R=0.181; P<0.001), and the results of both analyses were generally consistent. Therefore, the NMDS representing the Sørensen matrix is not displayed here.

Analyses of floral scent composition of species from the two subgenera *Salix* (N=5) and *Vetrix* (N=28) revealed no differences between these subgenera (CNESS, ANOSIM: R=-0.035; P=0.66). However, within the *Vetrix* subgenus, significant differences between species of section *Arbuscella* (N=4) and *Vetrix* (N=8) were found (CNESS, ANOSIM: R=0.274; P<0.005). A variance component analysis revealed *cis*-3-hexenyl acetate and 1,4-dimethoxybenze as the main variable compounds between these two sections. A relatively high amount of *cis*-3-hexenyl acetate was found in section *Arbuscella* and 1,4-dimethoxybenze in section *Vetrix*.

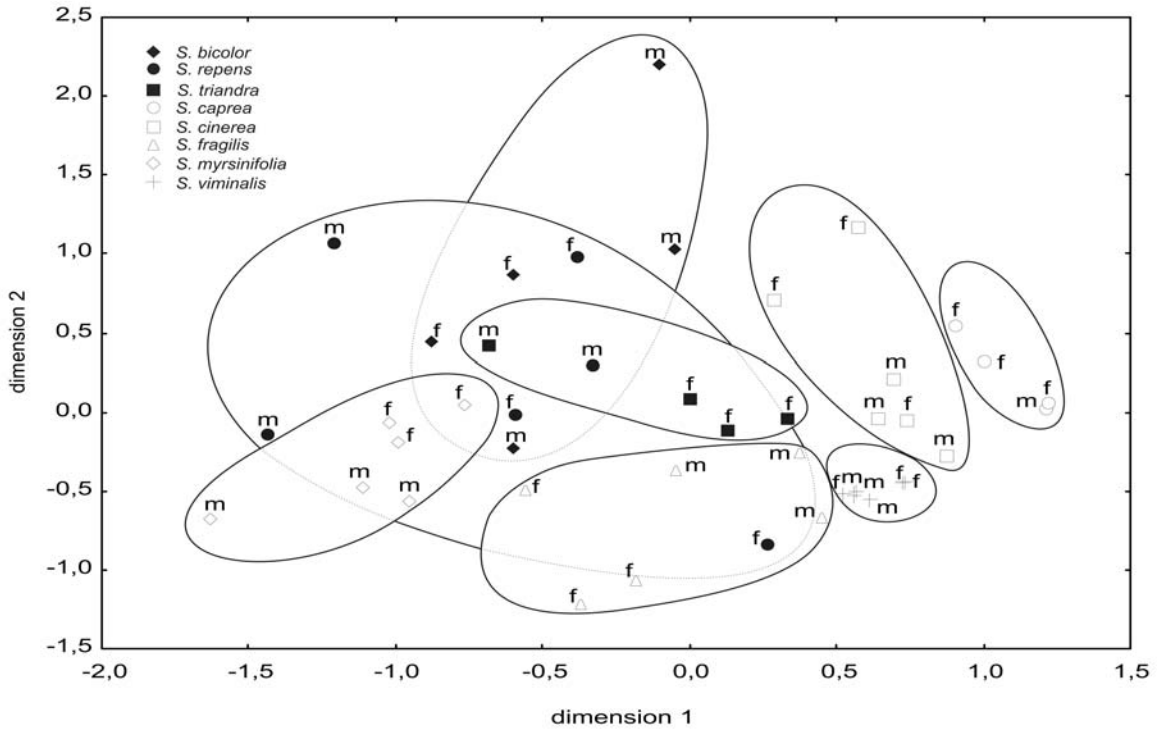

Fig. 2 Intraspecific comparison of floral scent between males (*m*) and females (*f*): nonmetric multidimensional scaling (NMDS) of eight *Salix* species based on the CNESSm=37 index (stress=0.18)

The variability of floral scent among and within the eight extensively sampled species is shown in Fig. 2. Variability within species (based on all samples from both sexes) was lower than variability among species (ANOSIM: R=0.598; P<0.001). When ignoring *S. bicolor* and *S. repens*, the two relatively variable species, the remaining six species had characteristic floral scent profiles, as revealed by grouping of individual samples of each taxon together in a NMDS analyses (Fig. 2). Out of 28 pairwaise species combinations, 14 revealed significant differences (Table 3). As already shown in the overall comparison of 34 species, differences were mainly based on the variability of 1,4-dimethoxybenzene and *trans*-β-ocimene. These two compounds explained 84% of the observed total variability among this subset of eight

Table 3 Test statistics (R) of pairwise species comparison (ANOSIM)

	S. caprea	*S. cinerea*	*S. myrsinifolia*	*S. fragilis*	*S. viminalis*	*S. repens*	*S. triandra*	*S. bicolor*
S. caprea								
S. cinerea	0.123							
S. myrsinifolia	**1**[a]	**0.728**						
S. fragilis	**0.976**	0.605	**0.872**					
S. viminalis	**0.969**	0.483	**1**	0.619				
S. repens	**0.743**	0.472	0.316	0.441	0.594			
S. triandra	**0.9**	0.214	**0.817**	0.573	**0.786**	-0.056		
S. bicolor	**0.728**	**0.709**	0.644	**0.745**	**0.781**	0.017	0.106	

[a] Bold values indicate significant differences between two species. All species are likely to grow sympatrically, except for the subalpine S. bicolor.

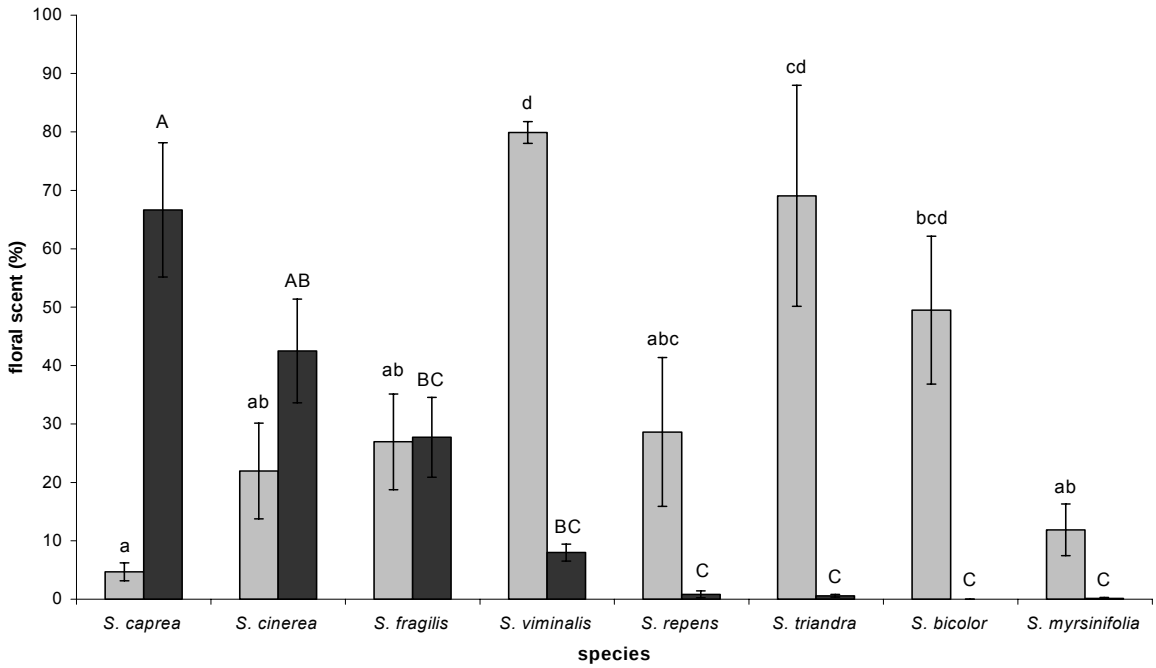

Fig. 3 Relative amount of 1,4-dimethoxybenzene (black) and *trans*-β-ocimene (*grey*) of the total floral scent in the most extensively sampled *Salix* species (ANOVA with Tukey-HSD test as post hoc procedure: $F_{df=7;55}$ 17.0; P < 0.001). *Different small letters* indicate significant interspecific differences in the amount of *trans*-β-ocimene and, *different capital letters* indicate significant interspecific differences in the amount of 1,4-dimethoxybenzene

species. For example, *S. caprea* and *S. cinerea* emitted much higher amounts of 1,4-dimethoxybenzene compared to *trans*-β-ocimene. Others, e.g., *S. viminalis*, *S. triandra*, and *S. bicolor*, were dominated by *trans*-β-ocimene (Fig. 3). *S. fragilis* was characterized by equally high amounts of 1,4-dimethoxybenzene and *trans*-β-ocimene. In *S. repens* and *S. myrsinifolia*, there was no clear predominance of a single compound; *trans*-β-ocimene content was below 30% and 1,4-dimethoxybenzene occurred only in traces.

Intraspecific Variation The variability within species in at least three out of eight species can be explained by sex differences (ANOSIM: R=0.405; P<0.001; Fig. 2). In *S. fragilis* (N=6), males emitted higher relative amounts of *trans*-β-ocimene and 1,4-dimethoxybenzene, whereas female samples contained more D-limonene and D-verbenone. In *S. myrsinifolia* (N=6), males emitted higher amounts of α-and β-pinene, while females emitted higher amounts of *cis*-3-hexen-1-ol, *cis*-3-hexenyl acetate, and *trans*-β-ocimene. In *S. triandra* (N=5), females emitted higher amount of *trans*-β-ocimene while males released more β-pinene, *cis*-3-hexenyl acetate, D-limonene, and linalool. In the remaining five species, intraspecific variation as shown in Fig. 2 cannot be explained by sex differences.

Discussion

Floral scent emission as found in *Salix* is typical for entomogamous species. Indeed, willows are visited during the day and also at night by many insect species, e.g., bees, flies, beetles, butterflies, and moths (Vroege and Stelleman, 1990; Tollsten and Knudsen, 1992; Tollsten and Sottocoornola, 2001; Karrenberg et al., 2002), and floral scents are probably important attractants.

Many floral scent compounds identified in *Salix* species are typical floral odors (compare e.g., Knudsen et al., 2006) and several are effective attractants for different insects (see below). This supports that in most willow species, flower-visiting insects are probably attracted by floral scents thereby promote pollination. However, some of the detected components have been described as typical green leave volatiles (e.g., *cis*-3-hexen-1-ol, *cis*-3-hexenyl acetate, and (*E*)-4,8-dimethyl-1,3,7-nonatrien; Andersen et al., 1988; Whitman and Eller, 1990; Pare and Tumlinson, 1999; Ruther, 2000; Tholl et al., 2006) or have been found in leaves and/or other vegetative parts of different *Salix* species (Füssel et al., unpublished data). Green leave volatiles are likely to be produced in vegetative parts of the inflorescences, e.g., rhachis, flower bracts, and especially the leaves at the base of the catkins, which are, depending on

species, more or less developed during flowering. Nevertheless, pollinators may detect, especially from long distances, the odor emitted from a whole plant and can use it as an olfactory cue to find their host plant and its flowers (e.g., Grison-Pigé et al., 2002). Therefore, in terms of pollinator attraction, we did not discriminate among compounds emitted by vegetative parts and by flowers, and refer to both as flower scent.

Compared with Tollsten and Knudsen (1992), who investigated floral scents in three species that we studied also – i.e., *Salix caprea*, *S. cinerea*, and *S. repens*, the results are similar considering both qualitative and quantitative aspects of scent composition. Tollsten and Knudsen (1992) identified 31 compounds, while we detected 34. Both studies found that *S. caprea* and *S. cinerea* are dominated by 1,4-dimethoxybenzene, while *S. repens* is dominated by a set of isoprenoids. However, despite these similarities, small differences exist. Tollsten und Knudsen (1992) identified four components (myrcene, 1,8-cineole, an oxygenated monoterpene, 2-phenyl ethyl methylether), which we did not detect. We identified α- und β-phellandrene, D-verbenone, and indole, which were not reported by Tollsten und Knudsen (1992). Surprisingly, we found one compound, benzaldehyde, in 31 of 34 species including *S. caprea*, *S. cinerea* and *S. repens*, that was not reported by Tollsten and Knudsen (1992). However, all these differences concern only minor components of the total floral scent bouquet of a species. They might have been found in one study but not in the other because they fall below detection limits in some samples. In particular, benzaldehyde may be an artefact built by heating Tenax TA during desorbtion of the volatiles in the injector of the gas chromatograph (Peters et al., 1994). Several other factors also may be responsible for differences. First, different methods were used in the two studies (different adsorbents, thermodesorption vs. extraction of volatiles from filter using solvent). Second, Tollsten and Knudsen (1992) collected scent from cut twigs that were placed into water, whereas we collected scent from flowering twigs *in situ*. Some studies have shown differences in scent composition of flowers still attached to the living plant compared to that of flowers from cropped twigs (Mookherjee et al., 1990). Finally, geographic variability in floral scent of the three species could explain observed differences. Tollsten und Knudsen (1992) analyzed Swedish specimens growing wild while we anaylzed specimens growing in southern Germany. Studies of other plant species document that specimens originating from different populations emit differing relative amounts of compounds or even different compounds (e.g., Knudsen, 2002; Dötterl et al., 2005b; Svensson et al., 2005; Raguso et al., 2006).

Indeed, differing geographic origin might explain the intraspecific variability found in two of eight extensively sampled *Salix* species. The two species with samples originating from four

or more origins (*S. repens* and *S. bicolor*), show a similarly high variability, while the other six extensively sampled species originating from one or two origins are less variable. We cannot confirm Tollsten and Knudsen's (1992) finding that sex differences are responsible for the highly variable pattern of compounds in *S. repens* because plants studied in the Ecological-Botanical Garden of Bayreuth originated from five different geographic regions, thus masking possible sex differences within populations. *S. repens* is also morphologically a variable taxon, and floral scent might follow the same trend.

Relatively little is known about sex-specificity of floral scent in dioecious species. At least small differences in profiles between sexes have been found in some studies (e.g., Tollsten and Knudsen, 1992, Ashman et al., 2005). Ashmann et al. (2005) reported that pollinators discriminated in gynodioecious *Fragaria* the scent of hermaphrodite flowers over those of females primarily because of the scent of hermaphrodite anthers. The anthers emitted high amounts of 2-phenylethanol, a benzoid compound found only in small amounts in the female flowers. A comparison of the floral scent profiles of the three *Salix* species having significant sex differences with pollen scent profiles showed only differences in relative amounts, but no qualitative differences (U. Füssel et al., unpublished data) indicating that observed differences in scent between sexes cannot be explained by the emission of additional pollen-specific compounds in male flowers. Compared to male plants, which offer both pollen and nectar, female plants offer only nectar, and are, therefore, less attractive to insects collecting or eating pollen, such as beetles or bees (see also Ashman et al., 2005). Nevertheless, potential pollinators must be attracted to both male and female flowers for pollination to occur. The general view is that signals of male and female flowers have to correspond to promote successful pollen transfer. Consequently, it is usually assumed that flower visitors use similar cues to obtain rewards from female and male flowers (see reviews in Chittka and Thomson, 2001). This implies that insects seek similar rewards from both sexes. If this is not the case, e.g., when pollen is the desired reward (or females produce less or no nectar), nonrewarding female flowers are apparently pollinated by deceit due to their resemblance to rewarding male flowers (Baker, 1976). Contradictory to this intersexual mimicry hypothesis, the overall resemblance of male and female flowers in willows, especially with respect to visual cues, is low, and selection for resemblance of olfactory but not of visual cues seems to be unlikely, unless we assume that visual cues are of negligible importance for pollinator attraction. However, while nocturnal moths are probably more dependent on olfactory cues than day-active flower visitors, the situation might be completely different in day-active bees. For example, Galizia et al. (2004) found no evidence of olfactory mimicry in a (nectar)

food-deceptive flower mimicry system. Their results indicate that in a bee-visited orchid evolutionary pressure acts on visual, but not olfactory traits toward a higher similarity to its model. Odor mismatch did not prevent bees from landing on flowers that had the expected visual display.

An alternative hypothesis, the specialized female reward hypothesis offered by Hemborg and Bond (2005) challenges the idea that pollinators search for the same reward in all conspecific flowers. According to Hemborg and Bond, males and females both offer essential, but different, components to the pollinators, and these sex-specific rewards may be advertised by sexually dimorphic floral signals. Kay (1985) and Elmqvist et al. (1988) found that female *Salix* flowers produce more nectar than male flowers, and Katoh et al. (1985) reported that females tend to have hexose dominated nectar in contrast to sucrose dominated nectar in males. Our own observations (Füssel et al., unpublished data) support these findings. Moreover, in case of the pollen specific bee *Andrena vaga*, it is known that females mainly collect pollen (and nectar) on some days, and on other days they feed on and/or collect only nectar from *Salix* (see Bischoff et al., 2003). Bees could use differences in scent of sex morphs to distinguish sexes, and to visit primarily/exclusively females when focusing on nectar, and males when focusing on pollen. Additionally, nectar-seeking flower visitors in general could choose their preferred nectar source from the two sexes thus fulfilling their actual needs. However, studies of specialisation of female nectar rewards in entomogamous willows are scarce, and bioassays that prove if and how flower visitors differentiate between male and female attractants are lacking. Furthermore, pollen carry over only during occasional behavioural switches might be insufficient to ensure pollination. Therefore, from the plant point of view, similarity between the sexes is probably desirable to prevent pollinators from discriminating between male and female plants and to promote frequent cross-pollination.

It is interesting to note that *S. repens,* which emits a weak (Tollsten and Knudsen, 1992) and highly variable (Tollsten and Knudsen, 1992; present study) scent in comparison to other *Salix* species, seems to be primarily wind-pollinated (Vroege and Stelleman, 1990). Thus, the selective pressure to display a consistent pollinator-type specific floral scent profile across populations, sexes, and individuals might be lower, compared to species that are strongly dependent on insect pollination. Only in predominantly entomophilous species can distinct species-specific scent profiles promote flower constancy thus avoiding pollen waste, and functioning as reproductive isolation barriers between species.

On one hand, plants from quite different systematic positions have evolved the same pollination system involving the emission of similar floral scent spectra (e.g., Knudsen and Tollsten, 1993; Andersson et al., 2002). On the other, closely related plant species may have evolved quite different floral scent profiles that function as reproductive isolation barriers (e.g., Mant et al., 2005). If floral scent is to act as a willow specific attractant, pollinators must be able to perceive willow-specific floral scent compounds. If floral scent is to function as reproductive isolation mechanism among willows, a second assumption has to be fulfilled: species must emit species-specific scents that pollinators may use to discriminate.

Dötterl et al. (2005a) demonstrated that floral scent compounds of *Salix* can act as cues for the attraction of oligolectic bees that collect pollen for their larvae exclusively on willows. *A. vaga* Pz. (Andrenidae) responded in GC-EAD tests to 1,4-dimethoxybenzene and other willow compounds; 1,4-dimethoxybenzene, in particular, attracted female bees in bioassays. Also many other *Salix*-visiting bee taxa respond strongly in GC-EAD tests to 1,4-dimethoxybenzene (Füssel et al., personal observation). It might be that 1,4-dimethoxybenzene, one of the two most common floral volatiles in willows, is the specific cue that provokes the flower constancy in these bees. It would be interesting to test whether *A. vaga* prefers 1,4-dimethoxybenzene dominated willow species over *trans*-β-ocimene dominated species.

Several other compounds found in this study are known to be attractive also to different insects or are at least active in electrophysiological studies. Beetles can be attracted by 1,4-dimethoxybenzene (Ventura et al., 2000), phenylacetaldehyde is attractive to flies (Howse, 2003), butterflies (Honda et al., 1998; Omura et al., 1999; Andersson, 2003), and moths (Haynes et al., 1991; Cunningham et al., 2004), and lilac aldehydes are known to be detected by butterflies (Andersson, 2003) and to be highly attractive to noctuid moth species (Dötterl et al., 2006). Besides *Andrena vaga* (Dötterl et al., 2005a), many other *Salix*-visiting bee taxa respond strongly in GC-EAD tests to 1,4-dimethoxybenzene and other typical willow volatiles (Füssel et al., personal observation). Moreover, different willow-visiting moth species (e.g., *Orthosia* spp.) respond strongly in GC-EAD tests to several of the volatile compounds found in *Salix* (such as benzyl nitrile, lilac aldehyde A, and 4-oxoisopherone). It can be hypothesized that, besides 1,4-dimethoxybenzene and *trans*-β-ocimene that mainly distinguish species, several other flower scent compounds that are frequently found in *Salix* species may be effective specific attractants of potential pollinators. It is likely that they also provide species-specific signals that serve as reproductive isolation barriers. In a subset of eight extensively sampled species, we showed a characteristic floral scent composition for six

species, and half of the pairwise species comparisons confirmed significant differences. The observed differences are mainly based on the variability of only two compounds – 1,4-dimethoxybenzene and *trans*-β-ocimene that together explain 84% of variability among these eight species. Of special interest are compatible species that grow sympatrically in the same habitats. According to Lautenschlager-Fleury and Lautenschlager-Fleury (1994) and Rothmaler (2002), this is the case for seven of the eight; only the subalpine *S. bicolor* is unlikely to cooccur with any of the other species. Nevertheless, even though these willows show significantly different scent profiles, natural hybrids are observed between most (see Rothmaler, 2002); even the subalpine *S. bicolor* is known to hybridize naturally with *S. caprea* and *S. myrsinifolia*.

Our results confirm those of Tollsten and Knudsen (1992) who found no significant differences between *S. caprea* and *S. cinerea*. However, their hypothesis that this high similarity in scent is responsible for frequently occurring hybridization between these species is not supported by our data on six other extensively sampled species. It seems that neither different scent profiles hamper hybridization, nor that similar scent profiles imply a higher risk of hybridization. However, because quantitative data on hybridization events between species pairs are lacking, this hypothesis needs further investigation.

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2 *Salix caprea*: an Interaction Generalist and Multi-Specialist with Bimodal Adaptations of Floral Scent to Bees and Moths

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Summary

- Assuming that floral characteristics reflect adaptations to single effective functional pollinator groups, plant species associated with several effective pollinator groups are unlikely to develop specific adaptations to a multiple groups. To test this hypothesis we investigated *Salix caprea*, a species with a generalized pollination system where wind, bees, and moths are pollen vectors.

- Floral scent analysis revealed 38 compounds, of which 25 were physiological active in electroantennographic studies with 13 bee and moth species. Bees and moths responded to nearly identical sets of compounds, but patterns of response strength differed. In choice tests, bees preferred 1,4-dimethoxybenzene over lilac aldehyde, which is emitted in higher relative amounts during the day, whereas most moths preferred lilac aldehyde, which is emitted in higher relative amounts at night, over 1,4-dimethoxybenzene.Temporal scent emission patterns corresponded with flower visitor activity patterns.

- *S. caprea*, an interaction generalist, has evolved a temporally fine-tuned scent emission pattern adapted to attraction multiple pollinator types at different hours.

