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PLANT ANIMAL INTERACTIONS

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Floral scent emission and pollinator attraction in two species of *Gymnadenia* (Orchidaceae)

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Abstract We investigated scent composition and pollinator attraction in two closely related orchids, *Gymnadenia conopsea* (L.) R.Br. s.l. and *Gymnadenia odoratissima* (L.) Rich. in four populations during the day and night. We collected pollinators of both species using hand nets and sampled floral odour by headspace sorption. We analysed the samples by gas chromatography with mass spectrometry to identify compounds and with electroantennographic detection to identify compounds with physiological activity in pollinators. In order to evaluate the attractiveness of the physiologically active compounds, we carried out trapping experiments in the field with single active odour substances and mixtures thereof. By collecting insects from flowers, we caught eight pollinators of *G. conopsea*, which were members of four Lepidoptera families, and 37 pollinators of *G. odoratissima*, from five Lepidopteran families. There was no overlap in pollinator species caught from the two orchids using nets. In the scent analyses, we identified 45 volatiles in *G. conopsea* of which three (benzyl acetate, eugenol, benzyl benzoate) were physiologically active. In *G. odoratissima*, 44 volatiles were identified, of which seven were physiologically active (benzaldehyde, phenylacetaldehyde, benzyl acetate, 1-phenyl-2,3-butandione, phenylethyl acetate, eugenol, and one unknown compound). In field bioassays using a mixture of the active *G. odoratissima* compounds and phenylacetaldehyde alone we caught a total of 25 moths, some of which carried *Gymnadenia* pollinia. A blend of the active *G. conopsea* volatiles placed in the *G. odoratissima* population did not attract any pollinators. The two orchids emitted different odour bouquets during the day and night, but *G. odoratissima* showed greater temporal differences in odour composition, with phenylacetaldehyde showing a significant increase during the night. The species differed considerably in floral odour emission and this differentiation was stronger in the active than non-active compounds. This differentiation of the two species, especially in the emission of active compounds, appears to have evolved under selection for attraction of different suites of Lepidopteran pollinators.

Keywords Gas chromatography–electroantennographic detection · Floral volatiles · Phenylacetaldehyde · Orchid pollination · Reproductive isolation

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Introduction

Pollination of flowers by animals is often influenced by a wide variety of volatile floral scent molecules (Knudsen et al. 1993; Dudareva and Pichersky 2000). Floral scent plays an important role in long-distance attraction of pollinators and in their attraction at night, but also over short distances, odour is an important cue for pollinators (Van der Pijl and Dodson 1966; Dodson et al. 1969; Dobson et al. 1999; Knudsen 2002; Plepys et al. 2002a, b). Scent can be an important learning cue in insects (e.g. Daly and Smith 2000; Daly et al. 2001), and in bees odours are learnt more rapidly and with greater retention than colours or other visual cues (Menzel 1985; Dobson 1994). Floral scent may thus influence flower constancy of pollinators (Waser 1986; Dobson 1994), that ensures effective pollen transfer, reduces pollen loss and contributes to the maintenance of reproductive barriers among species (Pellmyr 1986; Grant 1994). In sympatrically flowering plants that may potentially attract the same pollinators, selection for different floral signals,

leading to floral constancy of pollinators or attraction of different pollinator species, is thus expected.

The ecological importance of floral odour is well known in some orchid species. In sexually deceptive orchids, floral scent, although not detectable by humans, is the most important signal in attracting pollinators (Kullenberg 1961; Schiestl et al. 1999, 2003). The food rewarding species of *Gymnadenia* are characterized by a strong fragrance emission during both the day and night, which is produced in osmophores on the surface of the labellum and lateral sepals (Stpiczynska 2001).

The two *Gymnadenia* species in this study are morphologically and genetically similar (Soliva and Widmer 1999; Bateman et al. 2003) and occur in sympatry, where F1 hybrids are only occasionally found (Hess et al. 1976). Therefore, a prezygotic isolation mechanism in pollinator-attracting signals, such as floral scent production, is expected to keep the two species reproductively distinct. Scent compounds, emitted during the day or night, may attract or repel certain pollinators. Additionally, quantitative variation of a combination of compounds could also result in specific pollinator attraction, as shown in orchids pollinated by euglossine bees in tropical America (Dodson et al. 1969). Quantitative differences were found to be prominent in the scents of *Gymnadenia conopsea* and *G. odoratissima*, that produce largely the same set of odour compounds (Kaiser 1993).

In this study, we investigated how pollinator attraction, reproductive success, and reproductive isolation is associated with the differing emission of physiologically and behaviourally active floral odour compounds in two species of *Gymnadenia*. We aimed to answer the following questions:

1. What are the pollinators and reproductive success of the species?
2. Which floral odour compounds are emitted by the species, and which of these compounds attract the pollinators?
3. How do the species differ in scent composition and/or diurnal scent production?

Materials and methods

Natural history

Gymnadenia conopsea (L.) R.Br. s.l. (Orchidaceae) has a wide Eurasian distribution and is common throughout Switzerland, whereas *Gymnadenia odoratissima* (L.) Rich. is more sparse in temperate Europe but is common in some calcareous areas in the Swiss Alps. Both species are found in the same habitats from lowland forests to subalpine meadows up to 2,600 m. *G. conopsea* has been divided into two subspecies, based on different plant and inflorescence size and flowering time. *G. conopsea* (L.) R.Br ssp. *conopsea* flowers from May to June, whereas *G. conopsea* (L.) R.Br ssp. *densiflora* (Wahlenb.) K. Richter (Möseler 1987) flowers from July to August; *G. odoratissima* generally flowers from June to mid August (Hess et al. 1976; Hegi 1980; Reinhard et al. 1991). *G. conopsea* is self compatible yet spontaneous autogamy or apomixis is absent, and significant inbreeding depression was detected in artificially selfed seeds (S. Birrer, unpublished data). Both *Gymnadenia* species produce nectar in their floral spur as reward for their pollinators and have relatively generalist pollination systems, with many species belonging to numerous Lepidopteran families being involved (Brantjes 1984; Van der Cingel 1995; S. Birrer, unpublished data; Vöth 2000). The spur length of the flowers ranges from 10 to 20 mm in *G. conopsea* and only 4–7 mm in *G. odoratissima* (Brantjes 1984; Reinhard et al. 1991).While the dark purple–violet flowers of *G. conopsea* are visited by medium-sized diurnal and nocturnal pollinators (Faegri and Van der Pijl 1979; S. Birrer, unpublished data), the variable but usually more light-coloured and smaller flowers of *G. odoratissima* are mainly visited by nocturnal moths and other small-sized insects (Brantjes 1984; Van der Cingel 1995; Soliva and Widmer 1999).

