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EVOLUTION OF REPRODUCTIVE STRATEGIES IN THE SEXUALLY DECEPTIVE ORCHID *OPHRYS SPHEGODES*: HOW DOES FLOWER-SPECIFIC VARIATION OF ODOR SIGNALS INFLUENCE REPRODUCTIVE SUCCESS?

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Abstract.—The orchid *Ophrys sphegodes* Miller is pollinated by sexually excited males of the solitary bee *Andrena nigroaenea*, which are lured to the flowers by visual cues and volatile semiochemicals. In *O. sphegodes*, visits by pollinators are rare. Because of this low frequency of pollination, one would expect the evolution of strategies that increase the chance that males will visit more than one flower on the same plant; this would increase the number of pollination events on a plant and therefore the number of seeds produced. Using gas chromatography–mass spectrometry (GC-MS) analyses, we identified more than 100 compounds in the odor bouquets of labellum extracts from *O. sphegodes*; 24 compounds were found to be biologically active in male olfactory receptors based on gas chromatography with electroantennographic detection (GC-EAD). Gas chromatography (GC) analyses of odors from individual flowers showed less intraspecific variation in the odor bouquets of the biologically active compounds as compared to nonactive compounds. This can be explained by a higher selective pressure on the pollinator-attracting communication signal. Furthermore, we found a characteristic variation in the GC-EAD active esters and aldehydes among flowers of different stem positions within an inflorescence and in the n-alkanes and n-alkenes among plants from different populations. In our behavioral field tests, we showed that male bees learn the odor bouquets of individual flowers during mating attempts and recognize them in later encounters. Bees thereby avoid trying to mate with flowers they have visited previously, but do not avoid other flowers either of a different or the same plant. By varying the relative proportions of saturated esters and aldehydes between flowers of different stem positions, we demonstrated that a plant may take advantage of the learning abilities of the pollinators and influence flower visitation behavior. Sixty-seven percent of the males that visited one flower in an inflorescence returned to visit a second flower of the same inflorescence. However, geitonogamy is prevented and the likelihood of cross-fertilization is enhanced by the time required for the pollinium deposited on the pollinator to complete its bending movement, which is necessary for pollination to occur. Cross-fertilization is furthermore enhanced by the high degree of odor variation between plants. This variation minimizes learned avoidance of the flowers and increases the likelihood that a given pollinator would visit several to many different plants within a population.

Key words.—*Andrena nigroaenea*, chemical communication, learning behavior, *Ophrys sphegodes*, pollination by sexual deception, reproductive success, scent variation.

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Pollination by sexual deceit is exclusive to the Orchidaceae (Ackerman 1986; Nilsson 1992), and has been documented in orchids growing in Australia (Peakall 1990), South America (van der Pijl and Dodson 1966) and Europe (Kullenberg and Bergström 1976; Paulus and Gack 1990). Orchids of the genus *Ophrys* grow in Europe, especially around the Mediterranean, and are pollinated by male insects, mostly bees (Andrenidae, Anthophoridae, Colletidae, Megachilidae, and Apidae), but occasionally by predatory (Sphecidae) and parasitic wasps (Scoliidae) and in a few cases by beetles (Scarabaeidae; Kullenberg 1961, 1973; Borg-Karlson 1990; Paulus and Gack 1990). Male insects are lured to the orchid by visual cues and volatile semiochemicals. At close range, chemical signals from the flowers elicit sexual behavior in males, which respond in a manner similar to that shown to sex pheromones of females, whereby the males try to copulate with the flower labellum. During these so-called pseudocopulations, a male touches the gymnostemium of the flower and the pollinarium may become attached to the insect's head or in some species to the tip of the abdomen; pollination takes place when the male visits another flower.

Numerous behavioral tests have shown that the *Ophrys*-pollinator relationship is highly specific: Each *Ophrys* species is pollinated by males of usually only one or a few pollinator species (Kullenberg 1961, 1973; Paulus and Gack 1986, 1990, 1994). Because *Ophrys* orchids are usually interfertile, the species-specific attraction of pollinators is important for the reproductive isolation of each species.