- Pollination experiments revealed that nocturnal pollinators (e.g. moths) and wind contributed less to *S. caprea* seed set than diurnal pollinators (e.g. bees), but plants still have a fitness gain by attracting moths, because diurnal pollinators alone do not achieve maximum seed set.

Key words: bees, flower scent, GC-EAD, moths, multi-specialist, pollination system

Introduction

Flowers are complex systems in which features such as shape, nectar, colour, and odour work together to attract pollinators and to manipulate their behaviour for the benefit of the plants' reproduction (e.g. Stensmyr *et al*., 2002; Schiestl, 2005; Raguso *et al*., 2007). Diversity in flower features has been interpreted since Darwin as adaptations correspondingly to different types of pollinators (e.g. Darwin, 1862; Vogel, 1954; Fenster *et al*., 2004). Similarities in floral features are interpreted to be a result of adaptations to pollinators with similar morphological, behavioural and physiological characteristics (Müller, 1883; Delpino, 1868-1875; Knuth, 1906; Baker, 1963; Grant & Grant, 1965; Fægri & van der Pijl, 1979; Stebbins, 1970; Johnson & Steiner, 2000). This relationship between pollinators and plant species is reflected in the classification of flowers in-to pollination syndromes (Faegri & van der Pijl, 1979).

However, abundant observational data show that many flowers are visited by a broad spectrum of animal visitors (Robertson, 1928; Ellis & Ellis-Adam, 1993; Ollerton, 1996; Waser *et al*., 1996; Memmott, 1999). This has led to an ongoing debate on the underlying mechanisms of specialization in flowers, in particular on the question of how flowers can specialize if a wide range of flower visitors with different behaviour, morphology and physiology are interacting with them (Waser *et al*., 1996; Johnson & Steiner, 2000; Fenster *et al*., 2004).

Two arguments explain specialization in flowers with a high number of flower visiting species: (1) flowers adapt to functional groups of pollinators and not to species and (2) flowers adapted to their pollinator fitness (Stebbin, 1970; Fenster *et* al., 2004). In other words, to understand floral specialization in a given plant species it is necessary to analyse the pollination efficiency. It seems logical that a high degree of specialization would be assumed in plants with only one functional pollinator group mainly responsible for the plants' pollination or in plants where different functional groups of effective pollinators select for similar floral characteristics. Floral characteristics are then, according to Stebbins (1970), the result of the most effective pollinators – which are exerting the strongest selective pressure. We might speak of a kind generalist that is a compromise with many functional groups of pollinators that all contribute to the plants seed set, but exert selective pressure in different directions. However, a presumption for this scenario is that floral traits for different pollinators usually oppose each other and that opposing traits cannot be displayed within the same plant or flower. Aigner (2001, 2006) has point out that there is little evidence for such

tradeoffs and flowers may be a mosaic of different adaptations to various co-pollinators. A high number of plant species show during their flowering anthesis considerable variation or change of floral traits within single flowers, such as movement or growth of floral parts that lead to modifications of shape and form, flower colour changes, or changes of floral scent emission over day and night.

Floral fragrance is important for the attraction of floral visitors (Proctor *et al*., 1996), and in a number of studies, correlations between the fragrance composition of flowers and the type of flower visitor have been found (Knudsen & Tollsten, 1993; Knudsen & Tollsten, 1995; Miyake *et al*., 1998; Andersson *et al*., 2002). Olfactory cues in terms of scent intensity as well as volatile composition have been shown to correspond to different pollinator assemblages (Dobson, 2006), for example bees versus moths. Moreover, many flowers show a rhythmic scent emission, which is controlled by a circadian clock and/or regulated by light (Jakobsen & Olsen, 1994; Helsper *et al*., 1998; Pott *et al*., 2003). In some species the dynamic nature of scent is not only reflected in quantitative changes in the emission of volatiles but also in qualitative changes in the odour composition (Baldwin *et al*., 1997; Hoballah *et al*., 2005). A rhythmic scent emission is often correlated with the corresponding temporal activity of flower visitors, and most studies on rhythmic floral scent emission patterns have been analyzed in species with specialized pollinator-plant interactions where the plant is associated with a single or dominant functional pollinator group (Dötterl *et al*., 2005a). However, in generalized pollination systems, where the activity times of the pollinators and their olfactory preferences differ, temporal dynamics in volatile emission might in principle allow for multiple adaptations to multiple pollinators.

One group of plants with generalistic pollination is the genus *Salix* (willows, Salicaceae), whose species show traits associated with plants pollinated by insects as well as wind (Stebbins, 1970; Faegri & van der Pijl, 1979). On the one hand, the lack of a perianth, the release of large amounts of small pollen grains, and precocious flowering fit with the wind pollination syndrome. On the other hand, erect, stiff inflorescences, highly visible sexual parts, nectar production, and floral scent are interpreted as signs for entomophily (Faegri & van der Pijl, 1979; Kay, 1985; Proctor *et al*., 1996). Reported ratios of insect to wind pollination, range from 20-70% wind pollination in *Salix repens*, to 50% insect pollination in *S. caprea,* and almost total insect pollination in *S. arctica* (Kevan, 1972; Vroege & Stelleman, 1990). Therefore, depending on ecological context, insects (Kevan, 1972; Sacchi & Price, 1988; Elmqvist *et al*., 1988; Douglas, 1997) as well as wind (Argus, 1974; Vroege & Stelleman, 1990; Fox, 1992) seem to be important pollen vectors.

In the present study we aimed to investigate the role of floral scents as olfactory signals in an interaction generalist, the willow species *Salix caprea* (commonly called sallow), and to relate the adaptations between different flower visitors and scent emission to the relative importance of different pollinator types for seed production. In *Salix*, data on the chemical composition of floral scent are available for several species (Tollsten & Knudsen, 1992; Dötterl *et al*., 2005b; Füssel *et al*., 2007), but temporal emission of floral scent has never been investigated.

Many *Salix* species are visited by bees, flies (van der Werf *et al*., 1982; Pellmyr & Kärkkainen, 1987), butterflies, and beetles (Vroege & Stelleman, 1990; Urban & Kopelke, 2004). Further, several moth species are known to visit *Salix* for nectar (Steiner & Ebert, 1998). Preliminary investigations on *Salix caprea* showed that moths were powdered with pollen and therefore we suppose that moths play a role in pollination in our study sytem.

In order to relate the adaptations between different flower visitors and scent emission to the relative importance of different pollinator types for seed production, we examined circadian floral scent emission in *Salix caprea*, monitored flower visitors over day and night and studied their contribution to reproductive success, and studied the responsiveness of abundant flower visitors to the different floral scent compounds.

Materials and Methods

Plant Material and Volatile Collection

To analyse temporal variation over a day, floral scent was collected from seven plants in full bloom (three males, four females) during a 24 h period in 2006. Results from female and male plants were pooled for analyses, as there is no sex difference in floral scent of this species (Tollsten & Knudsen, 1992; Füssel *et al*., 2007). Thirteen floral scent samples were collected from each plant during a 24 h period. During the sampling period, sunrise was at approximately 6 am and sunset at approximately 8 pm, and the samples were taken every two hours starting at 8 am. On each plant, one twig with four to 10 flowering catkins was enclosed for 10 min in an oven bag (Nalophan), and the floral scent was subsequently trapped for 2.5 min in an adsorbent microtube (filled with 3 mg of a 1:1 mixture of Tenax-TA 60-80 and Carbotrap 20-40) by using a membrane pump (G12/01 EB, Rietschle Thomas, Puchheim, Germany; air flow rate: 0.2 ml min^{-1}). After sampling, the microtubes were stored at -20 °C for further analyses.

The samples were analysed on a Varian Saturn 3800 gas chromatograph (GC) fitted with a 1079 injector, and a Varian Saturn 2000 mass spectrometer (MS). For the analyses a ZB-5 column (5 % phenyl polysiloxane, length 60 m, inner diameter 0.25 µm, film thickness 0.25 µm, Phenomenex) was used. The microtubes were inserted via Varians Chromatoprobe into the GC injector. The injector vent was opened (1/20) and the injector was heated at 40 °C to flush any air from the system. After 2 min the split vent was closed and the injector heated at 200 °C min^{-1}, then held at 200 °C for 4.2 min, after which the split vent was opened (1/20) and the injector cooled down. Electronic flow control was used to maintain a constant helium carrier gas flow (flow rate of 1.8 ml min^{-1}). The GC oven temperature was held for 7 min at 40 °C, then increased by 6 °C min^{-1} to 260 °C and held for 1 min at this temperature. The mass spectra were taken at 70 eV with a scanning speed of 1 scan s^{-1} from *m/z* 40 to 350.

The GC-MS data were analysed by using the Saturn Software package 5.2.1. To identify the floral scent compounds of the GC-MS spectra, the data bases NIST 02 and MassFinder 3 were used, and identifications were confirmed by comparison of retention times with published data (Adams, 1995). Identification of some compounds was also confirmed by comparison of mass spectra and retention times with those of authentic standards.

During sampling of volatiles, the air temperature was measured by the meteorological station of the Ecological-Botanical Garden (EBG). The air temperature varied greatly between the different sampling days of the seven individual plants. Therefore, to standardize

measurements of air temperature among sampling days the maximum air temperature of a sampling day was equated with 100 %. For any time on this day the air temperature is given as percentage of the maximum day value (Figure 5).

Gas Chromatography coupled to Electroantennographic Detection (GC-EAD)

To get samples for the electrophysiological analyses (see below) two samples were collected in 2005 using a dynamic headspace method. One of these samples was collected *in situ*, but due to bad weather conditions the other sample was collected from twigs cut from another plant that were placed in water. For each of the two samples two or three twigs with 10 to 12 catkins of each *Salix caprea* plant (only males, because there is no sex difference in floral scent of *S. caprea* (Tollsten & Knudsen, 1992; Füssel *et al*., 2007) were enclosed in a polyethylene oven bag and volatiles were trapped for ca. 8 hours between 9 am and 5 pm in large adsorbent tubes filled with 30 mg of a 1:1 mixture of Tenax-TA 60-80 and Carbotrap 20-40. The volatile collection was during the day time, because mainly the quantity is changing during the whole day. Volatiles were eluted with 70 µl of acetone (SupraSolv, Merck KgaA, Germany) for later use in the GC-EADs.

Electrophysiological analyses were used to identify the compounds in the floral scent of *Salix caprea* being detected by the abundant flower visitors. The two scent samples were tested on the antennae of six frequent diurnal flower bee species, and seven frequent nocturnal moth species. Bees were caught either at their nesting places or from *S. caprea*, and moths were mainly caught by light traps, which were placed in *S. caprea* trees (see below). These insects were not naïve to *Salix* flowers. For some bee species more than one specimen was tested. All measurements were performed with the GC-EAD system described by Dötterl *et al*. (2005b). The GC-EAD system consisted of a gas chromatograph (Vega 6000 Series 2, Carlo Erba, Rodano, Italy) equipped with a flame ionization detector (FID), and an EAD setup (heated transfer line, 2-channel USB acquisition controller) provided by Syntech (Hilversum, Netherlands). 1 µl of an odor sample was injected splitless at 60 °C, followed by opening the split vent after 1 min and heating the oven at a rate of 10 °C min^{-1} to 200 °C. The end temperature was held for 5 min. A ZB-5 column was used for the analyses (length 30 m, inner diameter 0.32 mm, film thickness 0.25 µm, Phenomenex). The column was split at the end by the four arm flow splitter GRAPHPACK 3D/2 (Gerstel, Mülheim, Germany) into two pieces of deactivated capillary (length 50 cm, inner diameter 0.32 mm) leading to the FID and EAD setup. Makeup gas (He, 16 ml min^{-1}) was introduced through the fourth arm of the

splitter. For measurements, an excised antenna was mounted between glass micropipette electrodes filled with insect ringer (8.0 g l^{-1} NaCl, 0.4 g l^{-1} KCl, 4 g l^{-1} $CaCl_2$), and connected to silver wires.

To identify the structure of the compounds eliciting signals in the insect antennae, 1 µl of the acetone samples was placed in a quartz vial in the injector port of the GC by means of the ChromatoProbe, and then analyzed by GC-MS as described above for samples taken to study circadian rhythmicity of scent emission.

Behavioral Experiments

To test the attractiveness of 1,4-dimethoxybenzene and lilac aldehyde two-choice bioassays were conducted in a flight cage (*Apis mellifera*) and in a wind tunnel (*Orthosia gothica*) in spring 2007. The two floral scent compounds of *Salix caprea* were chosen, because they were the most variable compounds comparing day (2 pm) and night (8 pm).

Two-choice bioassay with *Apis mellifera* - Before flowering of *S. caprea,* one bee hive with nine honeycombs of naïve honey bees was placed in a flight cage (7.20 m x 3.60 m x 2.20 m). One rubber GC septum impregnated with 10 µl of 1,4-dimethoxybenzene (99 %, Aldrich; 10 µl 1,4-dimethoxymethoxybenzene dissolved in 90 µl paraffin) and one rubber GC septum with 10 µl of lilac aldehyde (synthesized as described in Dötterl *et al*., 2006; 10 µl lilac aldehyde dissolved in 90 µl paraffin) were presented in the flight cage (distance of the septa: 1 m) around noon for 40 min, when the activity of bees was high. Every ten minutes the position of the rubber GC septa was changed. The reaction of bees was classified as "zigzagging" when the honeybees flew upwind toward one of the septa within 10 cm.

Two-choice bioassay with *Orthosia gothica* - A 160-cm by 75-cm by 75-cm wind tunnel (Dötterl *et al*., 2006) was used for bioassays. A Fischbach speed controller fan (D340/E1, FDR32, Neunkirchen, Germany) continuously circulated the necessary air through the tunnel with an airspeed of 0.35 m s^{-1}. The incoming air was passed through four charcoal filters (145 mm x 457 mm), with a carbon thickness of 16 mm (Camfil Farr, Laval, Quebec, Canada). The temperature and humidity were adjusted to 22-24 °C and 30-32 %, respectively. Experiments were done during the beginning of the dark period, under dim red light. A two-choice assay, the same as described for the honeybees, was used to investigate the attractiveness of the floral scent compounds 1,4-dimethoxybenzene and lilac aldehyde. The rubber GC septa were offered at the upwind end of the tunnel behind polyester gauze and metal grids, so that they were invisible to the moths. For the tests, moths were used singly.

Therefore, moths, which were caught with a light trap (see below) were released from a holding chamber at the downwind end of the tunnel, and their behavior was observed for 5 min. In this experiment, 22 male and 24 female moths were tested. Ten male and eight female flew in the flight cage, and were attracted by the scent compounds. A positive attraction (response) was counted when the moth was observed to zigzag within a 10 cm radius or to land on the gauze in front of the odour source.

Flower visitors

The spectrum of visitors to flowers on three male and four female *Salix caprea* trees were recorded in 2006. The total observation time was 60 min during day and 60 min during night. All flower visitors observed were caught with an insect net. Clearly identifiable flower visitors (e.g. honeybees) were recorded (species, number of individuals) and released alive, while the others were killed and stored at -20 °C for further preparation and determination. Only the most frequent floral visitors during day and night, i.e. diurnal bees and butterflies, and nocturnal Lepidoptera, respectively, were included in the analysis.

Nocturnal Lepidoptera were additionally caught with automatic light traps (model Weber, bioform; 12 V, 15 W). The light traps were attached directly in the centre of the trees. Each of the seven *S. caprea* trees was investigated on one to four days, depending on flowering duration and weather conditions. Only insect species carrying pollen were included in the analyses.

Flower visitor abundance counts - To determine the abundance of flower visitors of *S. caprea* at specific times over a whole day the “scan sampling method” according to Sowig (1991) was applied. In intervals of two hours (parallel with floral scent collection from the seven individuals in 2006), one randomly selected branch per individual (length = 30 cm) was observed for 30 s for their flower visitors and in the following 30 s these observations were recorded. This procedure was repeated 13 times and the mean of these observations was subsequently calculated. Because of the difficult identification of species during foraging, the observed visitors were classified into seven easily distinguishable groups: 1 = honeybee; 2 = bumble bees; 3 = medium sized bees [wild bees approximately the size of a honeybee]; 4 = small bees [wild bees smaller than a honeybee]; 5 = butterflies; 6 = moths; 7 = others like flies, beetles. The number of observed individuals of each flower visitor group was recorded.

Pollination Experiments

In 2006, five female *S. caprea* trees of similar size and age (same subset as for pollinator observations) were chosen for pollination experiments. Before stigmas became receptive four twigs with five to 25 female catkins per plant were selected for the following four pollination treatments: (1) day- and night pollination (control): no exclusion of insects; (2) day pollination: exclusion of insects during night (8 pm until 6 am); (3) night pollination: exclusion of insects during day (6 am until 8 pm); (4) wind pollination: exclusion of all insects during day and night pollinated only by wind. To exclude insects, twigs were enclosed with nylon net (unifilar fabric of gossamer).

To guarantee natural progress of fruit and seed development, all nylon nets were removed from twigs after they had ceased flowering. Shortly before seed maturity, one fruiting catkin were enclosed in dialysis tubing (cellulose, Visking, Type 1-7/8 diameter, 79 mm). When fruits opened inside the dialysis tubing, the catkins were harvested. The number of seeds and capsules per catkin were counted, and the number of seeds per capsule was calculated. Since the calculated numbers of seeds per catkin and seeds per capsule varied greatly within pollination treatments among the different plant individuals, the data were standardized as follows. The maximum number of seeds in open day- and night-pollination (control) of an individual was equated with 100 %, for the other pollination treatments (2-4) the amount of seeds per catkin and seeds per capsule is given as percentage of the maximum amount found in the corresponding control.

Statistics

Floral scent - To determine the amount of scent emitted by *S. caprea* over a day, the data were standardized, as the total amount of volatiles emitted at a specific time varied greatly among twigs of *Salix* individuals sampled.The maximum amount of total floral scent emitted by a particular twig over the 13 sampling times was equated with 100 %, and the amount of volatiles emitted at any given time from this twig is given as percentage of this maximum amount.

To determine differences in the diurnal and nocturnal total floral scent, the sums of the amounts determined during day (sum of six measurements; 8 am, 10 am, 12 am, 2 pm, 4 pm, 6 pm) was compared with the sums of the total amounts determined during night (sum of six measurements; 8 pm, 10 pm, 12 pm, 2 am, 4 am, 6 am) by a paired t-test. Normality of paired differences was tested with a Kolmogoroff-Smirnov test. Analysis of similarities (ANOSIM,

two-way crossed design with no replication; factors: time and plant individual) in the program package Primer 6.1.6 (see also Clarke & Warwick, 2001; Clarke & Gorley, 2006) was used to test the null hypothesis that there are no differences in scent pattern across times for the different individuals. A two-way layout was necessary as there were differences in scent across individuals for the different times. As the total amount of scent emitted varied greatly among individuals and across times, either the percentage amount of compounds (for semiquantitative differences) or the presence / absence of compounds (for qualitative differences) were used. As the scent of *S. caprea* is strongly dominated by one compound, i.e. 1,4-dimethoxybenzene (Füssel *et al*., 2007), 4^{th} root transformed data were used for the semiquantitative analysis, to avoid the results being simply a function of this main compound. The transformed semiquantitative data or the qualitative data were used to generate similarity matrixes by calculating the Bray-Curtis (Sørensen) indix, which is used in the ANOSIM analyses. SIMPER ("similarity percentages") was used (two-way crossed design; factors: time and plant individual) to determine the compounds responsible for differences in scent emitted at 2 pm, where activity of day-active visitors was highest, and at 8 pm, where activity of night-active visitors was highest. For these analyses the 4^{th} root transformed percentage amounts of compounds were used.

Electrophysiological measurements - Bray-Curtis similarities were calculated to determine semiquantitative differences in antennal responses patterns of the different insects measured. For these analyses, the percentage response amplitudes to the different compounds were used (the sum of the amplitudes of all responses were equated with 100 %). To visualize similarities in antennal response patterns of different flower visitors non-metric multidimensional scaling was used (Clarke & Gorley, 2006). SIMPER also was used (one-way layout; factor: insect group) to identify the compounds (the antennal responses) being responsible for the different antennal response patterns found between noctuids and bees.

Behavioral Experiment - To compare the attractiveness of 1,4-dimethoxybenzene and lilac aldehyde an observed vs. expected χ^2-test was conducted.

Pollination Experiment - The mean relative number of seeds per catkin and seeds per capsule where composed among pollination treatments 2-4, the data were tested for normality with the Kolmogoroff-Smirnov test. Homogeneity of variances was tested using the Levene test. In case of normality and homogeneity of variances, a repeated measures ANOVA was calculated as a global test and subsequently the Least Significance Difference test (LSD test) was applied as a post hoc test.

Results

Flower Visitors

Species and abundance of flower visitors of diurnal bees and butterflies as well as nocturnal Lepidoptera are listed in Table 1. During day, the willows were visited mainly by bees, such as *Apis mellifera*, *Bombus terrestris*, *Andrena praecox*, and *A. clarkella*, and by butterflies (*Aglais urticae*). After sunset, mainly Noctuidae, e. g. *Orthosia cerasi*, *O. gothica*, and *O. gracilis* were found. A lot of bees as well as moths were observed touching the anthers and stigmas while drinking nectar (bees and moths) or collecting pollen (bees only), thus suggesting that they function as pollinators. Altogether 45 species (Hymenoptera and Lepidoptera) of potential pollinators were found. Additionally, specimens of Coleoptera, Diptera, Megaloptera, Planipennia, and Rhynchota were observed visiting the flowers during day. Pollen could be found on the body of several of these animals indicating that these also may contribute to pollination. However, most animals groups and species were only found in small numbers, and therefore, they were not studied in more detail.

The abundance of different flower visitor groups (honeybee, bumblebee, medium sized bee, small bee, butterfly, moth, other insect) during the course of the day is shown in Figure 1. Activity was highest between 10 am and 4 pm (28 to 51 flower visitors per 15 min). Frequently observed insects during the day were honeybees, medium and small bees, butterflies, and other insects. With the beginning of twilight (8 pm) moths (six moths per 15 min) were the most common flower visitors, but other insects (three other insects per 15 min) could also be detected. However, the total number of flower visitors declined.

Identification of Floral Scent (GC-MS) and electrophysiological active compounds (GC-EAD)

The compounds found in the floral scent samples of seven *Salix caprea* plants are listed in Table 2. Mainly aromatics and monoterpenes were detected. The scent was dominated by few components, e.g., 1,4-dimethoxybenzene and *trans*-β-ocimene, while most other compounds were only found in relatively small amounts.

Two flower scent samples of *Salix caprea* were tested on the antennae of 13 different bee and moth species to identify the compounds being detected by the main diurnal and nocturnal flower visitors. Several of the compounds elicited signals in the antennae of the main flower visitors (Figure 2). However, the tested insects did not respond especially to various cyclic

monoterpenes, e.g. different phellandrenes and pinenes. Compounds eliciting a signal in all tested antennae were the aromatics methyl salicylate and indole. All insects, except the two tested geometrids, most strongly responded to 1,4-dimethoxybenzene.