Localities, plants and pollinators

Three populations each of *G. conopsea* and *G. odoratissima* were studied at four locations in Switzerland during summer 2002 and 2003 (Fig. 1). We sampled two allopatric populations at Ofenpass (*G. odoratissima*, between 17 July 2002 and 14 August 2002) and Zürich (*G. conopsea*, 11 June 2003; 17 June 2003) and four sympatric populations at Münstertal (between 9 July 2002 and 20 July 2002; 3 July 2003) and Davos (24 July 2002; 15 August 2002), where both species occurred within the same area and habitat type and were in bloom at the same time.

Pollinator insects were collected from flowers during the day and at dusk and in the areas surrounding the

Fig. 1 Sampling localities of *Gymnadenia conopsea* and *G. odoratissima* populations in Switzerland. Zürich, allopatric *G. conopsea*; Ofenpass, allopatric *G. odoratissima*. Davos, Münstertal, sympatric populations

plants. Any insects visiting the flowers were caught using hand nets and examined for pollinia before being transferred to the laboratory. Pollinators and scent were collected at the same locations at the same time. Pollinators collected from plants were kept in the refrigerator at 4°C and used for the gas chromatography and electroantennographic detection (GC-EAD) experiments. Thereafter, the insects were identified by dissecting genitalia and comparing their morphology with that of a reference specimen.

Volatile collection, reproductive success

Plants used for odour collection were selected randomly from the populations and individually marked. At least 27 individual plants per population were sampled. For each individual, the total number of flowers per spike at the time of sampling was counted or estimated if >20 flowers were present. Approximately 2 weeks after the end of flowering, reproductive success was measured by determining the percentage of fruits formed per total number of flowers (relative pollination success). Volatiles were sampled by headspace sorption for 1–2 h during the day, between 1000 and 1400 hours, and during the night, between 2100 and 2400 hours. Inflorescences were inserted into polyethylene terephtalate (PET) cooking bags [Nalophan]; air was extracted from the bags by a battery-operated vacuum pump (SKC) at a rate of approximately 100 ml/min. Volatiles were trapped on 5 mg of Porapak Q in a glass tube. Before use, the Porapak was cleaned with 200 µl dichloromethane. Ambient air was collected as control samples to identify background contamination. After odour sampling adsorbed volatiles were eluted from the Porapak with 50 µl of a hexane:acetone (9:1) (Merck, Uvasol) mixture. Samples were sealed in glass vials and stored at −20°C.

Quantitative GC analyses and GC–mass spectrometry

Before analysis, 100 ng *n*-octadecane was added to all samples as an internal standard. One microlitre of each odour sample was injected in split-less mode at 40°C (1 min) into a gas chromatograph (Agilent 6890N) followed by opening the split valve and programming to 300°C at a rate of 10°C/min. The GC was equipped with a HP5 column [30 m×0.32 mm internal diameter (Ø)×0.25 µm film thickness] and a flame ionisation detector (FID); helium was used as carrier gas. Absolute amounts of odour compounds were calculated using the internal standard method (Schomburg 1990). Sampling times and sampling volumes were used to calculate the absolute amount per litre sampled air and hour and plant. Amounts of individual compounds were divided by the sum of all compounds to calculate relative amounts.

For identification of compounds, samples were analysed directly by injecting splitless 1.5 µl into a GC (Carlo Erba Fractovap 4160) or GC-MS (Carlo Erba Mega 5160 coupled to a Finnigan MAT 212 instrument with INCOS computer system) at 40°C (3 min) followed by opening the split valve and programming to 230°C at a rate of 2.5°C/min. The analyses were made on a DB-WAX column (J & W Scientific; 30 m×0.32 mm Ø×0.25 µm film thickness). Compounds were identified by comparison of their mass spectra and retention times with those of authenticated reference samples. To match retention times of the compounds, some GC-MS analyses were done on a HP5 column.

Electrophysiological analyses (GC-EAD)

Physiological activity of individual compounds in the odour samples was examined by combined GC-EAD; (Schiestl and Marion-Poll 2002). GC-EAD analyses were done with pollinators caught in the field (see below). Antennae of pollinators collected from flowers were cut off at their base and mounted between two silver electrodes using electroconductive gel (Parker Laboratories). The preparation was shielded with a Faraday cage to reduce electrical interference. A GC effluent splitter (SGE Australia; split ratio 1:1) was used and the outlet was placed in a purified and humidified airstream. This air was directed over the antenna from which summed olfactory neuron responses (EAD) were recorded with software by Syntech (Hilversum). EAD signals and FID responses were simultaneously recorded.

Bioassays

To test the electrophysiologically active compounds for behavioural activity, attraction experiments with sticky traps were carried out in the *G. odoratissima* population on Ofenpass during the flowering season of 2003. The traps consisted of a white plastic disc 8 cm in diameter to which insect glue was applied (commercial insect exclusion adhesive; Temmen Insektenleim, Hattersheim) and covered with a plastic bowl (Fig. 2). Odour compounds were applied on a small rubber GC septum placed in the middle of the disc. Release rates of volatiles from the septa were determined in the laboratory by collecting emitted odour using headspace sorption for 1, 2 and 4 h, as well as 1 day, after application on the septum (results not shown). According to these results, we applied blends of synthetic odour compounds to each septum using 100 µl hexane as solvent, to match the ratios emitted by the flowers. For *G. conopsea* the blend contained 800 µg benzyl acetate, 260 µg eugenol and 8,000 µg benzyl benzoate. For *G. odoratissima*, 200 µg benzaldehyde, 11,200 µg phenylacetaldehyde, 800 µg benzyl acetate, 1,060 µg phenylethyl acetate and 260 µg eugenol. Traps were placed in an area within the population with few orchids, to avoid "competition" of traps and flowers and mixing of natural floral odour plumes with the plumes of the trap compounds.