Ophrys flowers tend to produce complex bouquets of volatiles typically consisting of more than 100 species-specific chemical compounds (Borg-Karlson et al. 1985, 1987; Borg-Karlson 1987, 1990). Behavioral experiments with synthetic copies of the compounds produced by *Ophrys* flowers have shown that only certain volatiles are active in stimulating mating behavior in the males (Kullenberg and Bergström 1976; Tengö 1979; Borg-Karlson 1990). Among the compounds produced by *O. sphegodes*, 14 hydrocarbons that were identified in similar proportions in cuticle extracts of the pollinators' females elicited copulation attempts in the males (Schiestl et al. 1999). Therefore, not all of the compounds may have a function in attracting male pollinators. The role of those compounds that do not trigger copulatory behavior in the males remains unknown.

Because flower visits in non-food-rewarding pollination systems are rare and brief (Peakall and Beattie 1996), food and sexual deceptive plant species often show low levels of

fruit set compared to food-rewarding species (Hutchings 1987; Gill 1989; Neiland and Wilcock 1995). In six investigated species of deceptive orchids, 75–98% of all plants lacked fruit set (Calvo 1990). In a population of *O. sphegodes* near Illmitz (Austria), only 4.9% of 887 plants were visited by a pollinator, as revealed by either pollinia removal, pollination, or both (Ayasse et al. 1997). A high specialization in *Ophrys* orchids to one or a very few pollinators may be the most important reason for the observed low pollination frequency. As an adaptation to this, flowers are relatively long-lived and the opportunity for pollination is maximally extended (Dafni and Bernhardt 1990). Furthermore, pollinated flowers produce a high number of seeds (van der Cingel 1995).

Such a low pollination frequency should lead to the evolution of strategies that increase the chance that males visit more than one flower on the same plant, increasing the number of pollination events and therefore the number of seeds produced. However, pollinators successively visiting several flowers within an inflorescence may increase the frequency of geitonogamy (pollination of a neighboring flower). Furthermore, if they return to an already visited flower, autogamy (self-pollination) may occur. Although autogamy or geitonogamy have an obvious advantage in maintaining a high seed production even if pollinators are scarce, inbreeding depression may occur. In *Orchis morio,* self-pollinated flowers showed a delimited fruit formation compared to outcrossed flowers (Neiland and Wilcock 1995), and in *Caladenia tentaculata* outcrossing seeds developed more quickly than selfed seeds (Peakall and Beatie 1996). Due to these effects on fitness, pollinators should visit several different plants in a population and selection should enhance reproductive success at the level of population as well as the individual plant.

Another reason for the low frequency of pollination in sexually deceptive orchids may be the learning capabilities of pollinators. Behavioral experiments have shown that male bees are able to learn the distinctive odor bouquets of individual female bees during mating attempts (Smith and Ayasse 1987; Dutzler and Ayasse 1996) and that they use this information to avoid females they have already mated with. Bees might respond in a similar manner to *Ophrys* fragrances and individual unpollinated flowers might vary in their odor bouquets both within populations and even within the same inflorescence to increase the frequency of pollinated flowers. The evolutionary mechanism could be frequency-dependent response of male pollinators to floral signals (Paulus 1988). This would promote selection for floral scent variation. However, in sexually deceptive orchids, it has not yet been determined whether different odors are produced by the flowers of different plants or by the flowers within a single inflorescence.

The hypothesis that variation in flower characteristics in non-food-rewarding flowers inhibits the formation of a search image by the pollinator (Heinrich 1975; Nilsson 1980; Ackerman 1986) and interferes with its associative learning process was tested by Moya and Ackerman (1993), who found high levels of flower fragrance variation in the nectarless, moth-pollinated orchid *Epidendrum ciliare.* Intraspecific variation in floral fragrances has also been documented for nectar-producing orchids, including *Platanthera stricta* (Patt et al. 1989) and *P. bifolia* (Tollsten and Bergström 1989). However, no behavioral tests were performed to show whether these variations of odor bouquets from various flowers of the same or of different plants can be learned by pollinators. These important aspects of the behavior of pollinators at deceptive flowers thus remain to be documented.