The two differently collected scent samples (floral scent collection *in situ* or from cut twigs) differed in their composition resulting also in different antennal response pattern (Figure 3). Some compounds (e.g. eugenol, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-ocimene, (*E*)-ocimene oxide) were found in higher amount in the sample of cut twigs, and only elicited antennal responses in this sample. Other compounds (e.g. benzyl alcohol, salicylaldehyde, anisaldehyde) were found in higher amounts in the sample collected *in situ*.

Diurnal (bees) and nocturnal (moths) visitors of *Salix* responded differently to the flower scent. Figure 2 displays the antennal responses of five species belonging to the five families listed in Table 2 (all tested on the same *Salix* scent sample *in situ*). The response patterns of the three bee species differed from the response pattern observed in the two moth species. The bee antennae responded most strongly to 1,4-dimethoxybenzene, whereas all other compounds elicited comparatively small signals. The noctuid moth *Orthosia cerasi* responded to 1,4-dimethoxybenzene, but also showed a strong response to the coeluting compounds benzyl nitrile, lilac aldehyde A, and 4-oxoisophorone. These coeluting compounds also elicited a clear response in the geometrid species, which responded most strongly to methyl salicylate.

When comparing the antennal response pattern (percentage response amplitude to individual compounds) of all individuals measured it becomes clear that the results presented in Figure 2 can be generalized. The diurnal bees differently responded to the scent compared to the nocturnal noctuids and to the nocturnal geometrids. In contrast to the noctuids and the bees, both geometrids did not respond clearly to 1,4-dimethoxybenzene, and therefore, the response pattern of the two geometrid species measured are isolated in the ordination presented in Figure 3. The noctuids are placed in between the geometrids and the bees, and a SIMPER analysis revealed the responses being responsible for separation of these two groups.

Three responses, explained one third of the differences observed between bees and noctuids. The percentage response to 1,4-dimethoxybenzene was most different between the two groups, and the mean relative response of bees (31 %) was almost twice the response of noctuids (18 %). On the other hand, the relative response to the coeluting compounds benzyl nitrile, lilac aldehyde A, and 4-oxoisophorone, and to methyl salicylate was almost twice in noctuids (10 % and 12 %, respectively) compared to bees (6 % each).

Behavioral Experiments

The bioassays showed that 1,4-dimethoxybenzene and lilac aldehyde not only elicited antennal responses but also triggered behavioral responses in bees (*Apis mellifera*) and moths (*Orthosia gothica*) (Figure 4).

In a two-choice test significantly more honeybees flew to the rubber GC septum impregnated with the benzenoid 1,4-dimethoxybenzene than to the rubber GC septum impregnated with lilac aldehyde (observed vs. expected χ^2-test: $\chi^2 = 44.29$; $df = 1$; $p < 0.001$). The attraction of moths to the two compounds was vice versa. Significantly more of the moths, *Orthosia gothica*, flew to the rubber GC septum impregnated with lilac aldehyde than the rubber GC septum impregnated with 1,4-dimethoxybenzene (observed vs. expected χ^2-test: $\chi^2 = 14.22$; $df = 1$; $p < 0.001$).

Variation in Floral Scent over a day

A time-dependent variation of the total amount of scent emitted is evident (Figure 5), and significantly more scent was emitted during the day than at night (*t*-test: $t_{df=6} = 2.93$; $p = 0.03$). Further, there was not only a change in the total amount of scent emitted but also in the realive qualitaties (2-way ANOSIM: $Rho = 0.21$, $p < 0.001$). This was more pronounced in the semiquantitative scent pattern (4^{th} root transformed data, 2-way ANOSIM: $Rho = 0.30$, $p < 0.001$). Some compounds emitted only in trace amounts could not be detected at night, where the total amount of scent emitted was low (e.g. salicylaldehyde), and other compounds were found in differing percentage amount over a day. Of special interest for the interaction with insects are those compounds being detected by the main flower visitors (see below), i.e. bees during day, and moths during night.

When using only EAD active substances for analyses, qualitative (2-way ANOSIM: $Rho = 0.15$, $p = 0.004$) and semiquantitative (4^{th} root transformed data, 2-way ANOSIM: $Rho = 0.21$, $p < 0.001$) changes in the scent pattern also becomes evident. When comparing the scent pattern emitted at 2 pm, where the abundance of day-active visitors is highest, with the scent pattern observed at 8 pm, where the abundance of night-active visitors was highest, the isomers of lilac aldehyde were most variable, and explained together almost 40 % of the semiquantitative differences (2-way SIMPER analysis). They were emitted at 8 pm in higher percentage amount than at 2 pm (8.6 % at night versus. 2.1 % during the afternoon; paired *t*-test: $t_{df = 6} = -3.9$, $p = 0.01$). Further; the total amount of lilac aldehyde differed at night and during day-time, with a higher emission at night (day: 1.31 ng $(20\ min)^{-1}$ per dry weight of all

catkins; night: 4.42 ng (20 min)$^{-1}$ per dry weight of all catkins; dependent *t*-test: $t_{df = 6} = 3.02$, $p = 0.002$).

In contrast, the total floral scent amount of 1,4-dimethoxybenzene was much higher during day than during night (day: 61.83 ng (20 min)$^{-1}$ per dry weight of all catkins; night: 15.24 ng (20 min)$^{-1}$ per dry weight of all catkins; paired *t*-test: $t_{df = 6} = 3.01$, $p = 0.003$).

Pollination Experiments

The pollination success differed between treatments as shown in Figure 6. Significant differences were found in the number of seeds per catkin (repeated measures ANOVA: $df = 2$; $F = 45.79$; $p < 0.001$) as well as in the number of seeds per capsule (repeated measures ANOVA: $df = 2$; $F = 37.56$; $p < 0.001$). In both cases, the contribution of daylight pollination to the reproductive success (approximately 80 % seed set) was significantly higher than the contribution of night and wind pollination (each approximately 20 % seed set). Differences between night and wind pollination were not detected.

Discussion

Flower visitors of Salix caprea

In our study we recorded 45 Lepidoptera and Hymenoptera species visiting the catkins of *S. caprea*, among them day- as well as night-active species. For several *Salix* species flower visitors have already been reported (e.g. Vroege & Stelleman, 1990; Hilty, 2006). In all investigations, including our study, *Apis mellifera* was observed as flower visitor during the day.

In this and other studies several species of *Andrena* and *Bombus* were observed (e.g. Hilty, 2006). In this study, many nocturnal moth species were found visiting catkins. Several of these night-active visitors, e.g. *Orthosia gothica*, were highly abundant and willow flowers are their most important nectar source in early spring (Steiner & Ebert, 1998). Both bees as well as Lepidoptera species are potential pollinators because when visiting male catkins they usually come into contact with pollen and may transfer pollen from male flowers to female flowers.

Floral scent and antennal responses of abundant visitors

The flowers of *S. caprea* produced altogether 38 scent compounds (Table 2). Most of the identified compounds are known to be typical compounds in floral scents (Knudsen *et al*., 2006). Main compounds in the scent of *S. caprea* were 1,4-dimethoxybenzene and methyl salicylate (Table 2), and these aromatics often occur in the early flowering *Salix* species like *S. caprea*, *S. cinerea*, and *S. atrocinerea* (Füssel *et al*., 2007).

In an electroantennographic study of six bee and seven moth flower visitors several of the compounds emitted by *S. caprea* flowers elicited signals in their antennae, and all these compounds could be important for attraction of these insects to *Salix*.

Interestingly, bees and moths responded nearly to the same set of compounds, differing in the strength of the response.

Interestingly, the moths strongly responded to the co-eluting compounds lilac aldehyde A, benzylnitrile, 4-oxoisophorone, while the response of the bees to these compounds was less pronounced. It is unclear, which of the three co-eluting compounds were responsible for the observed differences between moths and bees. However, until now only antennal responses of moths to different lilac aldehyde isomers (including lilac aldehyde A) have been shown (Plepys *et al*., 2002b; Dötterl *et al*., 2006), and it is unclear whether moths also respond to

benzylnitrile, and 4-oxoisophorone. Lilac aldehyde is often found in plants pollinated by moths (Knudsen *et al*., 2006; Dobson, 2006), and was shown in the present study as well as in previous studies to be highly attractive for moths (Plepys *et al*., 2002a; Dötterl *et al*., 2006).

In all tests with bee antennae, the strongest signal was elicited by 1,4-dimethoxybenzene, whereas the responses to the other compounds were comparatively weak. This benzenoid compound was found to be highly attractive to willow-visiting bees in a previous study (Dötterl *et al*., 2005a). It is further known as attractant to beetles (Ventura *et al*., 2000) and assumed to attract euglossine bees (Williams & Whitten, 1983). In GC-EAD studies we tested generalistic bees visiting many different plants emitting sometimes quite different compounds, including the generalist honeybee and *Andrena haemorrhoa* as well as specialized (oligolectic) bees, such as *A. praecox*, which collect pollen for their larvae on *Salix* species only. Our expectation that specialists show specific adaptations to the scent of their hosts, and respond more sensitively to willow compounds, was not fulfilled. Instead, generalistic and specialized bees responded quite similarly to the scents of *S. caprea*, and no specific adaptations in the periphery of the olfactory circuit of the oligolectic bees to the scent of their host-plants were found.

Several of the electrophysiological active compounds found in this study are known to be attractive for other insect species. Phenylacetaldehyde is attractive to brachyceran as well as nematoceran flies (Howse, 2003; Jhumur *et al*., 2006) as well as to moths (Huber *et al*., 2005; Olsson *et al*., 2005). It further elicits antennal responses in butterflies (Andersson & Dobson, 2003). Benzyl alcohol elicited antennal response in moths (Hoballah *et al*., 2005). Linalool can be detected by butterflies (Andersson, 2003; Andersson & Dobson, 2003), and is known as attractant for honeybees (Henning *et al*., 1992).

Diurnal changes in floral scent and adaptation to pollinators

A vastly higher total amount of floral scent was emitted during the day than at night. A strong correlation between floral scent emission and temperature (Figure 5) was found. Similar circadian rhythms have been reported in other plants (see e.g. Matile & Altenburger, 1988; Picone *et al*., 2004), and some authors explained differences of the quantity of fragrance emission by temperature effects (Jakobsen & Olsen, 1994; Wang & Pichersky, 1998; Dudareva & Pichersky, 2000). However, in our study, contrary to total scent emission, some single floral scent compounds (e.g. lilac aldehyde isomers) were emitted in higher amounts at 8 pm when the temperature was much lower compared to 2 pm. The increased emission of

lilac aldehydes at night may be the result of an upregulation of genes, which are involved in the biosynthesis of these monoterpenoids, in the evening. Such an upregulation of genes in the late day was demonstrated for example in *Petunia hybrida* line W115 (Mitchel), a plant emitting the highest amount of benzenoids at dusk (Verdonk *et al*., 2003). The emission of high amounts of volatiles at night is typically found in plants being pollinated by nocturnal insects (Dobson, 2006). In case of *Nicotiana attenuata* (Solanaceae), night pollinating insects such as *Manduca sexta* hawkmoths could be attracted by the high nocturnal emission of the compound benzylacetone (Kessler & Baldwin, 2006). Huber *et al*. (2005) showed that phenylaceataldehyde in *Gymnadenia odoratissima* (Orchidaceae) was emitted in higher relative amounts during night and attracted effectively nocturnal moths. Our data likewise suggest that the isomers of lilac aldehyde, which were emitted at 8 pm in higher relative as well as total amounts than at 2 pm, represent an adaptation for attraction of nocturnal moths, particularly *Orthosia* species which visit *S. caprea* flowers in highest numbers at the time of highest lilac aldehyde emission.

A two-choice bioassay with *Orthosia gothica* verified that noctuid moths preferred lilac aldehydes over 1,4-dimethoxybenzene (Figure 4), although moths were able to detect both volatiles (Figure 2). Thus lilac aldehyde seems to be an important key attractant for noctuid moths in the floral scent of *Salix caprea*, as there was previous evidence for *Hadena* and *Autographa*, now *Orthosia* (Plepys *et al*., 2002a, b).

The compound 1,4-dimethoxybenzene is emitted in much higher amounts during day-time (see Figure 5). Furthermore, 1,4-dimethoxybenzene is most effective in eliciting antennal signals in bees (see this study; Dötterl *et al*., 2005b). The role of this compound in honeybee attraction was verified in a bioassay where 80 % of *Apis mellifera* individuals flew to 1,4-dimethoxybenzene and only 20 % flew to lilac aldehyde. Although honeybees could detect both compounds, they strongly preferred 1,4-dimethoxybenzene. Thus, the high total amount of emitted 1,4-dimethoxybenzene during daytime and the investigated behavior of honeybees indicate an adaptation of *S. caprea* for bees as the presumably most important diurnal pollinators.

Pollination System

Results of floral scent analyses and behavioral tests are pointing towards an adaptation of *S. caprea* to bees during day, and moths during night. However, the pollination experiment revealed that mainly day-active visitors contributed to the reproductive success, while the

contribution of night-active visitors was relatively low (Figure 6). There was no difference between the wind and the night pollination treatment. However, in the wind pollination treatment we did not discriminate between day and night, and it may be that the pollination success in the night pollination treatment was primarily the result of insects visits, and not that of wind. The wind speed at night is generally lower than the wind speed during day-time (Foken, 2006), and therefore, wind pollination may be more important during day than during night. Further, the humidity at night is generally higher compared to the humidity during day-time. As pollen may be better drifted by wind when humidity is low (and pollen grains are isolated and airy), wind as pollen vector may be more important during day-time. Therefore, we assume that the pollination success found after flower exposure at night was primarily the result of insect pollinators and not that of wind. In conclusion besides day-active visitors also night-active visitors seem to contribute to the reproductive success of *S. caprea*. Further experiments could highlight the contribution of wind pollination during day and night, and the role of moths in pollination of *S. caprea*.

We found a positive correlation between number of flower visitors and seed yields. Diurnal flower visitors were most important for reproductive success of the plant. These results correspond to other studies where both nocturnal and diurnal potential pollinators have been found visiting flowers of the same plant species and where diurnal pollinators have been found to be more abundant than nocturnal ones, resulting in higher visitation rates leading to greater seed yields (Jennersten, 1988; Jennersten & Morse, 1991; Altizer *et al*., 1998; Miyake *et al*., 1998; Balmford *et al*., 2006). However, we also found that diurnal pollinators did not achieve maximal seed set, and that for maximal reproductive success additionally wind and/or nocturnal pollination were necessary.

In our study the effect of wind on pollination success was quite low compared to other studies on willows. Vroege and Stelleman (1990) reported that the ratio of insect to wind pollination of *Salix caprea* is up to 50 %, and Karrenberg *et al*. (2002) found that wind is responsible for 70-90 % of pollination success in this species. Differing geographic origin might explain the variability found between insect- and wind pollination of *S. caprea* (Karrenberg *et al*. (2002): Italy and Vroege und Stelleman (1990): Netherlands).

The pollination system of *Salix* is generally regarded as a generalized pollination system, with a diverse array of insect pollinators as well as wind as an additional ancestral pollen vector. Most willow species flower early in the season and encounter unpredictable weather conditions and varying insect populations. An open and generalized pollination system might

be a strategy to ensure reproductive success under such unstable conditions (see Douglas, 1997; Peeters & Totland, 1999; Tamura & Kudo, 2000). The actual prediction from Ollerton (1996) and Waser *et* al. (1996) is that selection should prevent small adaptation to specific visitors. The idea of Tollsten *et al*. (1994) with *Angelica* was that generalized flowers could still have nested signals for specific visitors, although they did not partition the temporal component. In our study we found a cryptic phenotype of temporal odour change, which has not been considered by Aigner (2001, 2006) in their arguments about generalized flower forms. The plasticity and flexibility of floral scent emissions, which is exceptional compared to other floral traits such as color and shape, makes this possible. During day-time floral scent is dominated by a compound (1,4-dimethoxybenzene) that is highly attractive for bees, and at night a scent with a higher relative amount of a moth attractant (lilac aldehyde) is emitted.

It seems that the changes in the floral scent emission of *S. caprea* reflecting the selection by the two different functional groups (bees and moths) that are both effective pollinators. The generalist *S. caprea* is an interesting example of Aigner's (2006) view that floral characteristics may represent adaptations to pollinators that are neither most numerous nor most effective, but which provide an additional marginal fitness gain. Aigner (2006) theories that “we should be prepared to find adaptations to relatively uncommon or ineffective floral visitors when there is no sacrifice in the ability to use more common and effective ones”. This is a view that differs from Stebbins’ (1970) position that “the characteristics of flowers will be moulded by those pollinators that visit it most frequently and effectively”.

In case of *S. caprea* the flower, although an interaction generalist, is fine-tuned for attracting different pollinator types. This is possible by temporal changes in the scent emission patterns that are synchronized with the activity times of the most important types of pollinators. It seems that sallow plants that produce compounds attractive for moths, that are neither the most numerous nor most effective pollinators, can receive a marginal fitness gain. It gets even more complicated if we include the wind as effective pollinating agent. Altogether, this example is challenging the existing concepts of specialization/generalization of plant-pollinator interactions. Regarding the aspect of interactions, *S. caprea* is a generalist, but looking at the aspect of adaptations, *S. caprea* can be regarded as a multi-specialist.

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Table legends:

Table 1: Diurnal and nocturnal flower visitors observed on catkins of *Salix caprea* in 2005 (three females and three males) and 2006 (five females and five males).

Table 2: All compounds emitted by two flower scent samples of *Salix caprea* and compounds eliciting antennal responses (x = 30) in GC-EADs in female (♀) and male (♂) bees and moths. Bees: *An. fla = Andrena flavipes*; *An. pra = Andrena praecox*; *An. vag = Andrena vaga*; *An. hae = Andrena haemorrhoa; Ap. mel = Apis mellifera*; *C. cun = Colletes cunicularius*; Moths: *Ag. mar = Agriopis marginaria*; *O. got = Orthosia gothica*; *O. mun = Orthosia munda*; *O .cer = Orthosia cerasi*; *O. inc = Orthosia incerta*; *P. fla = Panolis flammea*; *T. rup = Theria rupicapraria.*

Table 1:

Diurnal flower visitors	Nocturnal flower visitors
Andrenidae	**Chimabachidae**
Andrena bicolor Fabricius 1775**	*Diurnea fagella* ([Denis & Schiffermüller], 1775)*
Andrena cf. *fucosa* (Erichson 1835)*	**Depressariidae**
Andrena cineraria (Linnaeus 1758)*	*Agonopteryx arenella* ([Denis & Schiffermüller], 1775)*
Andrena clarkella (Kirby 1802)***	**Endromidae**
Andrena dorsata (Kirby 1802)*	*Endromis versicolora* (Linnaeus 1758)*
[a]*Andrena flavipes* Panzer 1799*	**Geometridae**
Andrena fulva (Müller 1766)*	[a]*Agriopis marginaria* (Fabricius 1776)*
[a]*Andrena haemorrhoa* (Fabricius 1781)**	*Lycia hirtaria* (Clerck 1759)*
Andrena minutula (Kirby 1802)**	*Selenia dentaria* (Fabricius 1775)*
Andrena nitida (Müller 1776)*	[a]*Theria rupicapraria* ([Denis & Schiffermüller], 1775)*
[a]*Andrena praecox* (Scopoli 1763)****	**Noctuidae**
Andrena ruficrus Nylander 1848***	*Cerastis leucographa* ([Denis & Schiffermüller], 1775)*
Andrena subopaca Nylander 1848*	*Conistra rubiginea* ([Denis & Schiffermüller], 1775)*
[a]*Andrena vaga* Panzer 1799**	*Conistra vaccinii* (Linnaeus 1761)***
Apidae	*Eupsilia transvera* (Hufnagel 1766)*
[a]*Apis mellifera* Linnaeus 1758****	*Lithophane furcifera* (Hufnagel 1766)*
Bombus sp. ***	*Lithophane ornitopus* (Hufnagel 1766)*
Bombus terrestris (Linnaeus 1758)***	*Lithophane socia* (Hufnagel 1766)*
Normada cf. *flava* Panzer 1798*	[a]*Orthosia cerasi* (Fabricius 1775)***
Colletidae	*Orthosia cruda* (Fabricius 1775)**
[a]*Colletes cunicularius* (Linnaeus 1758)*	[a]*Orthosia gothica* (Linnaeus 1758)****
Halictidae	*Orthosia gracilis* ([Denis & Schiffermüller], 1775)**
Lassioglossum pauxillum (Schenck 1853)*	[a]*Orthosia incerta* (Hufnagel 1766)***
Nymphalidae	*Orthosia miniosa* ([Denis & Schiffermüller], 1775)*
Nymphalis urticae (Linnaeus 1758)**	[a]*Orthosia munda* ([Denis & Schiffermüller], 1775)*
	[a]*Panolis flammea* ([Denis & Schiffermüller], 1775)*
	Nolidae
	Nycteola revayana (Scopoli 1772)**
	Ypsolophidae
	Ypsolopho ustella (Clerck 1759)*

* 1-5, **5-10, ***11-20, **** more than 21 visits of a species to *Salix caprea*

[a]species which were used for GC-EAD

Table 2:

		S. caprea [a]								*S. caprea* [b]									
		Diurnal					Nocturnal			Diurnal						Nocturnal			
	Relative amount	Andrenidae					Geometridae	Noctuidae		Andrenidae			Apidae		Colletidae	Geometridae	Noctuidae		
		An .fla ♀	*An .fla* ♂	*An. pra* ♀	*An. vag* ♀	*An .vag* ♂	*Ag. mar* ♂	*O. got* ♂	*O. mun.* ♂	*An. hae* ♀	*An. pra* ♀	*An. pra* ♀	*Ap. mel* ♀	*Ap. mel* ♀	*C. cun* ♀	*T. rup* ♂	*O. cer* ♂	*O. inc* ♀	*P. fla*[c]
Aromatics																			
Benzaldehyde	**																		
Benzyl alcohol[d]	**										x	x							x
Salicylaldehyde[d]	*									x	x	x					x	x	
2-Phenylethylmethylether[d]	*		x			x		x	x										
2-Phenylethanol[d]	*	x			x	x				x	x	x	x	x	x		x	x	x
1,4-Dimethoxybenzene[d]	****	x	x	x	x	x		x	x	x	x	x	x	x	x		x	x	x
Methyl salicylate[d]	****	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Indole[d]	**	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
4-Methoxyacetophenon[d]	+									x	x	x			x			x	x
Eugenol[d]	+		x	x	x	x	x	x	x										
Monoterpenoids																			
α-Phellandrene	*																		
α-Pinene	**																		
β-Pinene	**																		
β-Phellandrene	*																		
D-Limonene	*																		
(*E*)-Ocimene[d]	**	x	x	x	x	x		x	x										
Linalool[d]	**						x	x	x				x	x	x	x	x	x	x
(E)-Ocimene oxide[d]	*							x	x										
Lilac aldehyde B+C[d]	**							x	x					x	x				
Lilac aldehyde D[d]	*															x	x		
Lilac alcohol A	*																		
Lilac alcohol B+C[d]	*										x	x			x	x	x	x	
Lilac alcohol D[d]	*														x				
Sesquiterpenoids																			
Germacrene D[d]	**				x			x											
(*E,E*)-α-Farnesene[d]	*	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x		x
Nerolidol[d]	*				x														
Homoterpenoids																			
(*E*)-4,8-Dimethyl-1,3,7-nonatriene[d]	*		x	x	x	x	x	x	x										
(*E,E*)-4,8,12-Trimethyltrideca-1,3,7,11-tetraene[d]	*						x	x											
Coeluting compounds																			
Benzylnitrile/Lilacaldehyde A /4-Oxoisophorone	**			x	x	x				x	x	x	x	x	x	x	x	x	x
α-Copaene/Jasmone[d]	*		x			x		x	x	x	x	x				x			x
Phenylethylacetate/ *p*-Anisaldehyde[d]	+									x	x	x			x				
Unknowns																			
Unknowns[d]		x[2e]	x[4]	x[4]	x[4]	x[5]	x[3]	x[5]	x[2]	x[4]	x[3]	x[3]			x[5]	x[1]	x[2]	x[2]	x[1]

[a] flower scent was collected from cut flowering stems

[b] flower scent was collected from another individual plant *in situ*

[c] sex was not determined

[d] electrophysiologically active compounds

[e] unknown compounds were pooled with the superscript digit indicating the number of pooled compounds

* < 1 %; ** 1-5 %; *** 5-10 %; **** > 10 % relative amount of the single compound

Figure legends

Figure 1. Mean number of flower visits (type and number of observed flower visitor individuals per type) of *Salix caprea* ($n = 7$) per 15 minutes in the course of a day ($n = 6$).