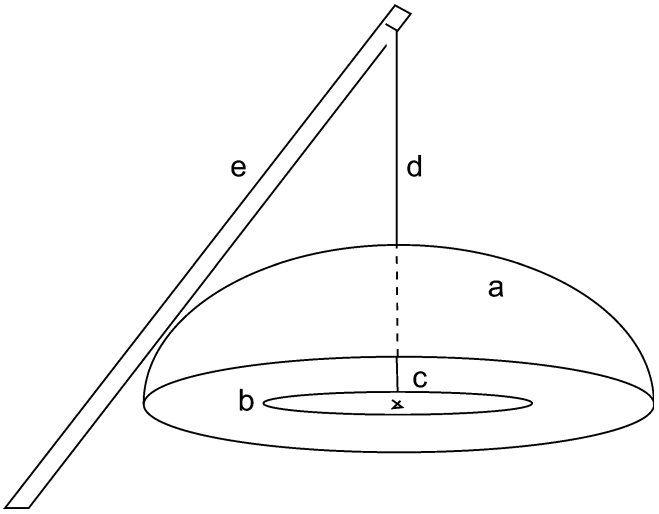

Fig. 2a–e Illustration of a trap used in the bioassays. **a** Blue covering bowl (diameter 15 cm); **b** white disc with glue on both sides (diameter 8 cm); **c** gas chromatography (GC) septum for scent application, on top of the white disc; **d** wire; **e** wooden stick fixed in the ground

The distance between traps was approximately 2 m and no flowering orchids were closer than 50 cm. The traps were left in the field for 4–6 days per trial for three trials. Traps baited with the five single compounds benzaldehyde (five traps), phenylacetaldehyde (15), benzyl acetate (five), phenylethyl acetate (five) and eugenol (five) and with the artificial blends of these compounds imitating the scent of each species (*G. conopsea*, 14; *G. odoratissima*, 15) were set up during each trial. Control traps (13) with no scent added were also set up in each of the three trials.

Statistical analyses

To investigate differences in inflorescences among the species, the numbers of flowers were compared using an independent samples *t*-test. Whether the number of flowers correlates with relative pollination success was assessed by calculating Pearson's correlation coefficient. To compare means of relative and absolute amounts of floral odour compounds emitted during the day and night, we used independent sample *t*-tests. To approach normal distribution and homogeneity of variances, relative amounts of all eight active compounds (benzaldehyde, phenylacetaldehyde, benzyl acetate, 1-phenyl-2,3-butandione, phenylethyl acetate, eugenol, unknown compound and benzyl benzoate) and of the eight most abundant non-active compounds [limonene, phenylethyl alcohol, (*Z*)-isoeugenol (and vanilline), benzyl alcohol, α-pinene, methyl eugenol, nonanal and one unknown compound] were ln $(1+x)$ transformed. To compare odour emissions during the day and night and among species, we calculated discriminant function analyses using the transformed relative amounts. For further comparison of the fragrance emission between species, we used the eight active and eight most abundant non-active compounds in a principal component analysis (PCA) with varimax rotation, extracting factors with an eigenvalue >1. We plotted the samples in a scatter plot using the two factors explaining the greatest proportion of the variance. For all analyses, we used SPSS 11 for Windows (SPSS).

Results

Pollinator insects

All pollinators caught on the orchids are listed in Table 1. We found no overlap in the pollinating insects of the two orchid species.

Number of flowers and pollination success

The two species did not have significantly different numbers of flowers per inflorescence (*G. conopsea*, 27.24 ± 1.7, $n=54$; *G. odoratissima*, 27.17 ± 0.91, $n=104$; $t=0.053$, $P=0.97$). However *G. conopsea* showed a significantly higher relative pollination success as measured by relative fruit set (*G. conopsea*, $86.24\% \pm 1.52$; $n=54$; *G. odoratissima*, $48.24\% \pm 2.54$, $n=101$; $t=12.84$, $P<0.001$). Comparison between the allopatric and sympatric populations of *G. conopsea* was not possible, because the meadow with the allopatric populations was mowed before pollination success could be measured. In *G. odoratissima*, pollination success did not differ between allopatric and sympatric populations (allopatric, $49.17\% \pm 3.14$, $n=72$; sympatric, $46.15\% \pm 4.32$, $n=32$, $t=0.55$, $P>0.05$).

In both species, relative pollination success correlated positively with the number of flowers per inflorescence (*G. conopsea*, $r=0.26$, $n=54$, $P=0.05$; *G. odoratissima*, $r=0.2$, $n=104$, $P=0.04$). Pollination success did not correlate with the absolute or relative amount of any of the physiologically active compounds (Pearson's correlation coefficient, not significant for any compound).

Flower volatiles

We found a total of 51 compounds in the headspace of both species, 45 of which were found in *G. conopsea* and 44 in *G. odoratissima* (Table 2). One compound in *G. conopsea* and two in *G. odoratissima* could not be identified with GC-MS analyses. There was a high degree of overlap in floral compounds produced, as 38 volatiles were found in both species, only seven in *G. conopsea* alone and only six in *G. odoratissima* alone. For further analysis and calculations of the floral scent, 12 compounds detected only in trace amounts were omitted and the remaining 39 more abundant compounds were included.

The mean absolute amount of all floral odour compounds emitted per plant is given in Table 3.