The aim of this study was to determine whether individual flower-specific olfactory recognition signals exist in the orchid *O. sphegodes* and if the pollinator males learn and recognize odors of individual flowers. We addressed these goals through: (1) behavioral learning experiments in the field with pollinating male bees of *Andrena nigroaenea*; (2) quantitative chemical analyses of the odors from individual flowers; (3) gas chromatography with electroantennographic detection (GC-EAD) analyses to identify biologically active compounds (compounds perceived by the males); and (4) behavioral experiments in the field to show how self-pollination is avoided when a pollinator visits more than one flower on the same inflorescence.

Materials and Methods

Study Species

Ophrys sphegodes sphegodes Miller grows on alkaline, dry to damp soils in meadows, grassland, and scrubland of the Mediterranean region and Central Europe (Delforge 1994). In Austria, *O. sphegodes* is mostly confined to the east and southeast. It flowers in April and May. Flower spikes are rarely more than 20 cm tall and one inflorescence is produced per stem. Most plants bear two to five flowers that open successively. Flowers are long-lived (14–21 days) if unpollinated, fading rapidly after pollination (Schiestl et al. 1997). *Ophrys sphegodes* is pollinated by *A. nigroaenea* males (Godfery 1925; Paulus and Gack 1986, 1990), a solitary bee species that is common in the area of our study and nests in the soil (Westrich 1989). Because females' nests are mostly dispersed, males search for and mate with females at non-resource-based landmarks or patrol along resources visited by females for self-nourishment (flowers). Sex pheromones emitted by females are important cues used by males to locate and/or recognize mates at these rendezvous sites (Tengö 1979). Typically, males engage in a scramble contest for virgin females.

Study Sites

We collected plants of *O. sphegodes* at two distinct populations: (1) the Bisamberg population, approximately 10 km to the north of Vienna; and (2) the Illmitz population, close to the eastern border of Lake Neusiedl. The distance between the populations is 30 km. All behavioral tests were conduced in Oberweiden and Weikersdorf (Eastern Austria). To avoid habituation of the males, we used several test locations (10–100 m apart) that were changed after a few tests.

Flower Odor Sampling

Individual *Ophrys* labella were cut from flowers and extracted in 0.5-ml pentane (Uvasol, Merck, Darmstadt, Germany) at 20°C for 2 days. The labella were then removed and the samples were stored at −20°C until chemical analyses

were performed. Because we could show that extracts of cut labella as well as mixtures of synthetic compounds mixed according to labella extracts modulate pollinator behavior (see Schiestl et al. 1999; this study), we can exclude effects of cutting off labella. Therefore, the results of our chemical analyses reflect the volatiles released by intact flowers.

Chemical Analyses

Labella solvent extracts were analyzed on a gas chromatograph HP 5970 (Hewlett-Packard, Palo Alto, CA) equipped with a DB5 capillary column (30 m × 0.32 mm internal diameter). The gas chromatograph was operated splitless at 120°C for 30 sec, followed by a programmed increase to 280°C at 4°C/min. For quantitative analysis, n-octadecane was added as an internal standard. Structure elucidation of individual compounds was based on gas chromatography–mass spectrometry (GC-MS) analysis (VG70/250 SE instrument, Vacuum Generators, Manchester, England, linked to a HP 5890; gas chromatographic condition as mentioned above); mass spectra were compared with those reported in the literature (Mc Lafferty and Stauffer 1989) and gas chromatographic retention times (coinjection) with those of authentic reference samples. Double-bond positions in unsaturated compounds were assigned according to Buser et al. (1983) and Dunkelblum et al. (1985). The stereochemistry of double bonds was determined by comparison of retention times using corresponding reference samples, including DMDS derivatives. The erythro- and threo-aducts can be well separated by GC.

Electrophysiology

To find chemical compounds within *Ophrys* odors to which male antennal olfactory receptor neurons are sensitive, the tip of an excised male antenna was cut off and the antenna was mounted between two pipet electrodes containing insect-Ringer, and grounded via an Ag-AgCl wire. The tip electrode was connected to a high-impedance DC amplifier with automatic baseline drift compensation. A Hewlett-Packard 5890 gas chromatograph equipped with a DB-Wax capillary column (30 m × 0.32 mm internal diameter) and effluent split was used for simultaneously recording flame ionization (FID) and electroantennographic (EAD) signals. Helium was used as carrier gas and the effluent split ratio was approximately 1:1. The gas chromatograph was operated splitless at 50°C for 1 min, followed by opening the split and programming to 230°C at 10°C/min. The outlet for the EAD was placed in a charcoal-filtered, moisturized airstream flowing over the antennal preparation at a speed of 0.5 m/sec. One microliter per sample was injected into the column. GC-EAD active compounds were identified by GC-MS (methods, see above) and synthesized. Additional GC-EAD analyses with these compounds were performed.