Figure 2. Coupled gas chromatographic and electroantennographic detection (GC-EAD) of a *Salix caprea* (male) flower scent sample (collected *in situ*) using antennae of different diurnal (bees) and nocturnal (moths) flower visitors of *Salix caprea*. **1**: Benzyl alcohol, **2**: Salicylaldehyde, **3**: Linalool, **4**: 2-Phenylethanol, **5**: Benzyl nitrile/Lilac aldehyde A/4-Oxoisophorone, **6**: Lilac aldehyde B+C, **7**: 1,4-Dimethoxybenzene, **8**: Lilac aldehyde D, **9**: Methyl salicylate, **10**: Lilac alcohol, **11**, **12**: unknowns, **13**: Lilac alcohol, **14**: Phenylethyl acetate/*p*-Anisaldehyde, **15**: unknown, **16**: Indole, **17**: unknown, **18**: 4-Methoxyacetophenon, **19**: unknown, **20**: α-Copaene/Jasmone, **21**: unknown, **22**: (*E,E*)-α-Farnesene.

Figure 3. Multidimensional scaling of the antennal response patterns of female (♀) and male (♂) diurnal and nocturnal flower visitors of *Salix caprea* to the floral scent samples a (*cut*), and b (*in situ*) based on Bray-Curtis similarities. For calculation of the similarity matrix relative responses (in %) were used. Stress = 0.09. Abbreviations: *An. fla = Andrena flavipes*; *An. pra = A. praecox*; *An. vag = A. vaga*; *Ag. mar = Agriopis marginaria*; *O. got = Orthosia gothica*; *O. mun = O. mundi*; *An. hae = A. haemorrhoa*; *Ap. mel = Apis mellifera*; *C. cun = Colletes cunicularius*; *T. rup = Theria rupicapraria*; *O. cer = O. cerasi*; *O. inc = O. incerta*; *P. fla = Panolis flammea.*

Figure 4. Attraction of *Apis mellifera* ($N = 101$) and *Orthosia gothica* ($N = 18$) to 1,4-dimethoxybenzene (black) and lilac aldehyde (grey).

Figure 5. Circadian emission of floral scent of seven *Salix caprea* specimens (mean ± SE, $n = 7$) and air temperature during scent collection (mean ± SE, $n = 2$ days).

Figure 6. Seeds per catkin and seeds per capsule of *Salix caprea* ($n = 5$) resulting from different pollination treatments (night-, day- and wind pollination; means ± SE) displayed as percentages in relation to open pollination (control). Capital letters indicate significant differences of seeds per capsules between pollination regimes (LSD test: $p < 0.001$) and small letters declare significant differences of seeds per catkin between pollination regimes (LSD test: $p < 0.001$).

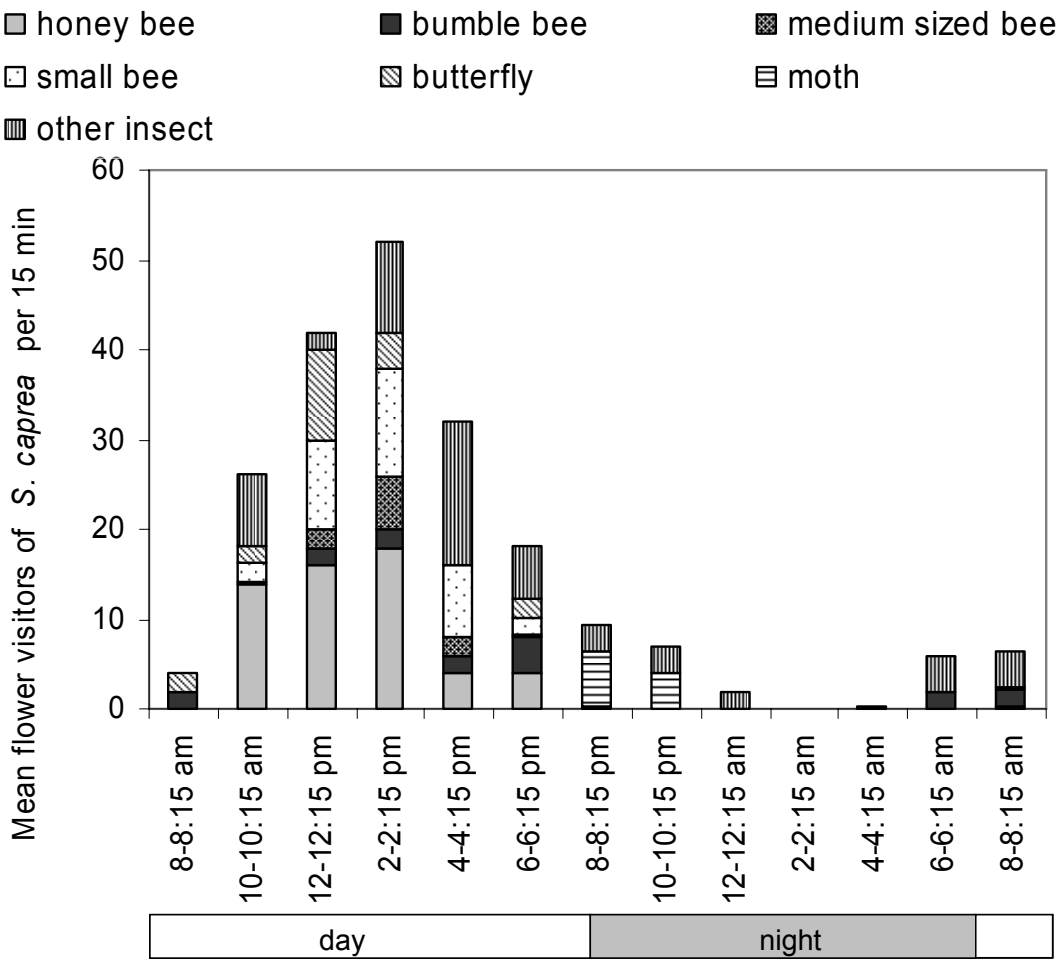
honey bee
bumble bee
medium sized bee
small bee
butterfly
moth
other insect
Mean flower visitors of S. caprea per 15 min
60
50
40
30
20
10
0
8-8:15 am
10-10:15 am
12-12:15 pm
2-2:15 pm
4-4:15 pm
6-6:15 pm
8-8:15 pm
10-10:15 pm
12-12:15 am
2-2:15 am
4-4:15 am
6-6:15 am
8-8:15 am
day
night

Fig 1

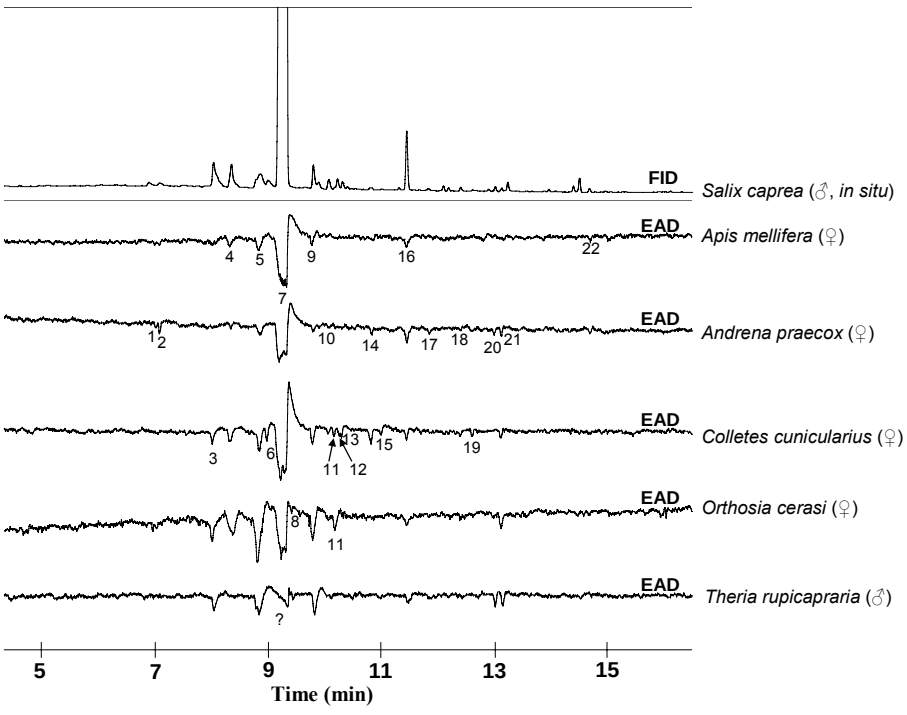
FID
Salix caprea (♂, in situ)
EAD
Apis mellifera (♀)
Andrena praecox (♀)
Colletes cunicularius (♀)
Orthosia cerasi (♀)
Theria rupicapraria (♂)
5
7
9
11
13
15
Time (min)

Fig 2

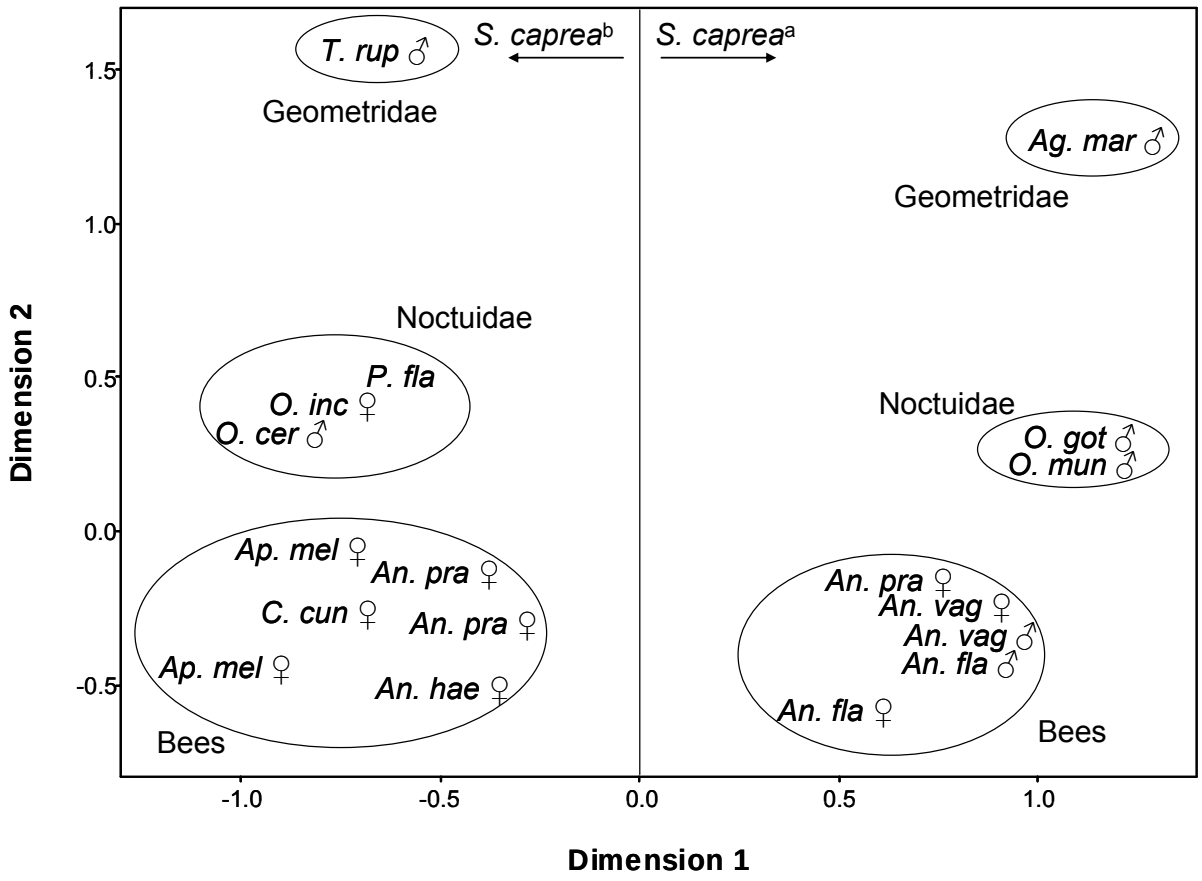
T. rup ♂
S. caprea^b
S. caprea^a
Geometridae
Ag. mar ♂
Geometridae
Noctuidae
P. fla
O. inc ♀
O. cer ♂
Noctuidae
O. got ♂
O. mun ♂
Ap. mel ♀
An. pra ♀
C. cun ♀
An. pra ♀
Ap. mel ♀
An. hae ♀
Bees
An. pra ♀
An. vag ♀
An. vag ♂
An. fla ♂
An. fla ♀
Bees
Dimension 2
Dimension 1
1.5
1.0
0.5
0.0
-0.5
-1.0
-0.5
0.0
0.5
1.0

Fig 3

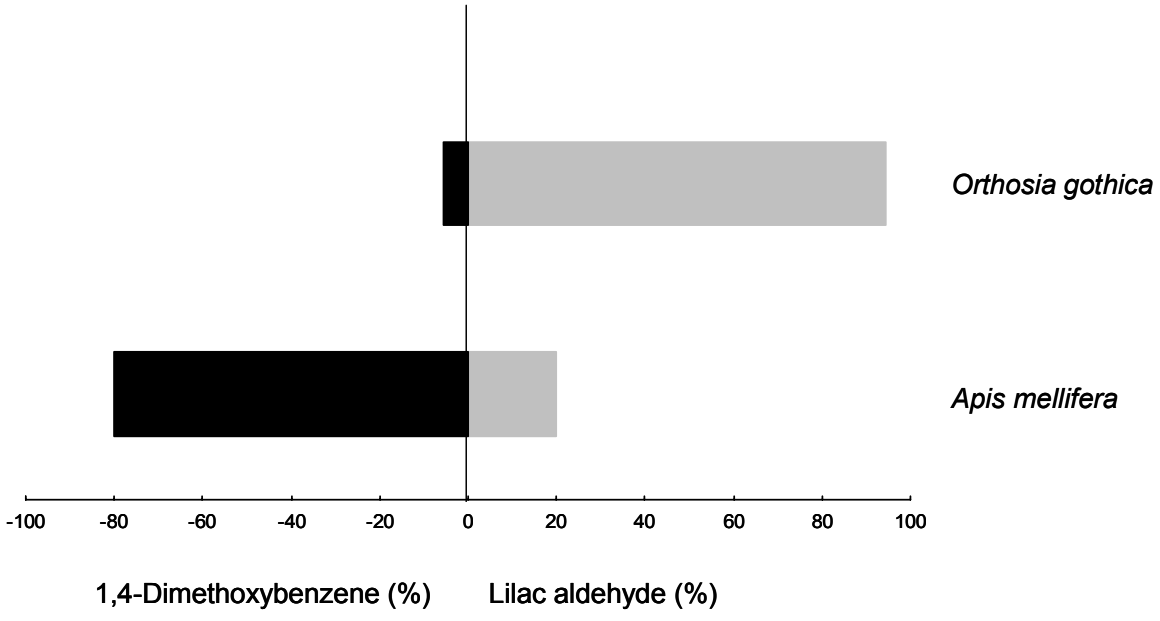
Orthosia gothica
Apis mellifera
-100
-80
-60
-40
-20
0
20
40
60
80
100
1,4-Dimethoxybenzene (%)
Lilac aldehyde (%)

Fig 4

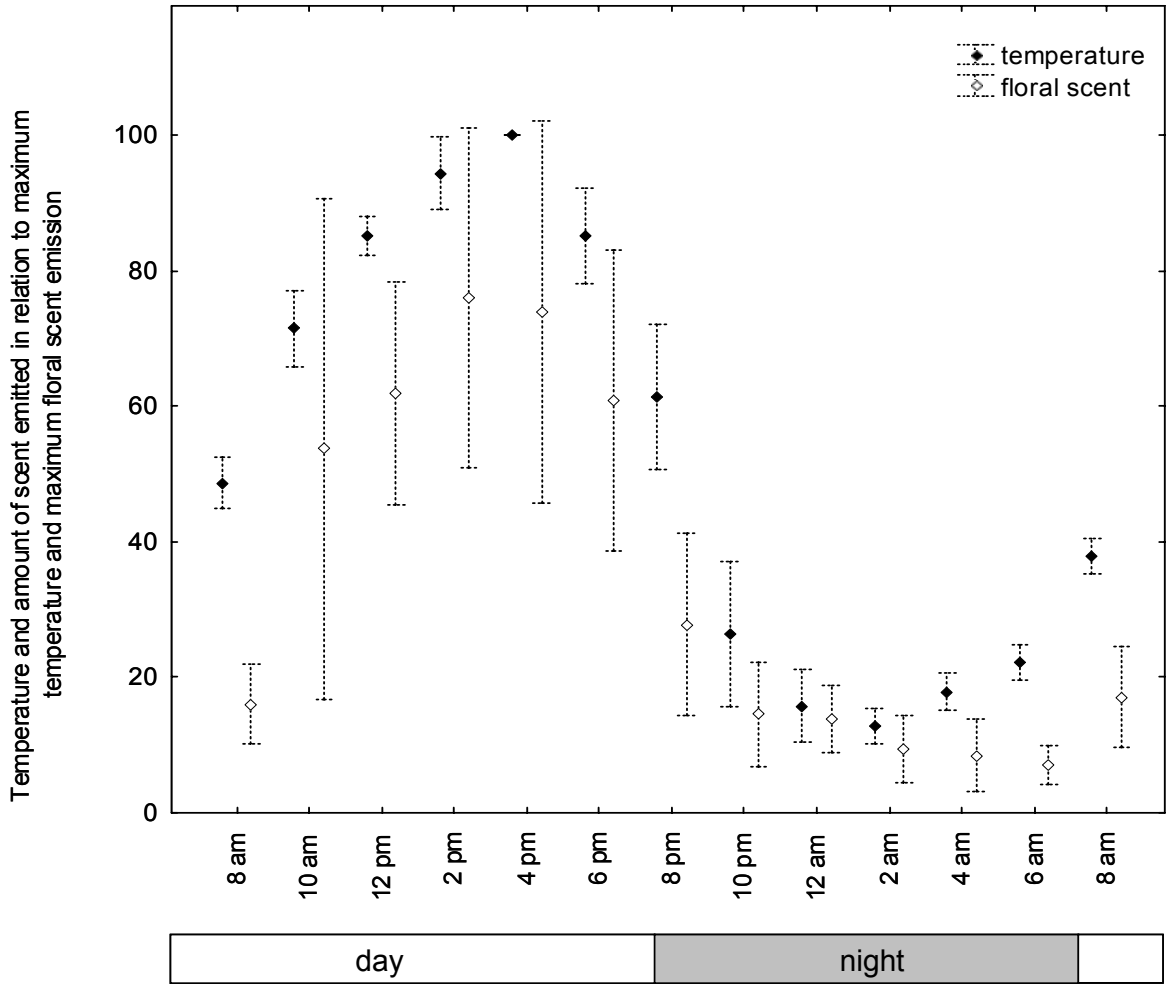
temperature
floral scent
Temperature and amount of scent emitted in relation to maximum temperature and maximum floral scent emission
0
20
40
60
80
100
8 am
10 am
12 pm
2 pm
4 pm
6 pm
8 pm
10 pm
12 am
2 am
4 am
6 am
8 am
day
night

Fig 5

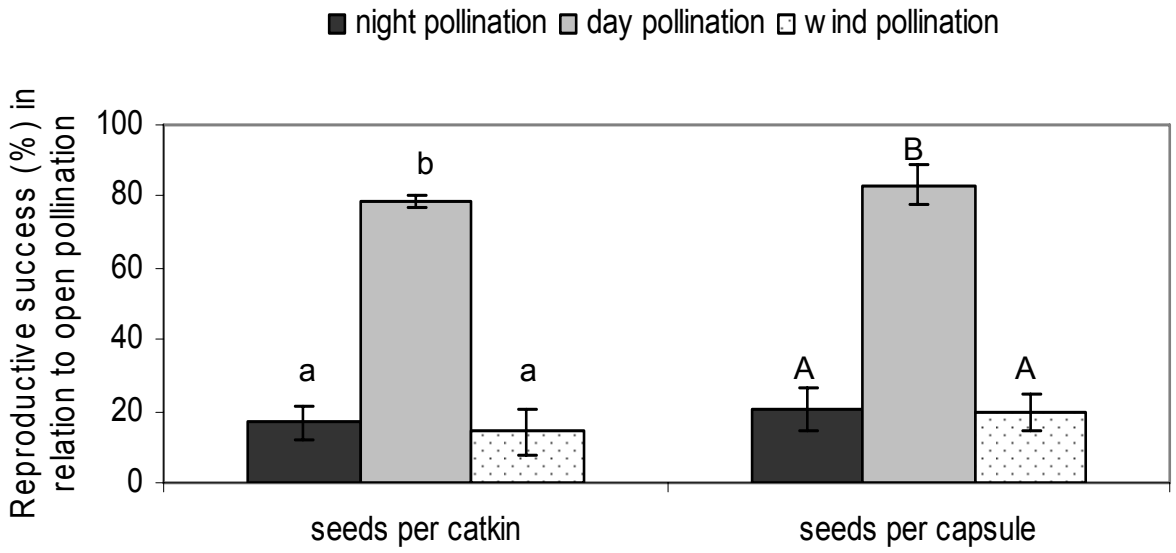
night pollination
day pollination
w ind pollination
Reproductive success (%) in relation to open pollination
0
20
40
60
80
100
a
b
a
A
B
A
seeds per catkin
seeds per capsule

Fig 6

3 1,4-Dimethoxybenzene, a Floral Scent Compound in Willows that Attracts an Oligolectic Bee

Rapid Communication

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Abstract

Many bees are oligolectic and collect pollen for their larvae only from one particular plant family or genus. Here, we identified flower scent compounds of two *Salix* species important for the attraction of the oligolectic bee *Andrena vaga*, which collects pollen only from *Salix*. Flower scent was collected using dynamic-headspace methods from *Salix caprea* and *S. atrocinerea*, and the samples were subsequently analyzed by coupled gas chromatographic-electroantennographic detection (GC-EAD) to detect possible attractants of *A. vaga*. EAD active compounds were identified by gas chromatography coupled to mass spectrometry. Both *Salix* species had relatively similar scent profiles, and the antennae of male and female bees responded to at least 16 compounds, among them different benzenoids as well as oxygenated monoterpenoids and sesquiterpenoids. The strongest antennal responses were triggered by 1,4-dimethoxybenzene, and in field bioassays, this benzenoid attracted females of *A. vaga* at the beginning of its flight period, but not at the end.