Table 1 The six pollinator species of *Gymnadenia conopsea* and the ten pollinator species of *G. odoratissima* caught with hand nets in the field. *M* Male, *UD* undetermined, *F* female, *N* no, *Y* yes

Species	No.	Sex	Location	Date	Pollinia
G. conopsea					
Hesperiidae					
Thymelicus lineolus (Ochsh.)[a]	1	M	Münstertal	20 July 2002	N
Ochlodes venata (Bremer and Grey)	3	UD	Zürich	17 June 2003	Y
Nymphalidae					
Cynthia cardui (L.)	1	UD	Davos	7 August 2002	Y
Sphingidae					
Macroglossum stellatarum (L.)	1	UD	Zürich	17 June 2003	Y
Noctuidae					
Mythimna conigera (Den. and Sch.)[a]	1	M	Davos	7 August 2002	N
Autographa bractea (Den. and Sch.)[a]	1	F	Davos	24 July 2002	Y
G. odoratissima					
Tortricidae					
Eana osseana (Scop.)[b]	5	FM	Ofenpass	22 August 2002	N
Pterophoridae					
Platyptilia gonodactyla (Den. and Sch.)[b]	1	M	Davos	24 July 2002	N
Pyralidae					
Eudonia sudetica (Z.)[b]	8	M	Davos, Ofenpass	24 July 2002	N
Catoptria speculalis (Hbn.)[b]	1	F	Ofenpass	21 July 2002	Y
Crambus hamellus (Thnbg.)[b]	1	M	Ofenpass	14 August 2002	N
Lycaenidae					
Polyommatus coridon (Poda)[b]	6	M	Ofenpass	14 August 2002	N
Geometridae					
Elophos dilucidaria (Den. and Sch.)[b]	7	FM	Ofenpass	21 July 2002	Y
Gnophos obfuscatus (Den. and Sch.)[b]	2	FM	Ofenpass	14 August 2002	Y
Perizoma verberata (Sc.)[b]	2	FM	Ofenpass	14 August 2002	Y
Entephria caesiata (Den. and Sch.)[b]	4	FM	Ofenpass	14 August 2002	Y

[a]New pollinator species of *G. conopsea*
[b]New pollinator species of *G. odoratissima* (Darwin 1862; Van der Pijl and Dodson 1966; S. Birrer, unpublished data; Vöth 2000 and references therein)

Physiologically active compounds

A total of 42 GC-EAD analyses were carried out on the floral scents of *Gymnadenia conopsea* and *G. odoratissima* using pollinators of the respective species (Tables 2, 4). We found eight substances that elicited a response in the olfactory receptors: three in *G. conopsea* (Fig. 3a) and seven in *G. odoratissima* (Fig. 3b). Two of the substances were active in both species (Table 2). There was no difference in the response between male and female antennae.

Seven of the physiologically active compounds belong to the chemical class of benzenoids and one, eugenol, to the phenyl propanoids. All eight active compounds were the most abundant constituents in the floral odours. In *G. conopsea*, benzyl acetate, eugenol and benzyl benzoate comprised approximately 70–77% of the volatiles. In *G. odoratissima*, benzaldehyde, phenylacetaldehyde, benzyl acetate, 1-phenyl-2,3-butandione, phenylethyl acetate, eugenol and an unknown compound comprised 77–82% of the volatiles.

Bioassays

In the scent-trapping experiments, we caught 26 potential pollinators, of which 30% carried pollinia. *Gymnadenia odoratissima* was the only orchid species flowering in the habitat, so the pollinia were most likely from this orchid. The moths without pollinia were either species already confirmed as pollinators or close relatives. We caught eight pollinators on traps with the artificial *G. odoratissima* floral blend, but as many as 17 on traps with only phenylacetaldehyde, and only one on a control trap (Table 5). None of the other substances and blends attracted any moths. During the 3 weeks of trapping, we observed a temporal change in caught insects from mainly a single species, *Glacies alpinata*, to a more diverse range of pollinators (Table 5).

Day–night change in odour emission

Both *G. conopsea* and *G. odoratissima* differed quantitatively in their floral odour emission between day and night (Tables 2, 3). In both species, the total amount of odour per plant and the amount of odour per flower was significantly lower during the night (Table 3). However, in our sampled populations, there was also a decrease in temperatures from up to 24°C during the day down to 3°C during the night, which may have influenced the amount of odour compounds emitted from the flowers. The relative amounts of active compounds also differed between day and night: *G. conopsea* emitted significantly more benzaldehyde, phenylacetaldehyde, benzyl acetate and significantly less benzyl benzoate during the night. *G. odoratissima* emitted significantly more phenylacetaldehyde, benzyl benzoate and significantly less benzaldehyde and eugenol during the night (Table 2). The increase in phenylacetaldehyde during the night in *G. odoratissima* was the most pronounced change in odour emission (9.4% day, 24.7% night). In a multivariate comparison, both species showed significant differences in odour emission between day and night; however, the differences were more pronounced in *G. odoratissima* (higher χ^2- and eigenvalues) (discriminant function

Table 2 Mean (±SE) relative amounts of the volatile compounds identified in the floral scent of *G. conopsea* and *G. odoratissima* emitted during the day and night. Within the chemical grouping of volatiles, physiologically active compounds in the pollinator of the respective orchid species are given *in italics* and listed first, followed by the other compounds presented in order of retention time. *n* Number of inflorescences sampled