Statistical Analysis

Three principal component analyses (PCAs) were performed on a set of 114 labella solvent extracts. We used the relative proportions of 24 GC-EAD active components for two PCAs and of 71 nonactive components for a third PCA. The resulting principal components (PCs) with an eigenvalue above one were used to test for differences in odor bouquets by means of a discriminant function analyses (DFA). We compared the uppermost flowers of different plants growing in different populations and the uppermost and the second uppermost flowers of an inflorescence using the calculated factor scores for each case on the principal axes (SPSS 1997). The standardized discriminant function coefficients and the factor loadings after varimax rotation were used to assess the importance of individual compounds. We considered a compound to have a high factor loading if the loading was above 0.5.

To compare the distances (dissimilarities) of odor bouquets produced by various flowers, we used the factor scores. Values for factor scores were first standardized to *Z*-scores (with a mean of zero and a standard deviation of one), then a dissimilarity matrix was computed by measuring the Euclidean distance values between cases. Measurements were based on the factor scores of two PCAs performed either with the relative proportions of 24 GC-EAD active or with 71 inactive compounds. Because the measurements of the active and inactive compounds were based on a different number of variables, the resulting Euclidean distance values had a different range. To make the data of the dissimilarity matrices of the active and nonactive compounds comparable, we rescaled the distance values to a range of zero to one.

Behavioral Tests

On the same day the behavioral experiments were performed, flowers were collected at random from the Bisamberg and Illmitz populations. Individual flowers were fixed on insect pins and offered to the males at separate locations. Copulation attempts were recorded during a 3-min test period. Between 10 to 20 males from each location (two locations in Weikersdorf and six locations in Oberweiden) were individually marked with enamel paint on the top of the thorax. Observations of individually marked males indicate that males do not move freely throughout the entire mating area. Most marked males could be observed at spots no more then 10 m away from where they were marked. Therefore, mostly the same males were tested within any one area.

Learning Experiments with the Pollinating Male Bees

To test the hypothesis that males can learn the distinctive odor bouquet of individual flowers, in a first behavioral experiment a flower was tested in one location and retested 2 min afterwards in the same location. After 2 min, the same flower was tested at another location of mostly other males patrolling. Choices of the two locations were random for each test. While performing the tests, we determined that two of 20 males we marked in one of the test locations could also be found in the neighboring test location. Because males that moved between both locations had been able to learn a tested flower offered at a second test location, we excluded the results of those tests.

A second experiment was performed to exclude both visual learning of a flower and odor marking by a pollinating male. Males were offered a flower and a dead, odorless female of *A. nigroaenea*. These dummies had been Soxhlet-extracted

TABLE 1. Comparison of the mean absolute amounts (μg) of GC-EAD active aldehydes and saturated esters in labellum extracts of the uppermost ($n = 20$) and the second uppermost flowers ($n = 20$) of an inflorescence.

Compounds	Flower stem position: Uppermost Mean ± SEM	Flower stem position: Second uppermost Mean ± SEM	Difference
Octadecanal*	0.14 ± 0.02	0.24 ± 0.02	0.10
Eicosanal*	0.40 ± 0.05	0.59 ± 0.05	0.19
2-Nonyl tetradecanoate	0.84 ± 0.07	1.02 ± 0.12	0.18
2-Nonyl hexadecanoate*[1] + Dodecyldodecanoate	0.80 ± 0.09	1.34 ± 0.16	0.54
Dodecyl tetradecanoate*	0.41 ± 0.04	0.94 ± 0.12	0.53

* Significant difference, *t*-test, $P < 0.001$. [1]Compounds that could not be separated by gas chromatography.

in dichlormethane for 48 h, dried and fixed on an insect pin. The flower was upside down and under the dummy bee, where it was out of reach of the incoming male. Therefore, males stimulated by the scent of a flower touched the dummy bee only. After 2 min, the same dummy bee was retested with a second flower of another plant. Our working hypothesis was that either marking a visited dummy with repellents (anti-aphrodisiac) or learning the visual cues of the two flowers should result in a decreased attractiveness during the retest of a dummy female.