Key Words—Floral scent, *Salix*, willows, GC-EAD, oligolectic bees, *Andrena*, GC-MS, flower visitor attraction.

Introduction

Bees are important pollinators of flowering plants. They visit flowers primarily to take nectar for their food and to collect nectar/oil and pollen for their larvae. Many of the about 30,000 bee species worldwide are oligolectic and collect pollen for the larvae only from a particular plant family or genus. So far, little is known about the cues used by the oligolectic bees to find host plants. However, it has been shown that naive bees of oligolectic *Chelostoma florisomne* (L.) rely on flower and especially pollen odors to recognize host plants (Dobson and Bergström, 2000), and floral scent compounds generally may be important for host plant finding by oligolectic bees.

Salix L. is a genus that hosts many different oligolectic bee species, probably because of its readily accessible pollen (Michener, 2000). *Salix* is a woody genus, distributed almost worldwide, but centered in the northern hemisphere (Newsholme, 1992). Plants are dioecious, and insects are important pollen vectors (e.g., Karrenberg et al., 2002). The often strongly scented flowers are borne in catkins. The scent is assumed to attract the pollinators (Tollsten and Knudsen, 1992). In Europe, several species of the genus *Andrena* are specialized on *Salix*, among them *Andrena vaga* Pz. (Westrich, 1989).

Here, we used coupled gas chromatography and electroantennography (GC-EAD) to elucidate the floral scent compounds of *Salix caprea* L. and *S. atrocinerea* Brot. that elicit signals in the antennae of female and male *A. vaga*. The compound that elicited the main signal in the antennae of bees was further tested for attraction in a field bioassay.

Methods and Materials

Plant Material and Volatile Collection. Floral scent was collected from male plants of *S. caprea* and *S. atrocinerea* in the Ecological-Botanical Garden of the University of Bayreuth. For each sample, floral scent was collected from 30 to 40 catkins during daytime for 8 hr by using dynamic headspace methods. Flowering branches were cut in the field and placed in water in the laboratory for immediate scent collections (compare with Tollsten and Knudsen, 1992).

Flowering branches were enclosed in an oven bag (Nalophan), and the emitted volatiles were trapped in an adsorbent tube filled with 20 mg of a 1:1 mixture of Tenax-TA 60-80 and Carbotrap 20-40. The air was sucked from the bag over the adsorbent by a membrane pump (G12/01 EB, Rietschle Thomas, Puchheim, Germany). Volatiles were eluted with 80 µl of

acetone. (SupraSolv, Merck KgaA, Germany) to obtain odor samples for the chemical and electrophysiological analyses (see below).

Electrophysiology. Electrophysiological analyses of the floral scent extracts were performed with a GC-EAD system. Antennae from two females and two males of *A. vaga* were tested. The bees were caught on the 4th and the 21st of April at a nesting site in the Ecological-Botanical Garden. The GC-EAD system consisted of a gas chromatograph (Vega 6000 Series 2, Carlo Erba, Rodano, Italy) equipped with a flame ionization detector (FID) and an EAD setup [heated transfer line, two-channel universal serial bus (USB) acquisition controller] provided by Syntech (Hilversum, Netherlands). One microliter of an odor sample was injected splitless at 60°C, followed by opening the split vent after 1 min and heating the oven at a rate of 10°C/min to 200°C. The end temperature was held for 5 min. A ZB-5 column was used for the analyses (length 30 m, inner diam 0.32 mm, film thickness 0.25 µm, Phenomenex). The column was split at the end by the four-arm flow splitter GRAPHPACK 3D/2 (Gerstel, Mülheim, Germany) into two pieces of deactivated capillary (length 50 cm, ID 0.32 mm) leading to the FID and to the EAD setup. Makeup gas (He, 16 ml/min) was introduced through the fourth arm of the splitter. For measurements, an excised antenna was mounted between glass micropipette electrodes filled with insect ringer (8.0 g/l NaCl, 0.4 g/l KCl, 0.4 g/l $CaCl_2$). The electrodes were connected to silver wires.

Chemical Analyses. To identify the EAD active compounds, 1 µl of the scent samples was analyzed on a Varian Saturn 2000 mass spectrometer and a Varian 3800 gas chromatograph fitted with a 1079 injector (Varian Inc., Palo Alto, CA, USA). Column and settings were as described in Dötterl et al. (2005). Component identification was carried out using the NIST 02 mass spectral database, or MassFinder 3, and was confirmed by comparison with retention times of authentic standards.

Behavioral Experiment. To test the attractiveness of 1,4-dimethoxybenzene, which elicited the main signal in the antennae of the bees, a two-choice bioassay was conducted in spring 2005 in the Ecological-Botanical Garden near the nesting site of *A. vaga*. One rubber GC septum impregnated with 10 µl of 1,4-dimethoxybenzene (99%, Aldrich) and one blank rubber GC septum were presented on a stand (distance of the septa, 1 m) around noon for 20 min, when activity of bees was high. The reaction of bees was classified as “zigzagging” when the bees flew upwind towards one of the septa up to within 10 cm and as “landing” when the bees had contact with a septum.

Results and Discussion

Floral odors of *S. caprea* and *S. atrocinerea* elicited clear signals in the antennae of both sexes of *A. vaga* (Figure 1). At least 16 EAD active compounds were found, of which 11 were present in both *Salix* species. The antennae of the bees responded especially to different benzenoids and isoprenoids, and 1,4-dimethoxybenzene consistently elicited the main antennal response. Clear signals were also triggered by different monoterpene oxides, by the nitrogen-bearing compound indole, and by the sesquiterpene (*E,E*)-α-farnesene. The antennal responses of female and male bees were similar; however, female bees were more strongly tuned to oxygenated sesquiterpenes such as (*E*)-nerolidol.

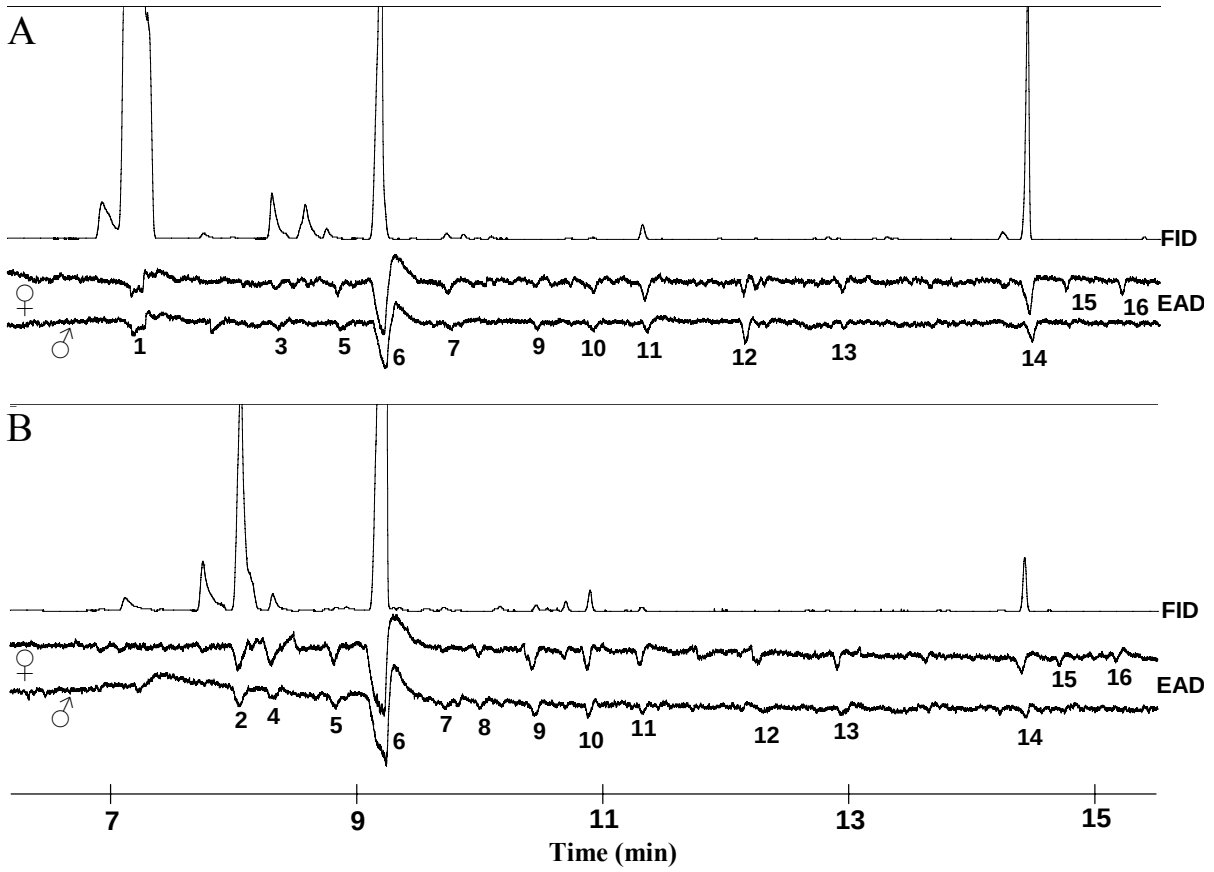

FIG. 1. Coupled gas chromatographic and electroantennographic detection (GC-EAD) of *Salix caprea* (A) and *S. atrocinerea* (B) flower scent samples using antennae of *Andrena vaga* males and females. **1**: (*E*)-*β*-ocimene; **2**: linalool; **3**: 4,8-dimethyl-1,3,7-nonatriene; **4**: 2-phenylethanol; **5**: 4-oxoisophorone/benzyl nitrile; **6**: 1,4-dimethoxybenzene, **7**: methyl salicylate; **8**: lilac alcohol isomer; **9**,**10**: monoterpene oxides; **11**: indole; **12**: eugenol; **13**: sesquiterpene; **14**: (*E,E*)-α-farnesene; **15**: sesquiterpene oxide; **16**: (*E*)-nerolidol.

TABLE 1. NUMBER OF FEMALES OF *Andrena vaga* ATTRACTED TO A SEPTUM IMPREGNATED WITH 1,4-DIMETHOXYBENZENE (DMB) AND TO A BLANK SEPTUM (CONTROL)

	"Ziggzagging"		"Landing"	
Date	DMB	Control	DMB	Control
5 April	6	0	0	0
11 April	7	0	3	0
13 April	4	0	1	0
14 April	4	0	0	0
26 April	0	0	0	0
6 May	0	0	0	0

The bioassay proved that 1,4-dimethoxybenzene not only elicits antennal responses but also mediates behavioral responses (Table 1), at least in female bees. They flew upwind towards the septum impregnated with this benzenoid, and some landed on it for a short period of time. After landing, they did not show the nectar drinking or pollen collecting behavior, which can be observed on *Salix* catkins.

However, in the first 2 weeks of April only, at the beginning of their flight season, females were consistently attracted to 1,4-dimethoxybenzene. End of April/beginning of May bees were not attracted at all. It seems that only freshly hatched, foraging-naive bees were attracted. This observation is consistent with the results of Dobson and Bergström (2000). They found that oligolectic *C. florisomne* relied on protoanemonin, the dominant pollen odor of its host plants (*Ranunculus*), only if the bees were foraging-naive, whereas foraging-experienced bees recognized their host plants on the complex volatile blend of a whole flower, which first has to be learned.

Not a single male of *A. vaga* was attracted by 1,4-dimethoxybenzene, although males also strongly responded to this substance in the GC-EAD study, and males were regularly observed on *Salix* species, probably drinking nectar. Because males of *A. vaga* hatch 2-3 weeks earlier than females (Westrich, 1989), we cannot rule out that they were already foraging-experienced when bioassays were conducted. Possibly, foraging-experienced bees have learned to recognize complex flower scents and do not rely on their innate preferences for single scent compounds (see also Dobson and Bergström, 2000).

1,4-Dimethoxybenzene is found in floral scents of all *Salix* species so far studied (this study and Tollsten and Knudsen, 1992). Furthermore, it is a common compound in orchids pollinated by perfume-collecting euglossine bees (Williams and Whitten, 1983; Gerlach and Schill, 1991), where it is suspected to be a good bee attractant (Williams and Whitten, 1983).

1,4-Dimethoxybenzene is also known to attract the chrysomelid beetle *Diabrotica speciosa* (Ger.) (Ventura et al., 2000).

To summarize, our results demonstrate that floral scent compounds of *Salix* can act as cues for the attraction of oligolectic bees, and that 1,4-dimethoxybenzene attracts female *A. vaga* bees. In oligolectic bees, males often search for females at the host plants; therefore, floral scent could be used by males in combination with female sex pheromones to find females. This is the first study analyzing antennal and behavioral responses of an oligolectic bee to the floral scent of its host plants.

Acknowledgments—Parts of the study were supportet by the German Research Foundation (Research Training Group 678). The GC for the electrophysiological study was provided by Konrad Dettner, and Andrea Beran provided advice on this system. Friderike Beyer and Irmgard Schäffler helped with biotests. Taina Witt and two anonymous referees gave valuable comments on the manuscript.

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4 Floral Reward and Advertisement in Dioecious *Salix caprea*

Running head: Attractiveness of male and female *S. caprea*

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Key words *Apis mellifera*, dioecy, olfactory signal, visual signal, nectar, pollen, *Salix caprea*

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Plant Biology

Abstract

The attraction of the honeybee (*Apis mellifera*, Hymenoptera) to olfactory and/or visual stimuli of dioecious *Salix caprea* (Sallow, Salicaceae) inflorescences was investigated in two-choice experiments. We showed that in *S. caprea* olfactory cues were stronger attractants than visual signals, however, honeybees were most responsive to a combination of both attractants. Male *Salix caprea* inflorescences were more attractive than female inflorescences. The higher visitation rate in male plants is less due to sex-specific olfactory stimuli than to the strong signalling effect of the yellow colour. The differing attractiveness of male and female plants might act as a guiding necessary to load pollinators first with sufficient pollen to ensure successful pollen transfer to female pollen receptors thereafter.

Introduction

Worldwide only 6 % of flowering plant species exhibit the separation of sexes on male and female plants, a phenomenon called dioecy (Renner and Ricklefs, 1995; Richard, 1997). Most likely dioecious plant species are so rare, because the risk for reproductive failure is relatively high due to their complete dependence on pollinating agents. As a consequence of the separation of male and female function, flower morphology of male and female flowers differs in their reproductive organs, which may have severe consequences for pollinator attraction. E.g., male flowers may often be more attractive to pollinators because they offer both nectar and pollen – according to Dafni (2005) the main rewards of flowers for visiting animals – whereas female flowers offer only nectar (Bawa, 1983; Lloyd and Bawa, 1984; Mayer and Charlesworth, 1991). Spatial separation of sexes in connection with differences in reproductive demands between the sexes of dioecious plants have often led to divergence between sexes, e.g. with respect to physiology (Laporte and Delph, 1996), flower size (Vaugthon and Ramsey, 1998), and floral advertisement and reward (Percival, 1961; Goukon et al., 1976; Kay et al., 1984; Nepi et al., 1996; Shykoff, 1997). As summarised by Costich and Meagher (2001), the evolution of sexual dimorphism, and the special characteristics of gender specialisation in plants can be explained through reproductive compensation (enhanced reproductive efficiency with gender specialisation), Bateman's Principle (sex-specific selection), and intersexual floral mimicry (mimicry of a reward-providing gender by a non-reward providing gender). Differences between males and females might cause the result of different flower visitor spectra as well as differing visitation rates at the two genders (e.g. Collison and Martin, 1979; Kay et al., 1984; Ashman, 2000). In various plant species it is known that the visitation frequency of female flowers is lower than that of males (Bawa, 1980a; Bierzychudek, 1987; Charlesworth, 1993; Vaughton and Ramsey, 1998). It was often hypothesised that this is of no disadvantage, because the male function (pollen dispersal) needs higher visitation rates than the female function (pollen receipt) to be accomplished (e.g. Harder and Wilson, 1994; Wilson et al., 1994; Delph and Ashman, 2006; Blair and Wolfe, 2007). However, if that is not the case, low visitation rates of female flowers could reduce reproductive success, because for successful pollination it is essential that sufficient pollen is transferred from male to female flowers (Bawa and Opler, 1975; Bawa, 1980b; Renner and Ricklefs, 1995; Howe and Westley, 1997). So it is not surprising, that in certain cases, female pollen-lacking non-food rewarding flowers mimick food-rewarding pollen-offering male flowers to enhance their visitation rate and reproductive success (Bawa, 1980b; Ågren et al.,

1986; Armstrong, 1997). Alternatively, in some plant species females and males offer different, but complementary essential resources for pollinators (Baker and Baker, 1983).

It is usually assumed that flower visitors use similar cues (floral signals) to obtain rewards from female and male flowers (see reviews in Chittka and Thomson, 2001). For example, floral scent composition in diclinous plants, e.g. dioecious species, generally show high similarity between male and female flowers, indicating that floral scent is an important cue guiding insects between the sexes to ensure pollination (Pham-Delegue et al., 1990; Tollsten and Knudsen, 1992; Ervik et al., 1999; Grison et al., 1999). Within the framework of a study on the pollination biology of dioecious willow species (*Salix* L., Salicaceae), we are interested in the floral gender differences of male and female willows and their effect on attraction, guidance and behaviour of its pollinators.

Salix L. (willow) is a genus of woody dioecious plants with numerous species (Fang, 1987; Skvortsov, 1999) distributed almost all over the world. Willows are mostly entomophilous with flowers arranged in catkins. Male catkins offer pollen and nectar as a reward, female inflorescences only nectar. For our investigations we chose *Salix caprea* L. (sallow), which is one of the most common *Salix* species in Central Europe. Male inflorescences are yellow due to the pollen presentation whereas female catkins are inconspicuously greenish; both sexes release a similar floral scent (Tollsten and Knudsen, 1992; Füssel et al., 2007). Insect pollination is predominant in *Salix caprea*, even though seed set by wind pollination may be up to 50 % (Vroege and Stelleman, 1990). *Salix caprea* is visited by many insect species, in particular social and solitary bees, flies, butterflies, moths and beetles (van der Werf et al., 1982; Vroege and Stelleman, 1990; Urban and Kopelke, 2004; Füssel et al., unpublished data). However, one of the most frequent visitors of *Salix caprea* is the honeybee (*Apis mellifera*). Therefore, we examined the role of olfactory and visual signals for the attraction of honeybees to male and female individuals, and determined the nectar quantity offered by both sexes. Further, floral scent, anther scent as well as nectar sugar composition were analysed by GC-MS and HPLC, respectively. The following hypothesis was tested: Due to the lack of pollen (which functions both as a reward and as a visual and olfactory attractant), female flowers of *Salix caprea* are less attractive for honeybees than male flowers. In combination with our previously published results on pollination success, and visitation rates in male and female sallow trees (Füssel et al., unpublished data), we discuss the relation between floral rewards/signals and differing visitation rates in male and female plants, and if these patterns are disadvantageous for the reproductive success of this species.

Material and Methods

Plant Material. All *Salix caprea* plants used for floral scent and nectar analyses as well as behavioural tests are located in the Ecological-Botanical Garden (EBG) Bayreuth, Germany.

Bioassay. To test the attractiveness of male and female *Salix caprea* to *Apis mellifera* a two-choice bioassay was performed. The experimental design (Fig. 1) consisted of three different test series (see point 1 to 3 below); each test series was conducted with three different arrangements (see Fig. 1-1, 1-2, 1-3):

1) Comparison of the attractiveness of different floral traits against a control: The attractiveness of olfactory and visual cues as well as both cues combined was tested separately against a negative control (Fig. 1-1).

2) Comparison of the attractiveness of different floral traits against each other: The attractiveness of floral scent vs. visual cues, floral scent and visual cues combined vs. floral scent, and visual cues vs. floral scent and visual cues combined was tested (Fig. 1-2).

3) Comparison of the attractiveness of sexes: The two genders of *Salix caprea* were compared regarding attractiveness of floral scent, visual cues, and olfactory and visual cues combined (Fig. 1-3).

Quartz glass cylinders were used to set-up the bioassays (Fig. 2). One cylinder consisted of two pieces of quartz glass (cap and body, thickness of glass: 0.3 cm) and a sleeve composed of macrolon® (thickness 0.8 cm), which connected and sealed cap and body. The macrolon® sleeve had 60 holes (diameter 0.2 cm), arranged in three horizontal lines to allow diffusion of floral scent. The cylinders were mounted with their bottoms on a PVC disc (diameter 11 cm) which was painted with a black, semi matte varnish. The disc was attached to a quadratic wooden table. A connecting element coupled the cylinder with a membrane pump (G12/01 EB, Rietschle Thomas, Puchheim, Germany).

The design of this standard cylinder construction was modified according to the requirements of the particular test series, as described below:

- A standard cylinder as described above was used for testing attraction to olfactory and visual stimuli in combination.

- A cylinder without holes was used for testing visual attraction only.

- A cylinder with holes, but totally painted black with semi matte varnish was used for testing olfactory attraction only.
- For the negative control in test series 1, we used for each arrangement the cylinder type corresponding to the cylinder loaded with willow branches.

For all three cylinder types all varnished surfaces were dried for one week at 50 °C in a drying oven to eliminate scent emission of the varnish.

Bioassays were performed during the flowering season in 2007 (from March 12th to March 30th). Flowering branches of seven male and four female plants were cut in the field and placed in the cylinders. Cut ends were wrapped in moist tissue paper and placed in polyester oven bags to prevent scent emission from damp tissues. In all arrangements of the tests series 1 and 2, four female and four male flowering branches of one plant individual (eight branches had altogether approximately 80 catkins) were enclosed together in one cylinder. In all arrangements of test series 3, either eight male or eight female branches with approximately 80 catkins each, were enclosed in different cylinders. If possible, for each arrangement and replicate (see below) of the tests, branches from different plant individuals were used.