Compound	*G. conopsea*		*G. odoratissima*	
	Day ($n=94$)	Night ($n=27$)	Day ($n=118$)	Night ($n=85$)
Benzenoids				
Benzaldehyde[a]	*1.84 ± 0.17*	*2.75 ± 0.28**	*8.07 ± 0.37*	*5.77 ± 0.43***
Phenylacetaldehyde[a,b]	*0.28 ± 0.03*	*1.03 ± 0.28**	*9.37 ± 0.70*	*24.71 ± 1.57***
Benzyl acetate[a,c]	*50.59 ± 1.94*	*65.32 ± 2.70***	*20.89 ± 1.33*	*17.22 ± 1.43*
1-Phenyl-2,3-butandione[a]	*0*	*0*	*7.69 ± 0.73*	*8.44 ± 0.68*
Phenylethyl acetate[a]	*0*	*0*	*24.78 ± 1.54*	*20.89 ± 1.47*
Benzyl benzoate[c]	*10.64 ± 0.68*	*6.21 ± 0.96***	0.02 ± 0.00	0.24 ± 0.05**
Benzyl alcohol	5.40 ± 0.64	2.17 ± 0.20**	3.36 ± 0.40	0.86 ± 0.08**
p-Cresol	0.68 ± 0.13	0.21 ± 0.03**	0.48 ± 0.08	0.42 ± 0.10
Phenylethyl alcohol	0	0	6.29 ± 0.72	2.72 ± 0.29**
1-Phenyl-1,2-propandione[b]	0.42 ± 0.04	0.33 ± 0.04	0.18 ± 0.02	0.21 ± 0.03
Benzyl propionate	0.26 ± 0.02	0.06 ± 0.02**	0	0
Benzyl butyrate + 3-oxy-4-phenyl-2-butanone	0.27 ± 0.02	0.28 ± 0.05	1.20 ± 0.21	0.29 ± 0.03**
Benzyl 2-methylbutyrate	0.01 ± 0.00	0.02 ± 0.01	0.03 ± 0.02	0.03 ± 0.01
Benzyl isovalerate[b]	0.28 ± 0.04	0.13 ± 0.03*	0.13 ± 0.02	0.21 ± 0.03
Styrol[b,d]	+	+	+	+
2-Phenylpropenal[d]			+	+
Fatty acid derivatives				
Nonanol	0.19 ± 0.02	0.45 ± 0.05**	0.18 ± 0.02	0.22 ± 0.03
Nonanal	0.62 ± 0.08	0.54 ± 0.06	0.82 ± 0.07	0.99 ± 0.15
Decanal	0.55 ± 0.06	1.09 ± 0.12**	0.50 ± 0.07	0.86 ± 0.20
Heptanal[b,d]	+	+		
Octanal[d]	+	+		
(*Z*)-3-Hexenyl acetate[d]	+	+	+	+
Hexyl acetate[d]	+	+		
Pentadecane[d]	+	+		
Phenyl propanoids				
Eugenol[a,c]	*8.91 ± 0.68*	*6.12 ± 1.29*	*4.65 ± 0.43*	*3.11 ± 0.31**
Phenylpropyl alcohol	0.16 ± 0.02	0.08 ± 0.02*	0.06 ± 0.01	0.01 ± 0.00**
(*Z*)-Cinnamic alcohol[b]	0.32 ± 0.04	0.05 ± 0.01**	0.79 ± 0.11	0.11 ± 0.03**
Cinnamic aldehyde	0.18 ± 0.02	0.09 ± 0.02*	0.41 ± 0.05	0.07 ± 0.01**
(*E*)-Cinnamic alcohol	0.78 ± 0.08	0.72 ± 0.16	0.21 ± 0.04	0.13 ± 0.03
Phenylpropyl acetate	0.84 ± 0.07	0.82 ± 0.11	0.40 ± 0.05	0.19 ± 0.03**
(*Z*)-Cinnamyl acetate[b]	0.62 ± 0.05	0.25 ± 0.08**	0.16 ± 0.03	0.09 ± 0.02*
Methyl eugenol	9.83 ± 0.74	3.91 ± 0.34**	0.07 ± 0.02	0.18 ± 0.04*
(*Z*)-Isoeugenol[b] + vanilline	0.73 ± 0.08	1.90 ± 0.24**	1.90 ± 0.32	3.38 ± 0.59*
(*E*)-Cinnamyl acetate	0.25 ± 0.02	0.16 ± 0.03*	0.12 ± 0.02	0.05 ± 0.01*
(*E*)-Isoeugenol[b]	0.53 ± 0.04	0.37 ± 0.07	0.22 ± 0.03	0.08 ± 0.01**
(*Z*)-Methyl isoeugenol[b]	0.18 ± 0.02	0.24 ± 0.07	0.18 ± 0.08	0.29 ± 0.08
(*E*)-Methyl isoeugenol[b]	0.09 ± 0.01	0.02 ± 0.01*	0.43 ± 0.07	0.19 ± 0.03*
Acetyl eugenol[b]	0.17 ± 0.01	0.51 ± 0.05**	0.12 ± 0.02	0.35 ± 0.05**
Elemicine	2.89 ± 0.29	0.91 ± 0.12**	0.03 ± 0.01	0.06 ± 0.01
Isoelemicine	0.23 ± 0.02	0.19 ± 0.02	0.03 ± 0.00	0.11 ± 0.02**
Isoprenoids				
α-Pinene	0.18 ± 0.02	0.55 ± 0.05**	2.29 ± 0.26	2.30 ± 0.24
Limonene	0.44 ± 0.07	0.64 ± 0.05	0.60 ± 0.07	1.15 ± 0.13**
6-Methyl-5-hepten-2-one[d]	+	+	+	+
6-Methyl-5-hepten-2-ol[d]	+	+		
Geranylacetone[d]	+	+		
Miscellaneous				
Unknown (*active*)[a] MS: 162(45), 147(2), 119(40), 105(4), 91(100), 89(8), 65(14), 51(4), 43(37)	*0.09 ± 0.01*	*0.07 ± 0.02*	*2.05 ± 0.22*	*2.32 ± 0.28*
Unknown (non-active)	0.41 ± 0.07	1.67 ± 0.68	0.98 ± 0.33	1.55 ± 0.37
Benzofuran[d]			+	+
α-Hydroxyacetophenone[d]			+	+

*$P < 0.05$, **$P < 0.001$ significantly different from day (independent samples *t*-test)

[a] Active in *G. odoratissima*

[b] Compounds new for *G. conopsea* (Kaiser 1993)

[c] Active in *G. conopsea*

[d] Compounds not used in calculations but listed as present (+) or absent in each species

Table 3 Calculated mean (±SE) absolute amounts of total scent emitted per inflorescence and per flower, in ng/l per h for each species. *n* Number of inflorescences sampled

	G. conopsea		*G. odoratissima*	
	Day ($n=60$)	Night ($n=25$)	Day ($n=110$)	Night ($n=76$)
Per inflorescence	196.20 ± 16.45	155.58 ± 17.2**	158.79 ± 14.44	56.63 ± 5.52**
Per flower	10.45 ± 0.98	8.02 ± 0.99**	9.28 ± 0.96	2.9 ± 0.3**

**$P < 0.001$ significantly different from day (independent samples *t*-test)

analysis, *G. conopsea*, $\chi^2 = 55.52$, $df = 6$, eigenvalue, 0.61, $P < 0.001$; *G. odoratissima*, $\chi^2 = 214.06$, $df = 8$, eigenvalue, 1.96, $P < 0.001$).

Species differences

In a comparison of floral odours, we found clear differences between the two species, shown in the plot of factor loadings of the PCA conducted on both active and non-active compounds (Fig. 4). The differentiation between the species was more pronounced in the active than in the non-active compounds. A discriminant function analysis for the eight active and the eight most abundant non-active compounds showed that χ^2- and eigenvalues were considerably higher in the analysis using the active compounds (discriminant function analysis, active compounds, $\chi^2 = 727.29$, eigenvalue, 33.14, $df = 8$; non-active compounds, $\chi^2 = 440.10$, eigenvalue, 7.47, $df = 8$).