To test if males can discriminate between odor bouquets in flowers of the same or different plants, a flower was tested in one location, and 2 min afterwards either a flower of the same inflorescence or a flower of another plant of the same population or of a plant of a second population was tested in the same location.

Another experiment was performed to test the hypothesis that males in successive visits of an inflorescence differentiate between flowers by learning the flower-specific patterns of chemical compounds. Among the compounds that were found to be GC-EAD active, esters and aldehydes did not elicit mating behavior in the male pollinators (F. P. Schiestl, unpubl. data). Our chemical analyses showed the differences between the uppermost and the second uppermost flowers of an inflorescence were mainly in the concentrations of these compounds. Therefore, our hypothesis that the males learn flower-specific patterns of esters and aldehydes was tested by offering the uppermost flower of an inflorescence to the males in one location. Thereafter, we impregnated the labellum surface with 20 μl of a mixture of synthetic esters and aldehydes. The mixture contained only those compounds whose total amount of odor in the labellum extracts was higher in the second uppermost then in the uppermost flowers of an inflorescence (Table 1). From each compound, we added exactly the differential amount between both groups of flowers. Because 2-nonyl hexadecanoate and dodecyl dodecanoate could not be separated by GC analyses, our test mixture contained 50% of both compounds. The impregnated flowers were tested in the same location with the same males patrolling. In a control experiment, flowers were retested after impregnation with solvent only (20 μl pentane Uvasol).

Behavioral Experiments to Prove How Self-Pollination Is Prevented

Ten plants with three flowers each were picked on the day of the behavioral experiments by sampling at random from the Bisamberg population. Plants were kept in glass tubes filled with water and transported to Oberweiden. Single plants were offered to male bees at two separate locations 30 m apart. The number of individually marked males that visited one flower of an inflorescence and returned to visit another flower of the same inflorescence were recorded. We compared the time spent on any visited flower and the lapse between leaving a visited flower of an inflorescence and arriving on another flower of the same inflorescence (bee flying time).

In all *Ophrys* species, the pollen is packed in one mass, the pollinarium (van der Cingel 1995). During visits by pollinators, the pollinia become attached to the insect's head or tip of the abdomen, and a bending mechanism subsequently takes place, whereby the pollinium swings forward through an angle of about 90°. After this, the pollinium is in the correct position to fit into the stigma of another flower (Darwin 1862). This bending, which takes place within a matter of seconds or several hours, decreases geitonogamy and enhances the likelihood of cross-fertilization. To do so, the time required for the pollen stalk to complete its bend should be longer than the amount of time that a pollinator spent on a plant (Catling and Catling 1991). In *O. sphegodes*, we recorded the time needed by a removed pollinium to complete its bend.