The two-choice bioassay was performed in a flight cage (7.20 m x 3.60 m x 2.20 m) which was set up in a greenhouse. A bee hive with nine honeycombs of *Apis mellifera* had been placed in the flight cage two weeks before the first flowers of *Salix* opened end of February 2007. Until the beginning of the experiment on March 12th, the bees were fed with sugar solution. The sugar solution feeder had been located at the same place where later on the test cylinders for the bioassay were built up. For each experimental arrangement both test cylinders were built up 3 m apart from the bee hive and 1 m apart from each other. All experiments were performed only on days with comparable weather conditions (sunny, at least 10 °C air temperature) between 12 p.m. and 3 p.m., when the activity of bees was highest according to previous field observations (Füssel et al. unpublished). According to these field observations, bee activity was higher on male sallows than on females around 12 p.m., but at 2 p.m. honeybees usually visited both male and female catkins with comparable frequencies. Each test was conducted for 40 min; 20 min after the beginning the arrangement of the cylinders are exchanged. For all three test series each test was repeated once. Usually, about 50 bees were attracted by the setup at the time during the bioassays. All active bees that flew to within 10 cm of a cylinder and continued “zigzagging”, or contacted after “zigzagging” either the macrolon® sleeve (positive “landing” response to floral), or the

cylinder where the catkins where visible (positive “landing” response to visual stimuli) were counted and classified into two behavioural groups: bees that zigzagged only = Z, and those that landed after zigzagging = ZL. For later comparison we also summarized both groups (Z+ZL).

Analysis of Floral Scent. Floral scent was analysed to test for differences between male and female individuals. In the flowering periods 2006 and 2007 scent samples were collected using two different dynamic headspace methods. In 2006 the floral scent of six male and five female individuals was collected. From each individual, scent was sampled at 2 p.m. from one twig with 4 to 10 flowering catkins. In 2007, floral scent samples were collected from a different set of individual plants than in 2006. Scent was sampled from branches of eight male and eight female individuals immediately after they had been used for release of olfactory cues in the behavioural experiments of test series 3.

For scent collection, the catkins were enclosed for 10 min in an oven bag (Nalophan), and floral scent was subsequently trapped for 2.5 min in an adsorbent micro tube (filled with 3 mg of a 1:1 mixture of Tenax-TA 60-80 and Carbotrap 20-40) using a membrane pump (G12/01 EB, Rietschle Thomas, Puchheim, Germany). Samples were analysed and compounds were identified using a combination of gas chromatography and mass spectrometry (GC-MS) as described earlier in Füssel et al. (2007). For each compound, the percentage amount compared to total scent amount in respective sample was calculated based on peak area.

Anther scent was collected from three different male *S. caprea* individuals in the flowering season 2005. For each sample, 20 anthers from one catkin were put in a quartz microvial for direct analysis via thermal desorption and coupled gas chromatography and mass spectrometry (GC-MS, see also Jürgens and Dötterl, 2004). The samples were analysed on a Varian Saturn 2000 mass spectrometer and a Varian 3800 gas chromatograph with 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 (Amirav and Dagan, 1997; Wilkinson and Ladd, Undated). The ChromatoProbe microvial 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 min and the injector was heated at 200 °C/min to 150 °C, and held at this temperature for 2 min, after which the split vent was opened (1/20) and the injector cooled down. The GC oven temperature was held for 4.6 min at 40 °C, then increased by 6 °C per min to 260 °C and held for 1 min. After each run the column was cleaned by heating at

100 °C/min to 300 °C. The MS interface was 260 °C and the ion trap worked at 175 °C. The mass spectra were taken at 70 eV (in EI mode) with a scanning speed of 1 scan/s from *m/z* 40 to 350. The compounds were identified as described earlier in Füssel et al. (2007).

For male and female catkins used for bioassays in test series 3, and anther volatiles, we estimated total scent emission as follows: For quantification of compounds known amounts of lilac aldehydes, *trans*-β-ocimene, *cis*-3-hexenylacetate, benzaldehyde, phenylacetaldehyde, and veratrole were injected, and the mean response of these compounds was used for quantification.

Analysis of Nectar. Nectar volume, nectar sugar concentration and composition were analysed to determine differences and similarities in the floral reward common to male and female flowers.

In 2006, 25 nectar samples were collected from flowers of fully abloom inflorescences of 11 female and 14 male individuals of *Salix caprea*. Sampling took place between 11 a.m. and 2 p.m. on sunny days with at least 10 °C air temperature. Nectar samples were taken with 0.5 µl capillaries ("Minicaps" from Hirschmann Laborgeräte). From each individual plant, one nectar sample, containing nectar from five to 15 flowers of a single catkin was taken. Nectar volume was determined and nectar was transferred into an Eppendorf reaction tube filled with 200 µl Milli-Q-Water. All samples were immediately frozen at -80 °C until further analysis.

The samples were analysed by using high performance liquid chromatography (HPLC – Jas.co PU-1580) equipped with a CarboPac PA 100, 4x250 mm column. Frozen nectar samples were thawed and diluted appropriately 1:10 to 1:100 with Milli-Q-Water, and a 2-µl-subsample was injected for analysis. Elution took place in Milli-Q-Water with a 0.5 M NaOH gradient from 3 to 70 % at a flow rate of 1 ml min^{-1}. An electrochemical detector (Dionex ED 40) was used for sugar detection. Borwin Chromatogram software created the respective chromatograms. Nectar sugar composition of *Salix caprea* was determined by comparison with standards (glucose, fructose, and sucrose). Sugar amount per single flower (µg), nectar sugar concentration (mol l^{-1}), and nectar sugar composition (proportion % of single sugars in relation to total sugar content) were calculated.

Statistics.

Bioassay. To compare the numbers of bees that showed a specific response to the different cylinders in a particular two-choice test, an observed vs. expected Chi-Square (χ^2) test was conducted, if the expected frequencies were bigger than five. We compared numbers of bees that zigzagged (= Z+ZL; summarising bees that zigzagged only = Z, and those that landed after zigzagging = ZL), numbers of bees that landed after zigzagging (= ZL), and numbers of bees that zigzagged only without further landing trials (= Z), separately.

Analysis of Floral Scent. The Bray-Curtis index was calculated (PRIMER 6.1.6 package) to determine semiquantitative differences in floral scent patterns between male and female individuals. For these analyses, the percentage amounts of the floral scent compounds were used. To visualise the similarities/dissimilarities in floral scent patterns among samples nonmetric multidimensional scaling was used (Clarke and Gorley, 2006).

Analysis of similarities (ANOSIM, two-way crossed design; factors: sex and year) in the program package Primer 6.1.6 (see also Clarke and Warwick, 2001; Clarke and Gorley, 2006) was used to test the null hypothesis that there are no differences in scent pattern between sexes (male and female) for different flowering years. A two-way layout was necessary as there were differences in scent within sexes for the different flowering years. Similarity percentage (SIMPER) was used, again in the PRIMER package (two way crossed design; factors: sex, year) to determine the compounds responsible for differences between sexes.

The procedure (ANOSIM and SIMPER: two-way crossed design; factors: total floral scent emission, anther volatile) was repeated to compare the scent patterns between total floral scent emission and anther volatiles (male and female floral scent from the flowering year 2006 and 2007 vs. anther volatile; male floral scent from the flowering year 2006 and 2007 vs. anther volatile).

Analysis of Nectar. For comparison of nectar volumes of male and female flowers we conducted a Mann-Whitney-U test. The same test was used to determine differences of concentration (mol l^{-1}) of glucose, fructose, and sucrose between samples collected from male and female plants, and to compare differences of total sugar content of samples collected from male and female plants.

Results

Floral Scent. The relative amount of compounds found in female and male floral scent samples of *Salix caprea* in the year 2006 and 2007 are listed in Table 1. The average total floral scent emitted by either eight male or eight female branches used in the behavioural test was 350.61 ng in males, compared to only 79.88 ng in females (Mann-Whitney-U-Test: $Z = 2.29$; $p = 0.022$). Volatile component of 20 anthers filled with pollen was 35.05 ng.

We found altogether 37 floral scent compounds, of which 36 occurred in samples of male catkins and 34 in female catkins. Together with linalool, both 1,4-dimethoxybenzene and methyl salicylate were found in all 19 inflorescence scent samples analysed, whereas *trans*-β-ocimene and phenylethylacetate were missing in two samples.

The similarity of floral scent composition of female and male individuals of *Salix caprea* (relative amounts) is shown in Figure 3, using nonmetric multidimensional scaling (stress = 0.08). In general, a significant separation between male and female individuals was found (ANOSIM: $R = 0.623$; $p < 0.001$). Four compounds (1,4-dimethoxybenzene, methyl salicylate, *trans*-β-ocimene, and phenylethylacetate) explained more than 60 % of the observed variability between male and female floral scent. Scent from female inflorescences was dominated by 1,4-dimethoxybenzene (48.4 %) and methylsalicylate (15.2 %), followed by *trans*-β-ocimene (5.5 %) and phenylethylacetate (3.4 %). The preponderance of 1,4-dimethoxybenzene was much stronger in males (71.0 %). Proportions of methylsalicylate (7.0 %) and *trans*-β-ocimene (0.7 %) were much lower in males than in females.

Comparing the composition of floral scent collected from plants in 2006 and 2007, significant differences could be found (ANOSIM: $R = 0.343$; $p < 0.005$; Figure 3). In 2006 higher amounts of 1,4-dimethoxybenzene, methylsalicylate, and linalool were detected, while in 2007 more *trans*-β-ocimene, and phenylethylacetate were emitted.

When comparing absolute scent emission of the main components, emission of 1,4-dimethoxybenzene was much lower in females than in males (40.7 ng vs. 242.9 ng; Mann-Whitney-U-Test: $Z = 2.45$; $p = 0.014$). Also with respect to other components such as methylsalicylate, phenylethylacetate, linalool, and benzylnitril females emitted lower amounts than males (18.0 ng vs. 10.2 ng; 13.4 ng vs. 4.7 ng; 13.1 ng vs. 1.1 ng; 4.2 ng vs. 1.1 ng). However, the biggest difference was due to 1,4-dimethoxybenzene and it can be concluded that the weaker scent of females is not exclusively but mainly due to the differences in 1,4-dimethoxybenzene.

Anther volatile composition differed significantly from inflorescence scent composition (male and female floral scent in flowering seasons 2006 and 2007 vs. anther volatile ANOSIM: $R = 0.48$; $p < 0.001$ / male floral scent from the flowering year 2006 and 2007 vs. anther volatile ANOSIM: $R = 0.78$; $p < 0.001$). Only 13 of these substances found in the headspace of inflorescences were present in the volatile spectra from anthers. In contrast to whole inflorescences, anther volatiles were dominated by *trans*-β-ocimene (50 %), (*E*)-geranylacetone (23 %), and (*E,E*)-α-farnesene (9 %). The relative proportion of 1,4-dimethoxybenzene was only 5 % and of methylsalicylate only 3 %. While all scent samples collected from male and female inflorescences contained a significant proportion of linalool (3.8 % in males compared to 1.9 % in females), linalool was not found in any anthers sample.

Based on peak areas, the ranking of main anther volatiles gained from 20 anthers was: *trans*-β-ocimene (13.9 ng), (*E*)-geranylacetone (8.2 ng), (*E,E*)-α-farnesene (6.8 ng), 1,4-dimethoxybenzene (2.8 ng).

Bioassay:

Visual and olfactory cues versus a negative control. Separate olfactory and visual signals as well as the combination of both attracted more honeybees than the control (Figure 4). In all three combinations more honeybees approached and landed on the cylinder loaded with female and male willow branches than the control (Olfactory signals vs. control: Z+ZL: $\chi^2_{df=1} = 41.29$, $p < 0.001$; ZL: $\chi^2_{df=1} = 41.29$, $p < 0.001$; Z: $\chi^2_{df=1} = 28.88$, $p < 0.001$. Visual signals vs. control: Z+ZL: $\chi^2_{df=1} = 14.88$, $p < 0.001$; ZL: $\chi^2_{df=1} = 10.24$, $p < 0.001$; Z: $\chi^2_{df=1} = 6.57$, $p < 0.001$. Olfactory and visual signals vs. control: Z+ZL: $\chi^2_{df=1} = 34.66$, $p < 0.001$.; ZL: $\chi^2_{df=1} = 27.46$, $p < 0.001$; Z: $\chi^2_{df=1} = 16.36$, $p < 0.001$).

Visual and olfactory cues compared. Olfactory signals attracted significantly more honeybees than the visual signals (Figure 5) (Z+ZL: $\chi^2_{df=1} = 30.04$, $p < 0.001$. ZL: $\chi^2_{df=1} = 6$, $p < 0.01$. Z: $\chi^2_{df=1} = 25.19$, $p < 0.001$). The combination of olfactory and visual stimuli was more attractive than either signal alone (Combined stimuli vs. floral scent alone: Z+ZL: $\chi^2_{df=1} = 29.25$, $p < 0.001$; ZL: $\chi^2_{df=1} = 5.44$, $p = 0.02$; Z: $\chi^2_{df=1} = 25.04$, $p < 0.001$. Combined stimuli vs. visual signal alone: Z+ZL: $\chi^2_{df=1} = 25.04$, $p < 0.001$; ZL: $\chi^2_{df=1} = 16$, $p < 0.001$; Z: $\chi^2_{df=1} = 13.47$, $p < 0.001$).

Gender comparison. With respect to olfactory signals female and male flowers attracted nearly the same numbers of zigzagging *Apis mellifera* (Figure 6), but the landing response

was stronger to male flowers. However, neither differences were significant (Z+ZL: $\chi^2_{df=1} = 0.34$, $p = 0.6$; ZL: $\chi^2_{df=1} = 1.64$, $p = 0.2$; Z: $\chi^2_{df=1} = 0.01$, $p = 0.9$). In response to visual signals, significantly more honeybees approached and contacted the cylinder with male branches than with female branches (Z+ZL: $\chi^2_{df=1} = 7.57$, $p < 0.001$; ZL: $\chi^2_{df=1} = 5.4$, $p = 0.02$; Z: $\chi^2_{df=1} = 4.32$, $p = 0.04$). Also when combining both signal types, male flowers were more attractive than female flowers (Z+ZL: $\chi^2_{df=1} = 5.76$, $p < 0.01$; ZL: $\chi^2_{df=1} = 5.4$, $p < 0.02$; Z: $\chi^2_{df=1} = 2.65$, $p = 0.5$).

Analysis of Nectar: Female flowers offer similar nectar volumes as male flowers (Table 2) (Mann-Whitney-U-Test: $Z = 1.29$, $p = 0.198$). Further, no differences in total sugar amount per single flower between female individuals (mean value: 11.70 µg) and male individuals (mean value: 7.60 µg) were found ($Z = -1.42$, $p = 0.155$).

Nectar samples from female flowers had a significantly higher concentration of glucose (1.9 M) and fructose (1.5 M) than male flowers (both sugars 0.3 M) (Mann-Whitney-U-Test, $N_{female} = 11$; $N_{male} = 14$: glucose, $Z = -4.05$, $p < 0.001$; fructose, $Z = -3.67$, $p < 0.001$), whereas samples from male flowers had tendential higher sucrose concentrations (female flowers 0.9 M, male flowers 1.9 M; Mann-Whitney-U-Test, $N_{femal\ e} = 11$; $N_{male} = 14$: $Z = 1.80$, $p = 0.07$).

According to the classification of Baker and Baker (1983), nectar sugar composition was hexose-rich in females ($S/(F+G) = 0.52$), but sucrose-dominant in males ($S/(F+G) = 5.22$).

Discussion

Insects use both olfactory and visual signals to find pollen or nectar in flowers (e.g. Dobson 2005). The relative importance of olfactory and visual cues to find flowers depends on the species of the plant and the species of flower visitors. For dioecious species potential pollinators need to be attracted to both male and female flowers for pollination to occur. However, little is known about the roles of olfactory and/or visual cues for the attraction of pollinators to separate sexes of dioecious plant species.

Attractiveness of olfactory and visual signals. In *Salix caprea* honeybees responded to both olfactory and visual cues. However, we found that floral scent is more attractive than visual cues alone. Furthermore, the combination of floral scent and visual signals attracts more honeybees than either cue alone. The stronger effect of scent, in our study is consistent with earlier studies in other plant species (Butler, 1951; Klostehalfen et al., 1978; Galen and Kevan, 1980). These authors found that under experimental conditions scent may be a more important determinant of honeybee floral choice than colour. In the future it will be necessary to conducte bioassay under natural conditions.

Attractiveness of male and female flowers.

Interestingly, floral scent of male and female *Salix caprea* catkins was similarly attractive to its main flower visitor *Apis mellifera*, despite the differing total scent emission and significant sex-specific differences of relative scent composition as demonstrated by NMDS. Thus, although scent of *S. caprea* is used by honeybees as a cue to find flowers and is advertising different sets of rewards in the gender (pollen and nectar in male, only nectar in female flowers), scent alone had no effect on flower choice of honeybees.

The significant gender differences in floral scent of *Salix caprea* found here are contradicting the data published in our first study on intra- and interspecific variability of floral scents in the genus *Salix* (Füssel et al., 2007), but also in the present study, most substances were found in scent samples of both genders, and differences were often only semiquantitative. Tollsten and Knudsen (1992) found also high resemblances in floral scent of male and female inflorescences, but they also demonstrated at least small differences in the floral scent profile between sexes for *Salix caprea*. These authors found dissimilarities of male and female scent of only 10.6 %, while we found 32.2 %. Different methods were used in the two studies (e.g., different adsorbents, thermodesorption vs. extraction of volatiles from filter using solvent), and perhaps these methodical differences were responsible for the differing results (see Füssel

et al., 2007). Both studies found that male flowers produced relatively more 1,4-dimethoxybenzene than other substances, but Tollsten and Knudsen (1992) detected methylsalicylate only in low relative amounts, whereas in our study methylsalicylate is one of the four main compounds explaining altogether more than 60 % of the observed variability between male and female floral scent composition. Altogether, floral scent alone is a relatively uncertain cue to discriminate male and female flowers of *S. caprea*: Total scent intensity is depending on other factors such as wind or distance, and composition is different but not consistently distinct enough across time and space.

In our bioassay a combination of olfactory and visual signals of male flowers attracted more honeybees than olfactory and visual cues from female flowers. Accordingly, these differing visitation rates to male and female sallows were repeatedly reported (Kay et al., 1984; Totland and Sottocornola, 2001), and our own field observations also support (Füssel et al., unpublished data) these findings.

Certainly, the different attractiveness of the sexes is due to the different rewards, but as our results show information about the different reward offers is better mitigated by visual than by olfactory cues. Besides pollen that is only offered by males, we found also differences in nectar (Table 2). Female *Salix caprea* flowers produce tentatively more nectar sugar per flower than male flowers. We found that females offer significantly higher concentrated nectar thus confirming the results of Elmqvist et al. (1988) and Katoh et al. (1985). Nectar composition also differs significantly between sexes. Similar results were reported from Elmqvist et al. (1988), Katoh et al. (1985), Percival (1961), and Goukon et al. (1976) from different willow species. Females have hexose-rich nectar ($S/(F+G) = 0.52$) in contrast to sucrose-dominated nectar ($S/(F+G) = 5.22$) in males. With respect to the single three sugars, nectar composition of females is relatively well balanced, a phenomenon that according to Percival (1961) is relatively rare in plants. It is known that honeybees prefer balanced nectars with more or less equal amounts of all three sugars (therefore usually hexose-rich nectars according to the classification of Baker and Baker, 1983) over sucrose-dominated nectars (Wykes, 1952). It may be hypothesised that female flowers compensate for the lack of pollen with higher concentrated nectar which matches the preferences of bees better than nectar from male catkins. Further behavioural tests in the field are necessary to determine if flower visitors, such as honeybees, link sex-specific visual cues to nectar quantity and quality of the genders. Greco et al. (1996) stated that the activity or rather the visitation rate of honeybees is associated with the circadian availability of resources. According to our own field observations, the visitation rate by honeybees on male *Salix* inflorescence is high in the

morning when activity in general is high, whereas female plants have a higher visitation rate in the afternoon when activity in general is decreasing (Füssel et al., unpublished data). Most likely, a combination of changing reward presentation and changing pollinator preferences in the course of the day account for this visitation pattern. In the afternoon, the pollen and nectar rewards of the highly preferred male *S. caprea* could run short due to the high visitation rate in the morning which is correlated with pollen release from anthers. If so, the visual stimulus of male plants and the reward found decrease, and in the afternoon honeybees are therefore stronger attracted to neighbouring female plants of *S. caprea* individuals to gather nectar. This naturally rhythmic interaction of reward offering and pollinator preference changes which mitigates pollen removal and pollen deposition could be enhanced by a gender-specific diurnal rhythm of floral scent emission (Füssel et al., unpublished data). *Salix caprea* has temperature-dependent maximum scent emission in the late morning, when honeybees visitation rate is usually highest (Füssel et al., unpublished data). Moreover, relative amounts of 1,4-dimethoxybenzene, a known bee-attractant (Dötterl et al., 2005; Füssel et al., unpublished data) increase in the afternoon in scent from female plants while in males the proportion of 1,4-dimethoxybenzene decreases (Füssel, unpublished data). Thus, the change in available resources could be advertised by scent composition. In conclusion, it is desirable to repeat the bioassays presented here at different times of the day, and e.g. with bee hives under different nutritional regimes of adult workers and larvae in order to characterise the influence of such internal factors on honeybee behaviour.

It is well known that honeybees can learn to distinguish among different floral scent compounds (Wright et al. 2005). In further studies it should be investigated if honeybees could discriminate between male and female floral scent after learning. It may be possible that experienced bees could fly specifically to male or female *Salix* individuals.

In summary, considering the results of the bioassay, where olfactory signals of male flowers triggered a similar response as olfactory signals of female flowers, the biological meaning of sex differences of inflorescence scent for different attraction of honeybees to male and female *S. caprea* seems to be low. Male flowers of *S. caprea* are more attractive to honeybees, due to visual signals. The typical yellow signalling colour of male flowers results from the large amounts of pollen, which are located in the anthers. Lepage and Boch (1968) found that visual pollen signals are responsible for behavioural reactions of flower visitors which feed on pollen, such as honeybees. Other authors found that the bias to males in pollination service could not be completely explained by visual cues (Ashman et al., 2005). These authors identified floral scent as a major driver of pollinator behaviour in gender dimorphic plants

(Ashman et al., 2005). Ashman et al. (2005) reported that in the gynodioecious *Fragaria virginiana* generalistic pollinators discriminated the scent of hermaphrodite flowers over those of females primarily because of the scent of anthers, which emitted high amounts of 2-phenylethanol, a benzoid compound found only in small amounts in the female flowers. This is contradicting our findings in *S. caprea*, where no male specific compounds were found.

To conclude, male sallow catkins were, despite the high importance of olfactory cues compared to visual cues for attraction in general, mainly due to their visual advertisement more attractive than females. However, it is unknown if other potential pollinators of *S. caprea* behave similar and how this affects pollination success. The pollination system of willows seems to be sufficiently effective despite or maybe just because of the higher attractiveness and visitation rates of male flowers. From a biological point of view, different visitation rates in both genders might be disadvantageous because a successful pollination requires a prior visit of a male willow by one and the same individual pollinator. But as a consequence of the higher visitation frequency of male willows the probability of a successful pollination of a female individual will increase, because it is more likely that the bee visiting a female flower is well loaded with pollen from several pollen donors.