Discussion

Pollinators of *Gymnadenia*

Surprisingly little is known about pollinator assemblages of most plant species, although this information is crucial for the ecological investigations of reproductive traits (Waser et al. 1996; Johnson and Steiner 2000). In our study we recorded a total of 18 Lepidoptera species carrying *Gymnadenia* pollinia; three species are here reported for the first time for *G. conopsea* and 12 for *G. odoratissima* (Tables 1, 5) (Darwin 1862; Van der Pijl and Dodson 1966; S. Birrer, unpublished data; Vöth 2000, and references therein). Interestingly, we did not find an overlap in the observed pollinator species. In agreement with this, Vöth (2000) reports no overlap of the insects carrying pollinaria of the two *Gymnadenia* species. These data suggest that the attraction of different suites of pollinators can act as a prezygotic reproductive barrier between the two orchid species.

Behaviourally active fragrances

Floral scents are often complex blends of secondary metabolites (Kaiser 1993; Knudsen et al. 1993). The floral scent of *G. conopsea* s.l. and *G. odoratissima* consisted of 51 odour compounds belonging mainly to the chemical classes of benzenoids and phenyl propanoids (Table 2). In *G. conopsea*, we found the same major compounds as Kaiser (1993); however, (*E*)-cinnamic alcohol and especially 6-methyl-5-hepten-2-ol, which were abundant in the samples of Kaiser (1993), were only minor compounds in our samples. Furthermore, we found 12 volatile compounds that have not been identified in *G. conopsea* before (Kaiser 1993). The differences between our results and those of Kaiser (1993) may be explained by differences among the sampled

Table 4 Pollinators used for gas chromatography–electroantennographic detection experiments and compounds that were physiologically active for the particular insect species. Pollinators were caught while they were visiting flowers in the field. *1* Benzaldehyde, *2* phenylacetaldehyde, *3* benzyl acetate, *4* 1-phenyl-2,3-butandione, *5* phenylethyl acetate, *6* eugenol, *7* unknown, *8* benzyl benzoate; for abbreviations, see Table 1

Pollinator species	No. animals analysed (F/M)	No. of analyses	Active compounds
G. conopsea floral odour			
Mythimna conigera (Den. and Sch.)	1 M	2	3, 6, 8
Autographa bractea (Den. and Sch.)	1 F	3	3, 6, 8
G. odoratissima floral odour			
Elophos dilucidaria (Den. and Sch.)	3 M	6	3, 4, 5
Eudonia sudetica (Z.)	3 M	7	1, 2, 3, 4, 5, 6, 7
Gnophos obfuscatus (Den. and Sch.)	1 F, 1 M	3	2, 3, 4, 5, 6
Perizoma verberata (Sc.)	1 F, 1 M	3	No response
Entephria caesiata (Den. and Sch.)	1 M	4	1, 2, 3, 4, 5, (6)
Eana osseana (Scop.)	2 F, 2 M	6	2, 3, 4, 5
Polyommatus coridon (Poda)	6 M	8	No response

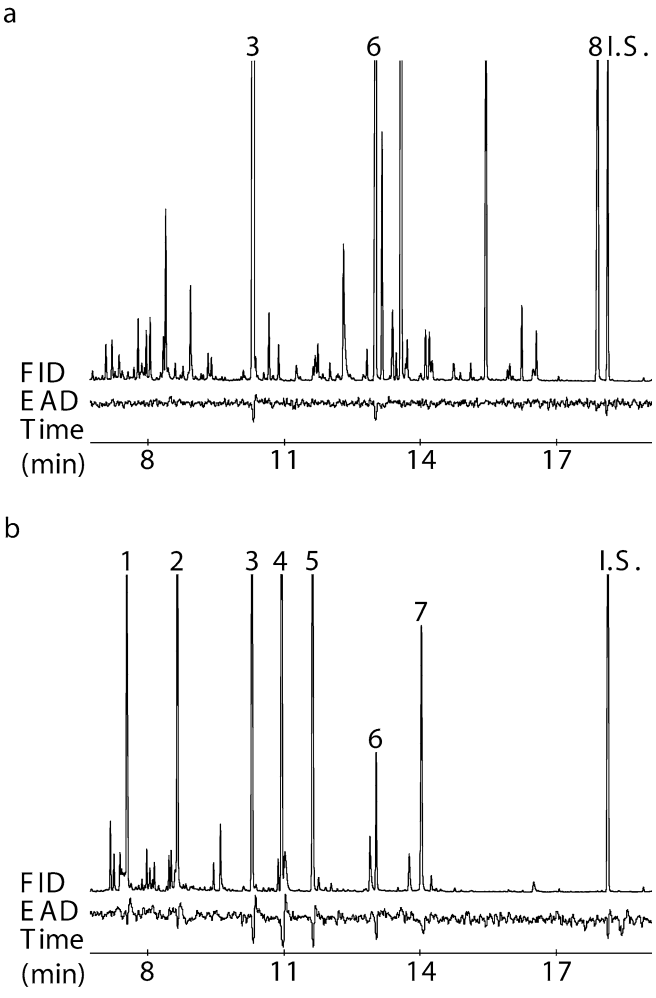

Fig. 3 Gas chromatographic analysis with simultaneous flame ionisation detection (*FID*) and electroantennographic detection (*EAD*) of **a** *G. conopsea* floral odour sample using an antenna of a pollinator, *Mythimna conigera* (Lepidoptera, Noctuidae) and **b** *G. odoratissima* floral odour sample using an antenna of *Eudonia sudetica* (Lepidoptera, Pyralidae). For names of active compounds see Table 1. *I.S.* Internal standard

populations, but also differences in sampling methods, since Kaiser collected scent from flowers placed in glass vials whereas we used PET bags. To our knowledge, there is as yet no extensive list of floral odour volatiles emitted by *G. odoratissima*. Overall, floral scent of *Gymnadenia* is a complex blend of compounds, originating from different biosynthetic pathways, which is generally true for many angiosperms (Knudsen et al. 1993; Raguso et al. 2003; Jürgens et al. 2003).