RESULTS

Chemical Analyses

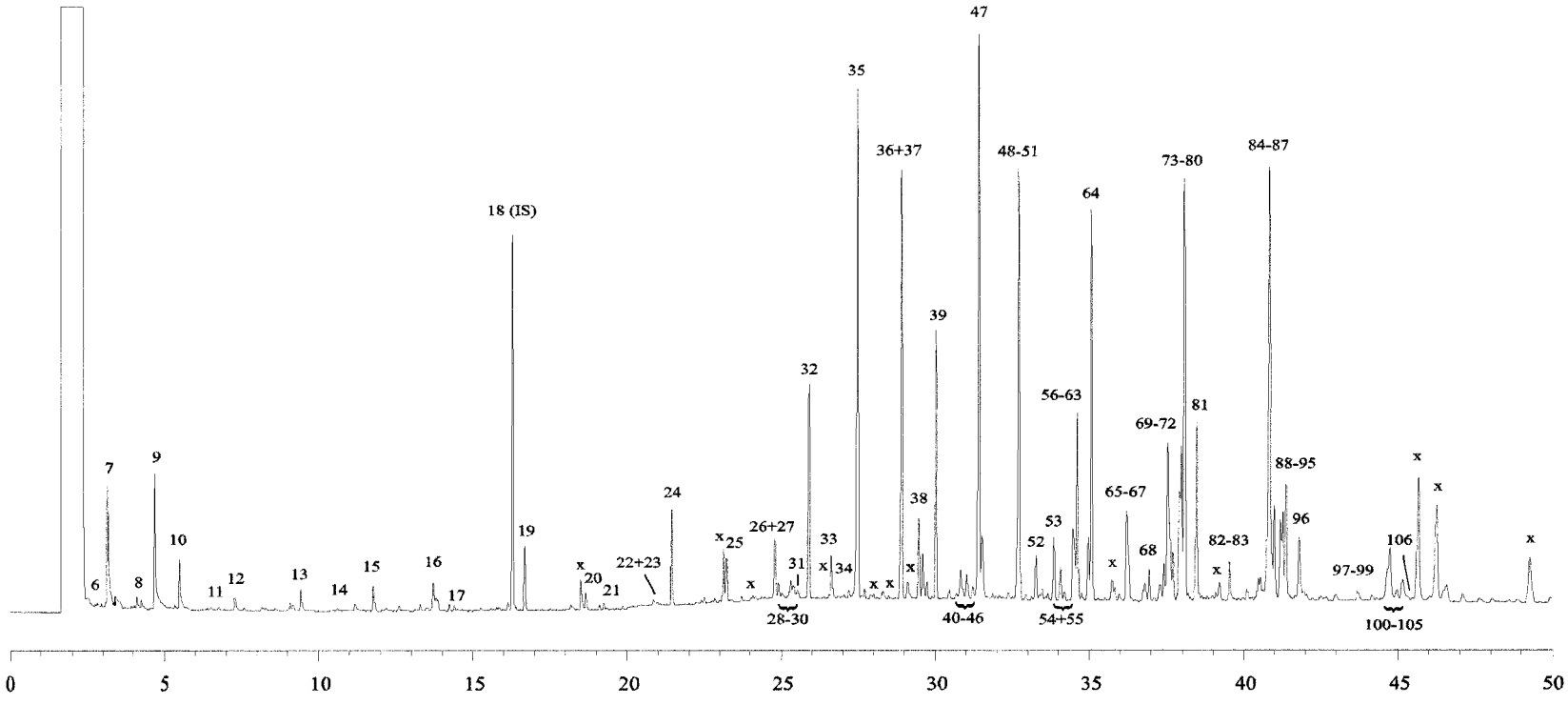
We identified 106 compounds in solvent extracts of *O. sphegodes* labella. The volatile bouquets are dominated by saturated nonterpenoic esters, n-alkanes, n-alkenes, and n-alkadienes with chain lengths of 19–33 carbons and aldehydes with chain lengths of 9–22 carbons. In addition, small amounts of fatty acids and terpenoic esters were identified (see Fig. 1).

Electrophysiology

The results of simultaneous FID and EAD analyses performed with labellum extracts of *O. sphegodes* showed that n-alkanes and n-alkenes, aldehydes, saturated nonterpenoic esters, E,E-farnesyl hexanoate, E,E-farnesol, and nonanoic acid triggered receptor potentials in the antennae of males (see Fig. 2). From 24 compounds that were found to be biologically active, 14 could be identified as n-alkanes or n-alkenes with the double bond in position 12, 11, or 9. Recently, the importance of these compounds in stimulating mating behavior in the males was shown by behavioral experiments (Schiestl et al. 1999).