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Table legends:

Table 1: Chemical composition of inflorescence scent and anther volatiles in *Salix caprea*.

Table 2: Nectar characteristics from male and female flowers of *Salix caprea* (median = Med). Nectar analysis was performed on 14 samples collected from 132 male (m) flowers and 14 samples from 101 female (f) flowers. Significant differences (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$) found by Mann-Whitney-U-tests are indicated by asterisk, no significant differences were indicated by ns.

Table 1:

Compound[a]	Relative amount[b]								
	anthers			Male inflorescences			Female inflorescences		
	Occurence[c]	Median	Min-Max	Occurence[c]	Median	Min-Max	Occurence[c]	Median	Min-Max
Aromatics									
Benzaldehyde	0	0	0	5/4	0.35	0-0.90	5/4	1.72	0.21-13.03
Benzyl Alcohol	0	0	0	6/1	0.38	0-0.99	5/0	0.83	0-3.44
Phenylethylacetate	1	0	0-1.05	5/4	0.49	0-6.02	4/4	3.40	0-9.62
Salicylaldehyde	0	0	0	2/1	0	0-0.37	0/1	0	0-0.15
2-Phenylethylmethylether	0	0	0	3/3	0.13	0-1.17	0/4	0	0-0.82
2-Phenylethanol	0	0	0	6/4	0.58	0.05-2.88	2/4	0.15	0-0.38
1,4-Dimethoxybenzene	3	5.28	5.08-62.70	6/4	70.96	60.17-84.26	5/4	48.35	38.73-59.09
Methyl Salicylate	3	3.09	1.64-3.16	6/4	6.97	1.92-8.44	5/4	15.21	7.22-19.78
Isoprenoids									
a-Phellandrene	0	0	0	1/3	0	0-0.54	2/4	0.30	0-6.51
a-Pinene	0	0	0	0/4	0	0-0.20	0/2	0	0-0.46
β-Pinene	0	0	0	5/2	0.47	0-3.21	5/2	1.75	0-8.07
β-Phellandrene	0	0	0	0/3	0	0-0.08	0/1	0	0-0.30
D-Limonene	0	0	0	6/4	0.07	0.01-0.40	4/4	0.26	0-1.91
cis-b-Ocimene	2	1.73	0-1.76	5/4	0.77	0-3.14	4/4	1.91	0-3.44
trans-b-Ocimene	3	52.13	1.19-53.09	5/4	0.73	0-12.03	4/4	5.49	0-19.23
Linalool	0	0	0	6/4	3.87	0.63-13.05	5/4	1.87	0.32-13.57
Lilac Aldehyde A	0	0	0	4/2	0.19	0-2.37	2/1	0	0-1.16
Lilac Aldehyde B+C	0	0	0	4/4	0.32	0-2.27	3/4	0.34	0-0.58
Lilac Aldehyde D	0	0	0	4/0	0	0-0.81	3/3	0.03	0-0.58
Lilac Alcohol A	0	0	0	2/0	0	0-0.21	0/0	0	0-0
Lilac Alcohol B+C	0	0	0	2/4	0.04	0-0.51	0/1	0	0-0.81
Lilac Alcohol D	0	0	0	4/0	0	0-0.74	0/0	0	0-0
α-Copaene	0	0	0	0/3	0	0-0.11	0/0	0	0-0
(*E*)-Caryophyllene	0	0	0	0/2	0	0-0.06	0/1	0	0-0.06
(*E*)-Geranylaceton	3	23.60	23.18-23.82	6/4	0.18	0.01-1.72	5/3	0.22	0-3.55
Cubebene	3	0.36	0.33-1.02	0/4	0	0-0.06	0/2	0	0-0.08
(*E,E*)-α-Farnesene	3	9.42	2.80-9.59	3/4	0.05	0-0.89	4/4	0.56	0-7.45
N-bearing compounds									
Benzyl Nitrile	0	0	0	5/4	1.04	0-11.84	4/3	0.52	0-6.75
Indole	3	1.08	0.58-1.10	4/4	0.62	0-1.48	5/4	0.65	0.36-3.18
Fatty acid derivates									
cis-3-Hexen-1-ol	0	0	0	0/2	0	0-0	0/4	0.30	0-0.99
cis-3-Hexenyl aceatate	0	0	0	4/4	0.16	0-0.87	4/4	1.19	0-2.21
4-Oxoisophorone	0	0	0	5/2	0.50	0-11.83	3/4	0.17	0-0.59
(*E*)-4,8-Dimethyl-1,3,7-Nonatriene	2	0.84	0-1.05	6/4	0.81	0.22-4.59	3/4	1.40	0-3.37
Unknowns									
41, 45, 59, 73, 97	3	0.96	0.82-1.29	6/3	0.96	0-6.84	5/4	2.12	0.92-6.22
39, 77, 91, 119, 134	3	0	0-0.53	0/4	0	0-0.22	0/4	0	0-0.48
40, 55, 69, 119, 154	3	0	0	0/4	0	0-0.03	0/4	0	0-0.04
40, 55, 95, 123, 138	3	0.66	0.65-1.46	0/2	0	0-0.14	0/2	0	0-0.36

a = Detected compounds ordered according to substance classes.
b = Relative proportion % of compounds in scent samples.
c = Number of plants where a compound was found. Total sample sizes: Anther scent 2005 (n = 3); Male inflorescences 2006/2007 (n = 6 / n = 5); Female inflorescences 2006/2007 (n = 5 / n= 4).

Table 2:

	Gender	Nectar per flower (µg)	Sugar per flowers (µg)	Glucose (%)	Fructose (%)	Sucrose (%)	Glucose (M)	Fructose (M)	Sucrose (M)	Glucose (µg/µl)	Fructose (µg/µl)	Sucrose (µg/µl)	Glucose + Fructose + Sucrose (µg/µl)
Med	**m**	**0.010**	**6.3**	**9.5**	**9.5**	**80.9**	**0.28**	**0.18**	**1.76**	**50.8**	**31.5**	**601.0**	**683.3**
Min	**m**	**0.005**	**1.0**	**0.1**	**3.4**	**51.4**	**0.00**	**0.02**	**0.31**	**0.1**	**3.1**	**107.7**	**110.9**
Max	**m**	**0.017**	**16.0**	**29.9**	**23.2**	**96.5**	**1.18**	**1.15**	**4.80**	**213.0**	**208.1**	**1641.9**	**2062.9**
Med	**f**	**0.012**	**8.8**	**42.2**	**37.9**	**20.5**	**1.76**	**1.14**	**1.14**	**317.7**	**206.3**	**391.1**	**915.1**
Min	**f**	**0.005**	**6.0**	**32.9**	**5.7**	**3.8**	**1.03**	**0.28**	**0.09**	**186.1**	**49.6**	**32.3**	**267.9**
Max	**f**	**0.027**	**20.1**	**69.5**	**46.0**	**36.4**	**3.79**	**3.51**	**1.71**	**682.5**	**631.9**	**584.9**	**1899.3**
Mann-Whitney U-test		**ns**	**ns**	*******	*******	*******	*******	*******	**ns**	*******	*******	*******	**ns**

Figure legends:

Fig. 1: The cylinder arrangement of the three test seriesin the behavioural experiments: attractiveness of different floral traits against a control (1), relative attractiveness of the different floral traits against each other (2), attractiveness of males against females (3). Filled squares = olfactory traits; Open squares = visual traits, Dotted squares = olfactory and visual traits combined; Black squares with c (control) = empty cylinders; m = male branches, f = female branches used for the different tests.

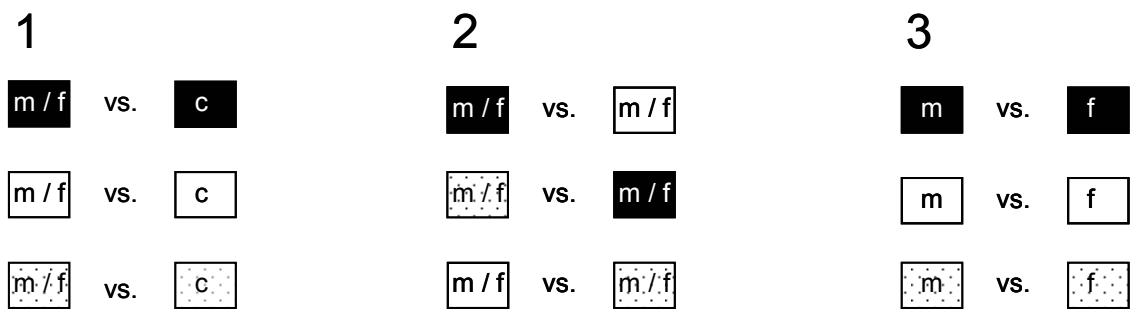
Fig. 2: Basic appearance of quartz glass cylinders used in the behavioural experiments.

Fig. 3: Nonmetric multidimensional scaling (NMDS) of floral scent composition of female (f) and male (m) individuals of *Salix caprea* in two different sampling years (2006 and 2007) (stress = 0.08).

Fig. 4: Proportion of active *Apis mellifera* showing a specific response to separate olfactory and visual signals or a combination thereof in comparison to an empty control cylinder. Black = bees that showed a landing response after zigzagging (ZL); Grey = bees that zigzagged only (Z) without further landing trials. The abbreviation Z+ZL summarises all bees that zigzagged either with or without landing thereafter. The numbers in the bars indicate absolute counts of bees showing a specific response. Significant differences (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$) found by observed versus expected tests are indicated by asterisk.

Fig. 5: Proportion of active *Apis mellifera* showing a specific response to (A) olfactory signal vs. visual signal, (B) olfactory/visual signals vs. olfactory signal, (C) olfactory/visual signals vs. visual signals. Black = bees that showed a landing response after zigzagging (ZL); Grey = bees that zigzagged only (Z) without further landing trials. The abbreviation Z+ZL summarises all bees that zigzagged independent of possible landing trials thereafter. The numbers in the columns indicate absolute counts of bees showing a specific response. Significant differences (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$) found by observed versus expected tests are indicated by asterisk.

Fig. 6: Proportion of active *Apis mellifera* showing a specific response to male and female *Salix* flowers. The single effects of olfactory and visual cues are compared with each other, and with the effect of both cues combined. Black = bees that showed a landing response after zigzagging (ZL); Grey = bees that zigzagged only (Z) without further landing trials. The abbreviation Z+ZL summarises all zigzagging bees. The numbers in the columns indicate absolute counts of bees showing a specific response. Significant differences (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$) found by observed versus expected tests are indicated by asterisk.


1
m / f vs. c
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m / f vs. c
2
m / f vs. m / f
m / f vs. m / f
m / f vs. m / f
3
m vs. f
m vs. f
m vs. f

Fig. 1:

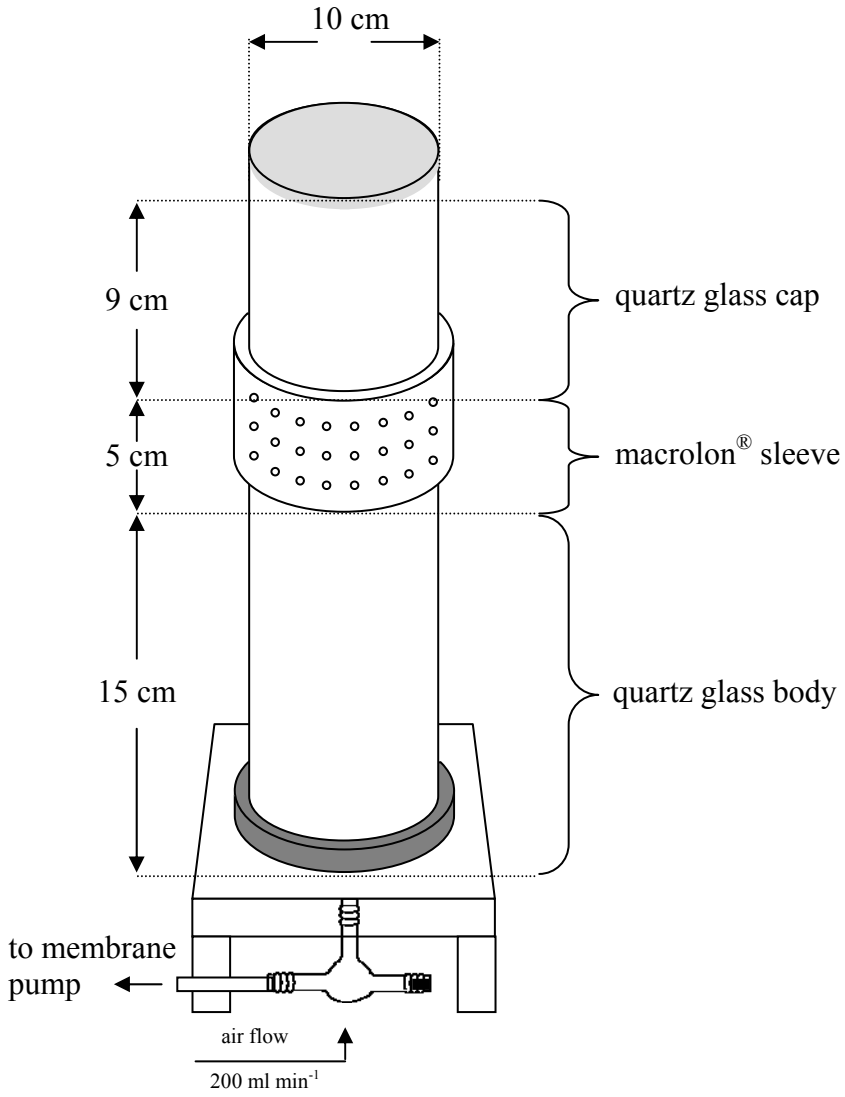
10 cm
9 cm
5 cm
15 cm
quartz glass cap
macrolon® sleeve
quartz glass body
to membrane pump
air flow
200 ml min-1

Fig. 2:

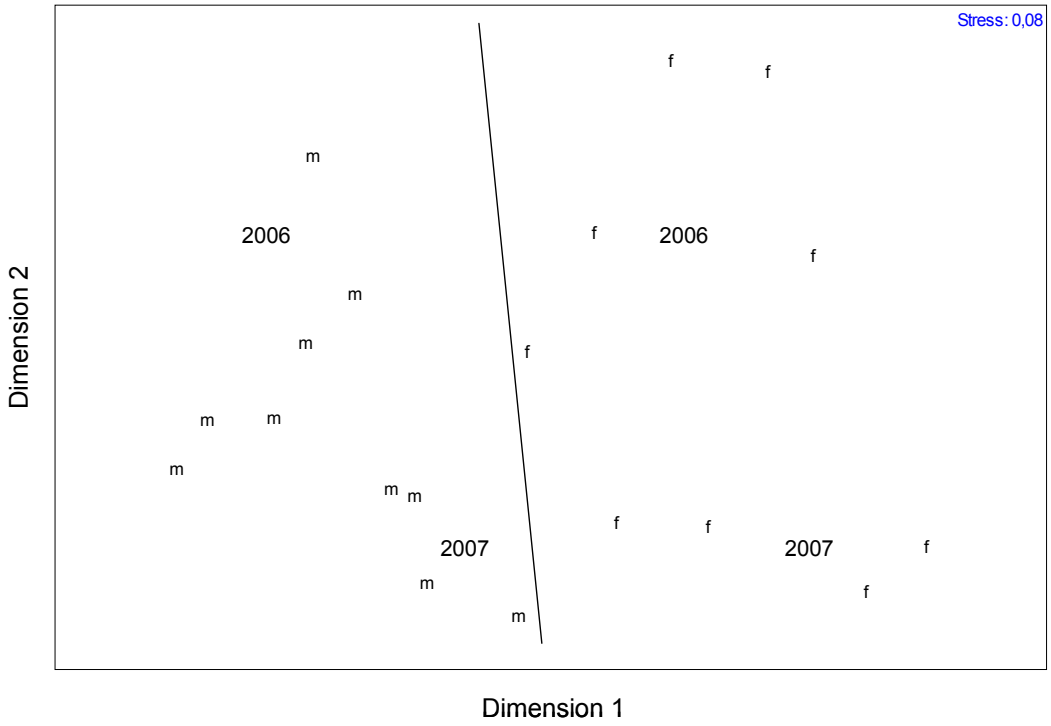
Stress: 0,08
Dimension 2
Dimension 1
2006
2006
2007
2007
m
f

Fig. 3:

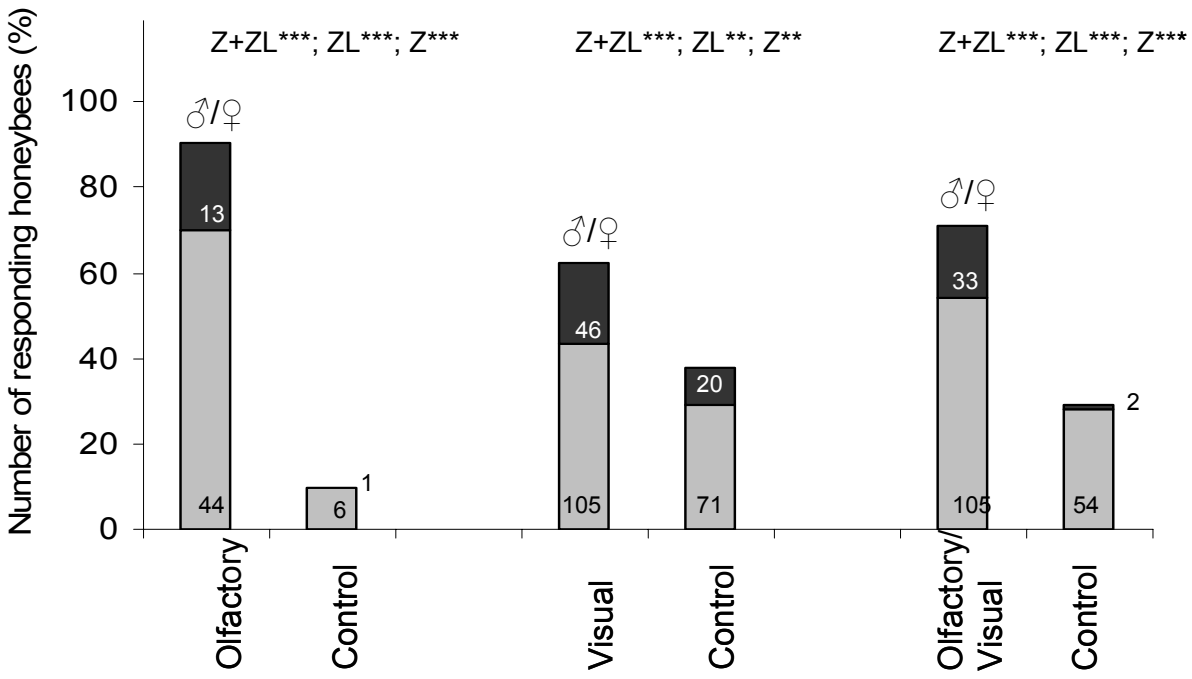
Number of responding honeybees (%)
Z+ZL***; ZL***; Z***
Z+ZL***; ZL**; Z**
Z+ZL***; ZL***; Z***
♂/♀
13
44
1
6
46
105
20
71
33
105
2
54
Olfactory
Control
Visual
Control
Olfactory/ Visual
Control

Fig. 4:

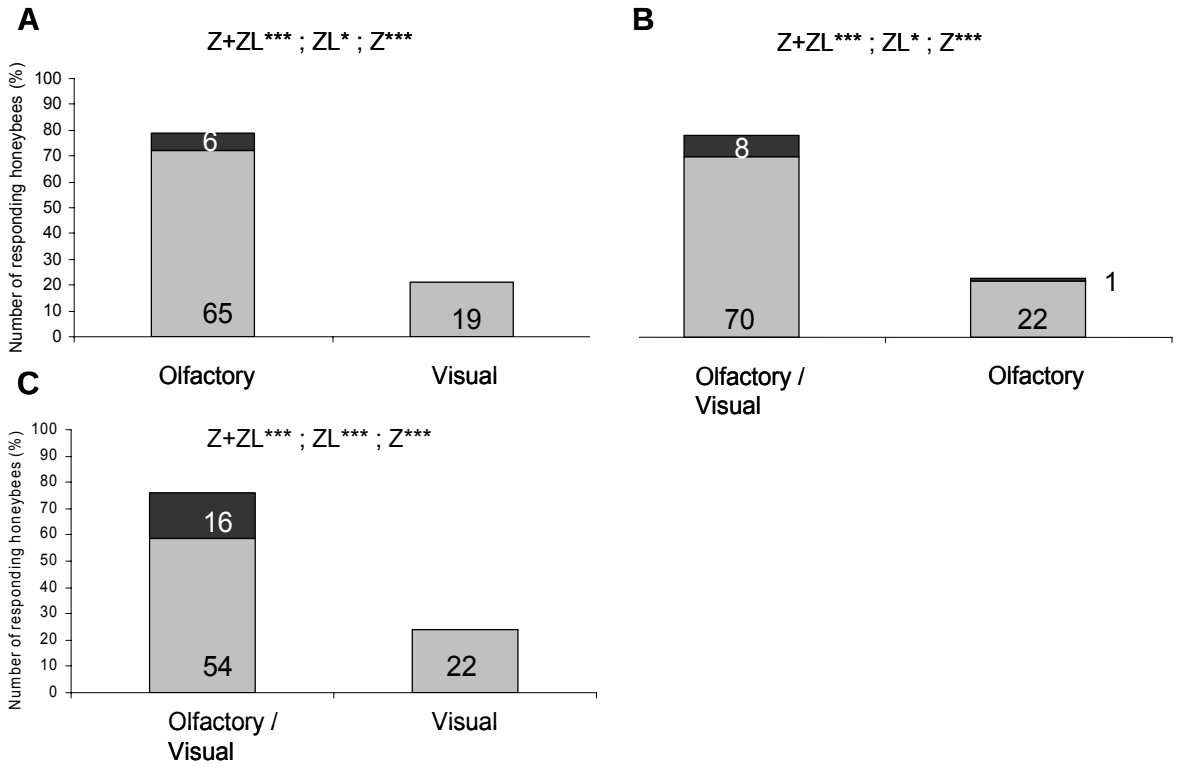
A
Z+ZL*** ; ZL* ; Z***
Number of responding honeybees (%)
100
90
80
70
60
50
40
30
20
10
0
6
65
19
Olfactory
Visual
B
Z+ZL*** ; ZL* ; Z***
8
70
1
22
Olfactory / Visual
Olfactory
C
Z+ZL*** ; ZL*** ; Z***
Number of responding honeybees (%)
16
54
22
Olfactory / Visual
Visual

Fig. 5:

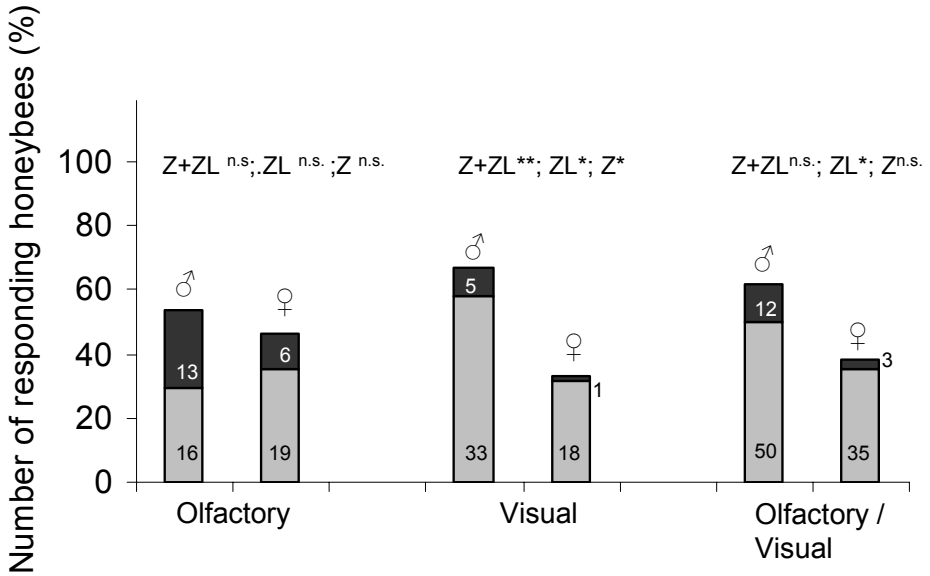
Number of responding honeybees (%)
100
80
60
40
20
0
Z+ZL n.s;.ZL n.s. ;Z n.s.
Z+ZL**; ZL*; Z*
Z+ZLn.s.; ZL*; Zn.s.
♂
♀
13
16
6
19
5
33
1
18
12
50
3
35
Olfactory
Visual
Olfactory / Visual

Fig. 6:

5 Summary

The present work was performed within the framework of the graduate college 678 "Ecological significance of natural compounds and other signals in insects – from structure to function" at the University of Bayreuth.