Given that often not all floral odour compounds have a signal function with respect to pollinators (Schiestl and Marion-Poll 2002), it is important to identify physiologically and behaviourally active compounds within the fragrance. In our investigations, the most abundant compounds in both orchid scents were also the ones that were physiologically active in the pollinator's antennae. All the active compounds have been reported in the floral scent of many other plants (Knudsen et al. 1993) and have been shown previously to elicit electrophysiological responses in Lepidoptera antennae (summarized in Schiestl and Marion-Poll 2002; Andersson 2003a). Electrophysiologically active compounds may attract but also repel pollinators (Omura et al. 2000), and thus, bioassays are necessary to evaluate the behavioural effect of scent compounds.

When we tested active *Gymnadenia* compounds in field experiments, we found that the artificial mixture of *G. odoratissima* attracted eight pollinator insects, and phenylacetaldehyde alone, attracted 17 insects. The lower catches of the whole blend of active compounds may be explained by the differences between the artificial blend and the natural floral odour, that might be readily discernible for most insects. Other factors, i.e. different shape of traps and flowers, may also have reduced the trap catches. Phenylacetaldehyde, the most attractive scent in our study, has been reported earlier as an attractant for diurnal and nocturnal Lepidoptera (Creighton et al. 1973; Cantello and Jakobson 1979; Haynes et al. 1991; Heath et al. 1992; Honda et al. 1998; Andersson and Dobson 2003) and honeybees (Blight et al. 1997). Andersson et al. (2002) describes phenylacetaldehyde as a characteristic compound of butterfly-pollinated flowers because of its widespread occurrence in such plants, but the compound has also been found in many other plants (Knudsen et al. 1993). In our study, none of the active compounds other than phenylacetaldehyde attracted pollinators when offered as single substances. Other studies also showed that only few constituents of floral fragrances may trigger behavioural responses in pollinators. Plepys et al. (2002b) found a mixture of four lilac aldehyde isomers to elicit a similar behavioural response as the complete blend of floral odour of *Platanthera bifolia* in the pollinator *Autographa gamma*. All other compounds elicited significantly lower responses. These data raise the question about the function of other compounds emitted by the flowers. In plants that are pollinated by a range of different insect species, selection on odour bouquets may vary considerably among populations, depending on the local preferences of pollinators. Additionally, pollinators may vary during the flowering period and among years (Maadt and Alexandersson 2004). In our study, we observed a change in trapped insects from mainly one single species (*Glacies alpinata* Sc.) to a more diverse pollinator composition during our 3-week scent-trapping period (Table 5). Widely distributed and long-lived plants like *Gymnadenia* may therefore be selected to produce a wider range of odour compounds with a potential attractiveness to many insects. Our field-trapping approach using synthetic compounds constitutes an attractive possibility to test this assumption in the future.

The lack of pollinators attracted to the artificial scent of *G. conopsea* in our study was most likely due to the location of the traps in the allopatric population of *G. odoratissima*. Although the *G. conopsea* population of Münstertal was only 2 km away and in a similar habitat

Table 5 Potential pollinator species caught with scented sticky traps in the allopatric population of *G. odoratissima* on Ofenpass. A total of 11 species were caught in 2003. For abbreviations, see Table 1

Species	No. of individuals caught (F/M)	Date	Pollinia (Y/N)
G. odoratissima blend			
Amphisbatidae			
Anchinia laureolella (H.-S.)	1 M	14 July 2003	N
Zygaenidae			
Zygaena exulans (Hochw.)[a]	1 M	14 July 2003	Y
Adscita geryon (Hbn.)	1 M	18 July 2003	N
Hesperiidae			
Hesperia comma (L.)	1 M	18 July 2003	N
Pyralidae			
Catoptria pyramidella (Tr.)	1 M	18 July 2003	N
Nymphalidae			
Eurodryas aurinia debilis (Obth.)	1 F	18 July 2003	N
Geometridae			
Glacies alpinata (Sc.)[a]	1 F, 1 M	8 July 2003	Y
Phenylacetaldehyde			
Zygaenidae			
Zygaena exulans (Hochw.)[a]	3 F	18 July 2003	N
Adscita geryon (Hbn.)	1 M	18 July 2003	N
Pyralidae			
Orenaia cf. *andereggialis* (H.-S.)	1 F	18 July 2003	N
Eudonia sudetica (Z.)	1 M	18 July 2003	N
Phycitodes saxicola (Vaugh.)	1 F	18 July 2003	N
Geometridae			
Glacies alpinata (Sc.)[a]	3 F, 7 M	8–18 July 2003	Y
Control			
Geometridae			
Elophos dilucidaria (Den. and Sch.)	1 F	18 July 2003	N

[a]First record as a pollinator of *G. odoratissima*

for insects, pollinators may have local preferences according to the abundances of food plants in their habitats (Pellmyr 1986). Future trapping experiments in sympatric populations are required to better assess the role of active floral compounds emitted by *G. conopsea*.

Diurnal changes in floral odour

Regarding the differences in floral odour between day and night, we observed differences both in absolute and relative amounts of floral volatiles. Diurnal rhythms in fragrance emission were found in *Odontoglossum constrictum* showing a pronounced nocturnal minimum, and *Hoya carnosa* emitting almost no volatiles during the day (Matile and Altenburger 1988). In our samples, there was a significant decrease in the absolute amounts of floral scent emitted during the night in both species, but the large temperature differences between day and night may have had an impact on the quantity of secreted volatiles by the flowers.