Intraspecific Variation of the Odor Bouquets of Labellum Extracts

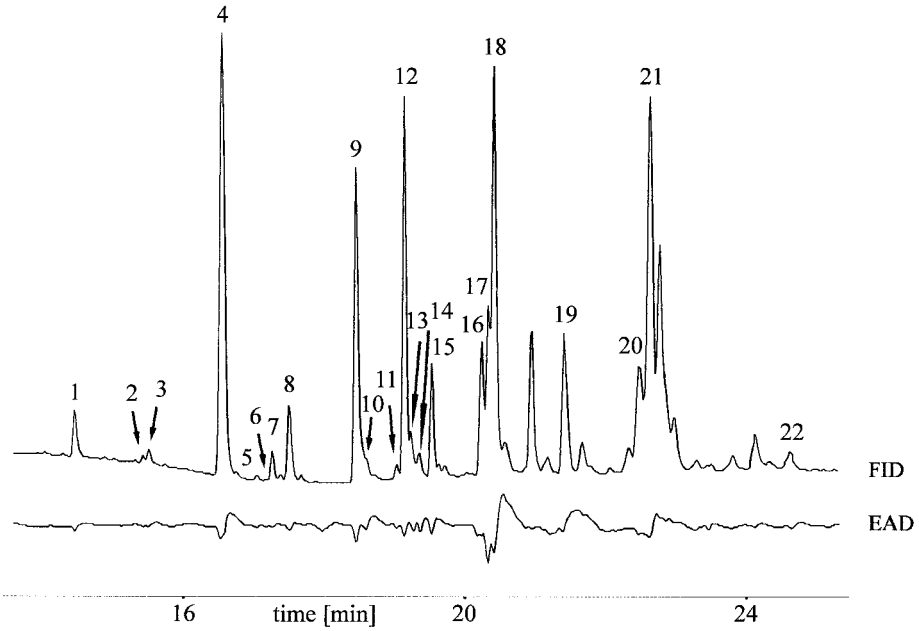
The first PCA included the relative proportions of 24 GC-EAD active compounds in 82 labellum extracts. We used only samples of the uppermost flowers from plants of the



List of compounds

1 α-Pinene
2 β-Pinene
3 Limonene
4 Caryophyllene
5 α-Humulen
6 Nonanal
7 Heptanoic acid
8 Decanal
9 Nonanoic acid
10 Undecanal
11 Decanoic acid
12 Dodecanal
13 Tridecanal
14 Dodecanoic acid
15 Tetradecanal
16 Heptadecane
17 E, E-Farnesol
18 Octadecane (IS)
19 Hexadecanal
20 Nonadecane
21 Hexadecanoic acid
22 1-Eicosene
23 Eicosane
24 Octadecanal
25 Heneicosane
26 2-Nonyl dodecanoate
27 Heptyl tetradecanoate
28 E, E-Farnesyl hexanoate
29 Octadecadienoic acid
30 Octadecanoic acid
31 Docosane + 1-Docosene
32 Eicosanal
33 9-Tricosene
34 7-Tricosene
35 Tricosane
36 2-Nonyl tetradecanoate
37 Octyl tetradecanoate
38 Tetracosane
39 Docosanal
40 5-Pentacosene
41 7-Pentacosene
42 8-Pentacosene
43 9-Pentacosene
44 10-Pentacosene
45 11-Pentacosene
46 12-Pentacosene
47 Pentacosane
48 Dodecyl dodecanoate
49 Tetradecyl decanoate
50 2-Nonyl hexadecanoate
51 Octyl hexadecanoate
52 Hexacosane
53 Tetradecanal
54 8,19-Heptacosadiene
55 9,17-Heptacosadiene
56 5-Heptacosene
57 7-Heptacosene
58 8-Heptacosene
59 9-Heptacosene
60 10-Heptacosene
61 11-Heptacosene
62 12-Heptacosene
63 13-Heptacosene
64 Heptacosane
65 Dodecyl tetradecanoate
66 Tetradecyl dodecanoate
67 Hexadecyl decanoate
68 Octacosane
69 8,19-Nonacosadiene
70 8,20-Nonacosadiene
71 9,15-Nonacosadiene
72 9,17-Nonacosadiene
73 7-Nonacosene
74 8-Nonacosene
75 9-Nonacosene
76 10-Nonacosene
77 11-Nonacosene
78 12-Nonacosene
79 13-Nonacosene
80 14-Nonacosene
81 Nonacosane
82 Tetradecyl tetradecanoate
83 Hexadecyl dodecanoate
84 8,22-Hentriacontadiene
85 9,15-Hentriacontadiene
86 9,17-Hentriacontadiene
87 9,21-Hentriacontadiene
88 7-Hentriacontene
89 8-Hentriacontene
90 9-Hentriacontene
91 11-Hentriacontene
92 12-Hentriacontene
93 13-Hentriacontene
94 14-Hentriacontene
95 15-Hentriacontene
96 Hentriacontane
97 8,23-Tritriacontadiene
98 9,15-Tritriacontadiene
99 9,17-Tritriacontadiene
100 7-Tritriacontene
101 9-Tritriacontene
102 11-Tritriacontene
103 12-Tritriacontene
104 13-Tritriacontene
105 15-Tritriacontene
106 Tritriacontane
107 Steroid MW 414
108 Steroid MW 412

FIG. 1. Gas chromatogram of the solvent extract of an *Ophrys sphegodes* labellum, with over 100 compounds identified (x, compounds not yet identified).