For the first time, the role of floral scents for the interaction of dioecious willows (*Salix* spp., Salicaceae) with their pollinators was examined in detail. Willows are mainly pollinated by wind and/or insects, but the flower visitor composition of specific willow species is mostly as unknown as the contribution of particular insect species or wind to the reproductive success of these willows. Flower-visiting insects are primarily attracted to the catkins by visual and olfactory signals of the flowers. However, up to now there are no thorough studies of the relative significance of olfactory and visual cues, and the importance of single floral scent compounds for pollinator attraction.

In this thesis, the chemical composition of floral scent of different willow species as well as its variability on the inter- and intraspecific level were analysed in general. In a subsequent case study (*Salix caprea*), the role of floral scent for attracting the identified flower visitors was examined in detail by means of electroantennographic studies and bioassays in the field, in a flight cage, and in a wind tunnel. The relevance of different pollen vectors for the reproductive success of this willow species was examined by pollination experiments.

Chemical composition and variability of the floral scent of *Salix*

For the determination of the chemical composition and the variability of floral scent within the genus *Salix* the inflorescence odour of 93 different individuals (male and female) from 34 species was examined. The floral scent of several catkins of an individual was collected using a dynamic headpace MicroSPE method and analysed by gas chromatography coupled with mass spectrometry (GC-MS). Isoprenoids (e.g. α- and β-pinene, D-limonene, *cis*- and *trans*-β-ocimene, and linalool) and aromatic compounds (e.g. benzaldehyde and 1,4-dimethoxybenzene) were identified as typical floral scent compounds of the genus *Salix*. Particularly 1,4-dimethoxybenzene and *trans*-β-ocimene were responsible for the interspecific variation (both qualitatively and semiquantitatively).

Eight out of 34 willow species were examined with higher sample sizes (at least in each case three male and three female individuals). In half of the 28 pairwise species comparisons differences in floral scent were significant. In three of these eight willow species differences

in the floral scent pattern between the two genders could be determined. For example, the floral scent of male *Salix fragilis* individuals emitted higher relative amounts of *trans*-β-ocimene and 1,4-dimethoxybenzene, whereas female individuals contained more D-limonene and D-verbenone.

The circadian rhythm of floral scent emission was exemplarily studied in *Salix caprea*. The floral scent emission changed both semiquantitatively and qualitatively in the course of the day. Generally, a larger quantity of floral scent was emitted during the day than at night. Thereby, the quantity of floral scent correlated positively with the air temperature. Primarily, the several isomers of the monoterpene lilac aldehyde were responsible for significant differences between day and night. Lilac aldehyde is produced in higher quantities at night, which could be interpreted as an adaptation to nocturnal pollinators – e.g. the moth *Orthosia gothica*, which responded strongly of lilac aldehyde in bioassays.

Flower visitors of *Salix caprea*

Insect species from different orders (Coleoptera, Diptera, Hemiptera, Hymenoptera, and Lepidoptera) were identified as flower visitors and are regarded as potential pollinators of *Salix caprea*. The visiting frequency was highest for Hymenoptera (primarily honeybees, bumblebees, and solitary wild bees such as *Andrena vaga*) and Lepidoptera (many nocturnal and only few diurnal species). Both frequency and species spectrum of flower visitors varied within a day: During the day primarily bees (honeybee, bumblebees and solitary wild bees) and butterflies dominated, whereas during and after dawn moths were the only flower visitors. Altogether, nocturnal flower visitors had clearly a lower frequency than diurnal visitors.

Responses of *Apis mellifera* and *Orthosia gothica* to 1,4-dimethoxybenzene and lilac aldehyde

1,4-Dimethoxybenzene and lilac aldehyde are main compounds of the floral scent of *Salix caprea*. 1,4-Dimethoxybenzene dominated the floral scent day and night, but its proportion decreased over night, while the proportion of emitted lilac aldehyde increased at night. Both compounds are electrophysiological active and elicited signals in antennae of both *Apis mellifera*, the most frequent diurnal visitor, and *Orthosia gothica*, the most frequent nocturnal visitor. The effect of the two floral scent compounds on these two insect species was examined in biotests. The biotests were performed in a flight cage (*Apis mellifera*) and in

a wind tunnel (*Orthosia gothica*), respectively. The honeybee was attracted most strongly by 1,4-dimethoxybenzene; in contrast, *Orthosia gothica* was attracted most strongly by lilac aldehyde.

Responses of *Andrena vaga* to single components of floral scent of *Salix caprea* and *S. atrocinerea*

Floral scent of *Salix caprea* and *S. atrocinerea* were analysed with GC-MS and tested for their physiological activity on the oligolectic wild bee *Andrena vaga* by gas chromatography coupled with electroantennography (GC-EAD). Altogether 16 floral scent components of both *Salix* species induced clear signals in the antennae of *A. vaga*. The main component of the examined extracts, 1,4-dimethoxybenzene, led to the strongest antennal signals. Interestingly, in the biotest 1,4-dimethoxybenzene attracted many female *A. vaga*, but no male individuals.

Behavioural responses of *Apis mellifera* to male and female individuals of *Salix caprea*

The attractiveness of male and female flowering twigs of *Salix caprea* for honeybees (*Apis mellifera*) was examined in biotests in a flight cage.

In the experiment, male willow inflorescences attracted more honeybees than female inflorescences. Considering the relatively high similarity of floral scent of both genders, this is most likely due to visual cues. Because of their conspicuously yellow-coloured pollen presentation, the male catkins of *Salix caprea* are obviously visually more attractive than the pollen-lacking insipid greenish female catkins. Male and female *S. caprea* individuals differed also in the sugar composition of nectar. While females produced hexose-rich nectar, in contrast males had sucrose-dominated nectar. Further investigation should highlight if these differences also contribute to the different attractiveness of both genders to *Apis mellifera*. The higher visit frequency to male sallows may be of ecological importance, since it increases the probability that flower visitors collect sufficient pollen – of possibly several male individuals – before visiting a female individual. Thus not only the probability for successful pollination and fertilisation, but also the genotypic variability might increase within a population.

Importance of diurnal and nocturnal insects as well as wind for the reproductive success of *Salix caprea*

In order to quantify the relative contribution of diurnal (primarily bees) and nocturnal insects (primarily moths) as well as wind to a successful pollination, pollination experiments were conducted at five selected female *Salix caprea* individuals. During flowering, insects were excluded from flower visits by covering inflorescences with nylon nets either at night (testing diurnal pollination), during the day (testing nocturnal pollination), or for the entire day (testing wind pollination). After the flowering season, seeds were counted to quantify the reproductive success. Exposure to diurnal flower visitors resulted in higher reproductive success, than exposure to nocturnal flower visitors. The reproductive success that can be attributed to wind pollination is also relatively low. Most likely, low nocturnal air temperatures in the investigation year and a consequently low activity of moths, were the main reason for the low contribution of nocturnal insects.

Altogether, the case study of *Salix caprea* is challenging the existing concepts of specialisation/generalisation of plant-pollinator interactions. Regarding the aspect of interactions (diurnal and nocturnal flower visitors vs. wind), *S. caprea* is a generalist, but looking at the aspect of adaptations, *S. caprea* can be regarded as a multi-specialist with respect to its floral scent emission.

In summary,

(1) The catkins of several species of the genus *Salix* (willows) emit a rich species-specific bouquet of floral scent compounds. Within some species also gender-specific differences were found.

(2) A high variety of diurnal and nocturnal insects (mainly bees and moths) visit the catkins of the sallow (*S. caprea*), but the frequency of diurnal visitors is essentially higher than those of nocturnal insects.

(3) Both diurnal and nocturnal flower visitors can detect a large number of floral scent compounds.

(4) The floral scent of *Salix caprea* is subjected to a circadian rhythm, which correlates with the change of the flower visitor spectrum over day and night.

(5) *Apis mellifera* (diurnal pollinator) is stronger attracted to 1,4-dimethoxybenzene than to lilac aldehyde, while *Orthosia gothica* (nocturnal pollinator) prefer lilac aldehyde over 1,4-dimethoxybenzene.

(6) Male *Salix caprea* individuals are more attractive to *Apis mellifera* than females, most likely due to the yellow pollen colour.

(7) Female *Salix caprea* produce hexose-rich nectar, while males had sucrose-dominated nectar.

(8) Diurnal insects play a larger role in pollination of *Salix caprea* than nocturnal insects and wind.

(9) In conclusion, the pollination system of *Salix caprea* (and probably also those of other willow species) is a generalistic one, but exhibits specific adaptations to different functional groups of pollinators.

6 Zusammenfassung

Die vorliegende Arbeit wurde im Rahmen des Gradiertenkollegs 678 „Ökologische Bedeutung von Wirk- und Signalstoffen bei Insekten – von der Struktur zur Funktion“ an der Universität Bayreuth durchgeführt.

Die Rolle der Blütendüfte für die Interaktion von diözischen Weiden (*Salix* spp., Salicaceae) und ihren Bestäubern wurde erstmals detailliert untersucht. Weiden werden überwiegend von Insekten, aber auch vom Wind bestäubt, wobei nur für wenige Arten die Bedeutung der Anemogamie genauer bekannt ist. Ebenfalls nur unzureichend erforscht ist für verschiedene Weidenarten das Artenspektrum blütenbesuchender Insekten. Welche dieser Blütenbesucher tatsächlich eine Rolle als Bestäuber spielen, ist bisher überhaupt nicht untersucht worden. Insekten werden vor allem durch olfaktorische und visuelle Signale der Blütenkätzchen angelockt. Konkrete Untersuchungen, welche Bedeutung der Blütenduft bzw. einzelne Duftkomponenten als Signal für potenzielle Bestäuber hat, gab es bislang nicht.

Im Rahmen der vorliegenden Dissertation wurde deshalb die chemische Zusammensetzung des Blütenduftes verschiedener Weidenarten sowie dessen Variabilität auf inter- und intraspezifischer Ebene analysiert. Anhand eines Fallbeispieles (*Salix caprea*) wurde die Bedeutung von Blütenduft für die Anlockung der gefundenen Bestäuber mittels Elektroantennographie und Biotests in Feld, Flugkäfig und Windtunnel ausführlich untersucht. Mithilfe von Bestäubungsexperimenten wurde die Bedeutung verschiedener Pollenvektoren für den Reproduktionserfolg dieser Weidenart bestimmt.

Chemische Zusammensetzung und Variabilität des Blütenduftes bei *Salix*

Zur Bestimmung der Zusammensetzung und der Variabilität des Blütenduftes innerhalb der Gattung *Salix* wurde der Duft von 93 verschiedenen Individuen (männliche und weibliche) von 34 Arten untersucht. Dazu wurde der Blütenduft von jeweils mehreren Blütenkätzchen eines Individuums mittels der „dynamic headpace MicroSPE“-Methode gesammelt und mit Hilfe von gekoppelter Gaschromatographie und Massenspektrometrie (GC-MS) analysiert. Typische Duftstoffkomponenten der Gattung *Salix* waren Isoprenoide (z. B. α- und β-Pinen, D-Limonen, *cis*- und *trans*-β-Ocimen und Linalool) und aromatische Verbindungen (z. B. Benzaldehyd und 1,4-Dimethoxybenzol). Besonders 1,4-Dimethoxybenzol und *trans*-β-Ocimen waren für die interspezifische Variation (sowohl qualitativ als auch semiquantitativ) verantwortlich.

Von den 34 Arten wurden acht eingehender untersucht (mindestens jeweils drei männliche und drei weibliche Individuen). Die Hälfte der paarweise verglichenen Arten unterschied sich signifikant im Duft. Bei drei von acht untersuchten Weidenarten konnten Unterschiede im Duft zwischen den beiden Geschlechtern festgestellt werden. Beispielsweise enthielt der Blütenduft männlicher *Salix fragilis*-Individuen höhere Anteile an *trans*-β-Ocimen und 1,4-Dimethoxybenzol, während weibliche Individuen dieser Art mehr D-Limonen und D-Verbenon emittierten.

Am Beispiel von *Salix caprea* wurde die tageszeitliche Rhythmik der Duftstoffemission untersucht. Der Blütenduft zeigte sowohl quantitativ als auch qualitativ eine tageszeitliche Variation. Generell wurde tagsüber mehr Duft emittiert als nachts. Die Duftstoffmenge korrelierte hierbei positiv mit der Lufttemperatur. Für die Unterschiede in der chemischen Zusammensetzung waren in erster Linie die verschiedenen Isomere des Monoterpens Lilakaldehyd verantwortlich, die nachts in höheren Mengen produziert wurden als tagsüber. Dies ist vermutlich als Anpassung an nachtaktive Bestäuber – z. B. den Nachtfalter *Orthosia gothica*, der in den durchgeführten Biotests stark von Lilakaldehyd angelockt wurde – zu interpretieren.

Blütenbesucher von *Salix caprea*

Als Blütenbesucher von *Salix caprea* und damit als potenzielle Bestäuber konnten zahlreiche Insektenarten aus unterschiedlichen Ordnungen (Coleoptera, Diptera, Hemiptera, Hymenoptera und Lepidoptera) nachgewiesen werden. Die Besuchsfrequenz war bei Hymenopteren (in erster Linie Honigbienen, Hummeln und solitäre Wildbienen wie z. B. *Andrena vaga*) und Lepidopteren (viele nachtaktive und nur wenige tagaktive Schmetterlinge) am höchsten. Sowohl die Häufigkeit als auch das Artenspektrum der Blütenbesucher variierten innerhalb eines Tages: tagsüber dominierten in erster Linie Bienen (Honigbiene, Hummeln und solitäre Wildbienen) und Tagfalter, nach Einbruch der Dämmerung dagegen waren Nachtfalter die Blütenbesucher. Nachtaktive Blütenbesucher wiesen insgesamt eine wesentlich geringere Frequenz auf als die tagaktiven Besucher.

Reaktionen von *Apis mellifera* und *Orthosia gothica* auf 1,4-Dimethoxybenzol und Lilakaldehyd

1,4-Dimethoxybenzol und Lilakaldehyd sind die Hauptkomponenten des Blütendufts von *Salix caprea*. Dabei dominiert 1,4-Dimethoxybenzol gegenüber Lilakaldehyd sowohl tagsüber als auch nachts. Nachts ist der relative Anteil von 1,4-Dimethoxybenzol geringer als tagsüber, während der relative Anteil von Lilakaldehyd ansteigt. Beide Substanzen sind elektrophysiologisch aktiv, sie lösten Signale in den Antennen sowohl bei *Apis mellifera*, dem häufigsten Blütenbesucher am Tag, als auch bei *Orthosia gothica*, dem häufigsten nachtaktiven Besucher, aus. In Biotests wurde die anlockende Wirkung der beiden Blütenduftstoffe auf diese beiden Insektenarten untersucht. Die Biotests wurden in einem Flugkäfig (*Apis mellifera*) bzw. im Windkanal (*Orthosia gothica*) durchgeführt. Die Honigbiene wurde am stärksten von 1,4-Dimethoxybenzol angelockt, *Orthosia gothica* dagegen von Lilakaldehyd.

Reaktionen von *Andrena vaga* auf einzelne Blütenduftkomponenten von *Salix caprea* und *S. atrocinerea*

Der Blütenduft von *Salix caprea* und *S. atrocinerea* wurden mittels GC-MS analysiert und die Reaktion der oligolektischen Wildbiene *Andrena vaga* auf den Duft mit Hilfe der gekoppelten Gaschromatographie und Elektroantennographie (GC-EAD) getestet. Insgesamt 16 Komponenten des Blütenduftes beider *Salix*-Arten riefen deutliche Signale in den Antennen von *A. vaga* hervor. Die Hauptkomponente der untersuchten Duftextrakte, 1,4-Dimethoxybenzol, führte zu den stärksten Signalen. In einem Biotest im Freiland lockte 1,4-Dimethoxybenzol weibliche *A. vaga* an, aber keine männlichen.

Verhaltensreaktionen von *Apis mellifera* auf männliche und weibliche Individuen von *Salix caprea*

Die Attraktivität von männlichen und weiblichen Blütenzweigen von *Salix caprea* für Honigbienen (*Apis mellifera*) wurde in mehreren Biotests in einem Flugkäfig untersucht.

Männliche Weidenzweige wurden mit höherer Intensität angeflogen als weibliche. Bei nur geringen Unterschieden im Blütenduft beider Geschlechter waren die Blütenkätzchen männlicher Sal-Weiden wegen des gelben Pollens attraktiver als die grünlichen weiblichen Blütenkätzchen. Männliche und weibliche *S. caprea*-Individuen unterschieden sich außerdem

in ihrer Nektarzusammensetzung. Während die Weibchen Hexose-reichen Nektar produzierten, erzeugten die Männchen Saccharose-dominierten. Ob diese Unterschiede ebenfalls zur unterschiedlichen Attraktivität beider Geschlechter beitragen, müssen weitere Untersuchungen zeigen.

Für die Bestäubung bei *Salix caprea* ist diese höhere Attraktivität männlicher Individuen eventuell von Bedeutung, da dadurch die Wahrscheinlichkeit erhöht wird, dass schon vor dem Anflug einer weiblichen Weide Pollen von – möglicherweise sogar mehreren – männlichen Individuen gesammelt worden ist und sich dadurch die Wahrscheinlichkeit einer erfolgreichen Bestäubung erhöht.

Anteil von tag- und nachtaktiven Insekten sowie Wind am Reproduktionserfolg von *Salix caprea*

Um den Beitrag von tag- (v. a. Bienen) und nachtaktiven Insekten (v. a. Nachtfalter) sowie des Windes zur erfolgreichen Bestäubung von *Salix caprea* zu quantifizieren, wurden an fünf ausgewählten weiblichen *Salix caprea*-Individuen Bestäubungsexperimente durchgeführt. Dazu wurden in drei Versuchsvarianten blühende Zweige nachts (Test auf Tagbestäubung), tagsüber (Test auf Nachtbestäubung) und Tag und Nacht (Test auf Windbestäubung) vor Insekten geschützt. Zur Quantifizierung des Reproduktionserfolgs wurden die Samen ausgezählt. Tagaktive Blütenbesucher hatten den größten Anteil am Reproduktionserfolg, während nachtaktive Blütenbesucher und Wind nur zu einem geringen Teil dazu beitrugen. Vermutlich spielten niedrige nächtliche Lufttemperaturen im Untersuchungsjahr und eine daraus resultierende geringe Aktivität von Nachtfaltern eine Hauptrolle für den geringen Anteil der Bestäubung durch nachtaktive Insekten.

Zusammenfassend lässt sich sagen, dass die herkömmlichen Konzepte bezüglich der Interaktionen zwischen Pflanzen und Bestäubern (Spezialisierung vs. Generalisierung) hinterfragt werden müssen. Hinsichtlich des Bestäubungssystems (tagaktive und nachtaktive Blütenbesucher vs. Wind) ist *S. caprea* ein Generalist, der jedoch spezifische Anpassungen (unterschiedliche Duftemission) an bestimmte Insektenarten als potenzielle Bestäuber aufweist und somit als Multispezialist charakterisiert werden kann.

Zusammenfassend lässt sich sagen:

(1) Die Blütenkätzchen der Arten der Gattung *Salix* (Weiden) geben ein reiches artspezifisches Bouquet an Duftstoffen ab. Bei einigen Arten sind auch geschlechtsspezifische Unterschiede nachweisbar.

(2) Eine Vielzahl tag- und nachtaktiver Insekten (hauptsächlich Bienen und Nachtfalter) sind Blütenbesucher bei *Salix caprea.* Tagaktive Insekten sind dabei wesentlich häufiger als nachtaktive.

(3) Verschiedene tag- und nachtaktive Blütenbesucher reagieren auf eine Vielzahl einzelner Komponenten des Blütenduftes von *Salix caprea*.

(4) Der Blütenduft von *Salix caprea* unterliegt einer tageszeitlichen Rhythmik, die mit dem rhythmischen Wechsel des Blütenbesucherspektrums korreliert.

(5) *Apis mellifera* (tagaktiver Blütenbesucher) bevorzugt 1,4-Dimethoxybenzol, während für *Orthosia gothica* (nachtaktiver Blütenbesucher) Lilakaldehyd attraktiver ist.

(6) Männliche *Salix caprea*-Individuen sind – vermutlich aufgrund der gelben Pollenfarbe – für *Apis mellifera* attraktiver als weibliche.

(7) Weibliche *Salix caprea*-Individuen produzieren Hexose-reichen Nektar, männliche Individuen dagegen Saccharose-dominierten.

(8) Für die Bestäubung von *Salix caprea* sind tagaktive Insekten von großer Bedeutung, während nachtaktive Insekten und der Wind nur eine geringe Rolle spielen.

(9) Als Schlussfolgerung ergibt sich, dass das Bestäubungssystem von *Salix caprea* (und vermutlich auch anderer Weidenarten) ein generalistisches ist, welches spezifische Anpassungen an bestimmte Bestäubergruppen aufweist.

7 Erklärung

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

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

Bayreuth, den 24. Oktober 2007

Ulrike Füssel