Relative amounts were also found to differ between day and night in both *Gymnadenia* species, and climatic factors should not have influenced the composition of relative amounts (Jakobsen and Olsen 1994). The overall magnitude of these difference was greater in *G. odoratissima*, but in both species, diurnal changes in odour bouquets influenced the compounds differently, with phenylacetaldehyde showing the most dramatic nocturnal increase in *G. odoratissima*. Previous studies on rhythms of fragrance emission have reported asynchronism in the emission of different volatiles and suggest that biosynthetic pathways may have different diurnal rhythms (Matile and Altenburger 1988; Loughrin et al. 1990). Loughrin et al. (1990) proposed that the increased nocturnal emission of aromatic compounds released by *Nicotiana sylvestris* may enhance the attraction of night pollinating insects such as moths. While our data likewise suggest that phenylacetaldehyde in *G. odoratissima* represents an adaptation to attract nocturnal moths, in our field bioassays, phenylacetaldehyde attracted both primarily nocturnal (*Eudonia sudetica*) and diurnal moths (*Zygaena exulans*, *Adscita geryon*, and *Glacies alpinata;* W. Sauter, unpublished observations). *G. conopsea*, also showed a minor nocturnal increase in phenylacetaldehyde, and this species has been shown earlier to be pollinated by both diurnal and nocturnal insects (S. Birrer, unpublished data). More trapping experiments during the day and night are necessary to better understand the role of nocturnal increase in phenylacetaldehyde emission.

Pollinator attraction and prezygotic isolation

Pollinators may function as isolation barriers among plant species by assortative transferring of pollen (Grant 1949). Common mechanisms of such prezygotic isolation are morphological barriers to hybridization, like different spur lengths, that have been shown to be under selection, e.g. in *Aquilegia* (Hodges 1997; Fulton and Hodges 1999) or *Platanthera* (Nilsson 1983). As shown in Nilsson (1983) and Brantjes (1984), differences in spur length in *Gymnadenia* may lead to unidirectional isolation, as gene flow

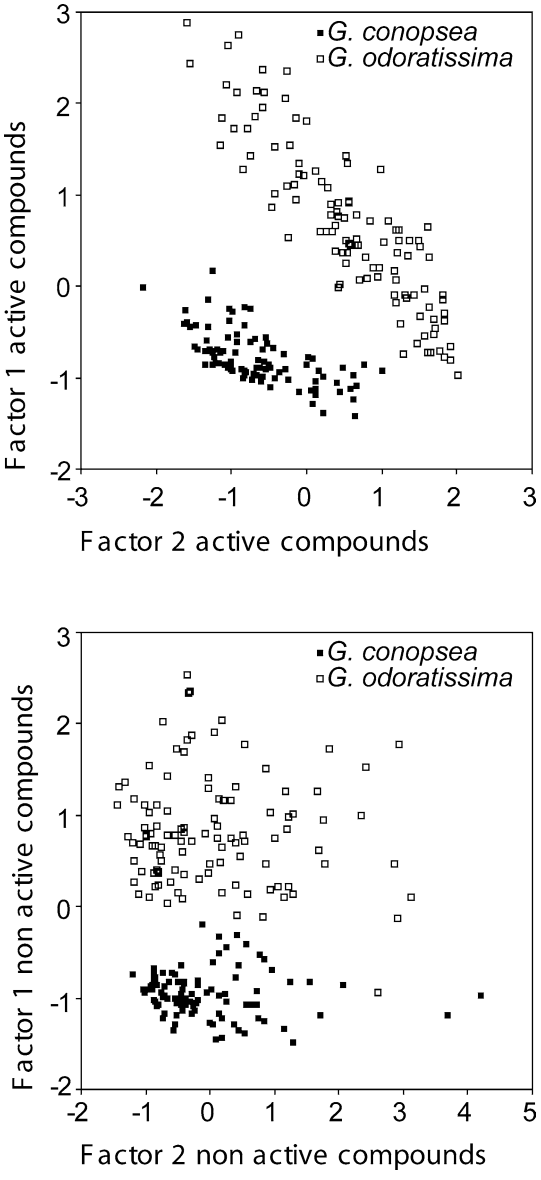

Fig. 4 Comparison of floral odour in *G. conopsea* and *G. odoratissima* by scatter plots of factor loadings explaining 82% of the total variance in a principal component analysis (PCA) of active compounds, and explaining 60% of the total variance in a PCA of non-active compounds

should only occur from the short- to the long-spurred species, in this case from *G. odoratissima* (spur length 4–7 mm) to *G. conopsea* (10–20 mm). Floral signals like colour and odour may also act as prezygotic isolation mechanisms in preferentially attracting certain pollinator species (Grant 1994; Hodges et al. 2002). In our study we found that an array of different diurnal and nocturnal Lepidoptera species pollinate the two *Gymnadenia* orchids; nevertheless, there seems to be little or no overlap in the insects attracted to the two orchid species. Although the role of floral colour in filtering pollinators has probably been overemphasised in earlier work (Johnson and Steiner 2000), colour differences in *Gymnadenia* may also contribute to different pollinator attraction, and/or reinforce the impacts of floral scent differences (Raguso and Willis 2002). In *G. conopsea*, flowers are mostly pink to red and much less variable in colour than flowers of *G. odoratissima*, which frequently vary from white to red (F. P. Schiestl and F. K. Huber, in preparation). Future trapping experiments could use coloured traps in combination with scent, to investigate the role of colour in pollinator attraction.

One important mechanism leading to differential pollinator attraction may be the differences in floral odour we found among the *Gymnadenia* species. Our trapping experiments clearly show that floral odour alone can attract the pollinators, stressing the importance of this floral trait. Different pollinators may have different innate preferences for certain odour compounds (Plepys 2002a, b; Andersson 2003b), but pollinators may also learn floral odour bouquets and use this to maintain flower constancy (Pellmyr 1986; Waser 1986). Interspecific differences in floral odour have been found in numerous investigations and have been interpreted as cues for attracting distinct pollinators (Gregg 1983; Nilsson 1983; Ågren and Borg-Karlson 1984; Knudsen et al. 1993; Raguso and Pichersky 1995; Raguso et al. 2003; Jürgens et al. 2003). Species differences, however, may also be a non-adaptive result of random genetic processes. Our data show that differences between the two *Gymnadenia* species were less pronounced in the non-active than in the active compounds, that are responsible for pollinator attraction. Since many active and non-active compounds are chemically related and stem from similar biosynthetic pathways, random processes alone cannot explain different pattern of differentiation of these compounds among the species (McKay and Lätta 2002). Our findings thus support the assumption that selection for differential pollinator attraction may act to set apart biologically active floral scent compounds. In conclusion, we propose that differences in floral odour among the two *Gymnadenia* species, in combination with other floral traits such as colour and differences in spur length, have evolved under selection as a means of attracting a different pollinator spectrum and hence enhancing prezygotic isolation.

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