List of GC-EAD active compounds:

1. Heneicosane
2. Nonanoic acid
3. Docosane
4. Tricosane
5. Octadecanal
6. E, E-Farnesol
7. 2-Nonyl dodecanoate
8. Tetracosane
9. Pentacosane
10. (Z)-9-Pentacosene
11. Eicosanal
12. 2-Nonyl tetradecanoate
13. E, E-Farnesyl hexanoate
14. Hexacosane
15. Linolenic acid ethylester
16. Heptacosane
17. (Z)-12+(Z)-11-Heptacosene*
18. (Z)-9-Heptacosene
19. Dodecyl dodecanoate + 2-Nonyl hexadecanoate*
20. (Z)-12+(Z)-11-Nonacosene*
21. (Z)-9-Nonacosene
22. Dodecyl tetradecanoate

FIG. 2. Simultaneous flame ionization/detection and electroantennographic analyses performed with an *Ophrys sphegodes* labellum extract. Mostly saturated and unsaturated hydrocarbons triggered receptor potentials in the antennae of males (*, compounds that could not be separated with the gas chromatography parameters used).

two populations (Bisamberg and Illmitz). Six PCs with an eigenvalue above one explained 73.4% of the total matrix variation. In most cases, compounds from the same chemical classes were found to be associated with each of the PCs.

A further PCA was based on the relative proportions of the GC-EAD active compounds of 40 labellum extracts from uppermost and second uppermost flowers collected in Illmitz. Six PCs with an eigenvalue above one explained 79.9% of the total matrix variation. As in the first PCA, predominantly compounds from the same chemical classes were found to be associated with each of the PCs.

The third PCA was performed with the relative proportions of the nonactive compounds from the same labellum extracts as the second PCA. It revealed highly variable odor bouquets produced by various flowers. Fifteen PCs with an eigenvalue above one explained 88.9% of the total matrix variance. The results of the PCA using varimax rotation showed various compounds from different chemical classes of compounds to be associated with each of the PCs.

Comparison of Odor Bouquets from Flowers of Different Populations

The odor bouquets comparisons based on the relative proportions of 24 GC-EAD active compounds showed significant differences between populations. The labellum extracts of the uppermost flowers from Bisamberg ($n = 33$) and Illmitz ($n = 49$) were well separated by a DFA performed with the calculated factor scores of six PCs ($\chi^2 = 47.3$, df = 6, $P < 0.001$). The standardized canonical discriminant function coefficients and the factor loadings showed that the differences between the populations were mainly in concentrations of n-alkanes, nonacosene (double-bond positions 11 and 12), (Z)-9-tricosene, as well as aldehydes (octadecanal, eicosanal), E,E-farnesol, and nonanoic acid.

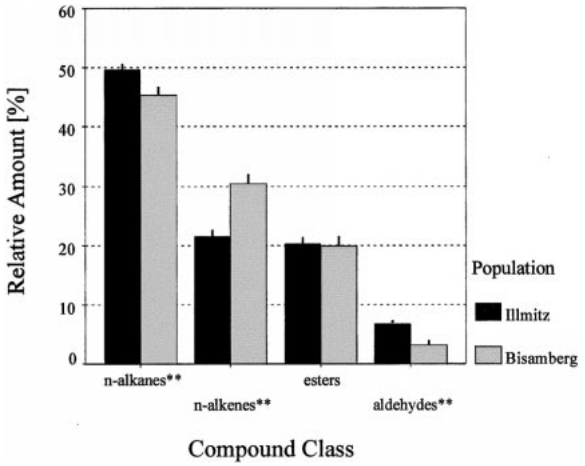


FIG. 3. Comparison of the relative proportions (mean + SEM) of the main chemical classes of compounds in labellum extracts of the uppermost flowers from Bisamberg (n = 33) and Illmitz (n = 49; **, significant difference, t-test, $P < 0.01$).

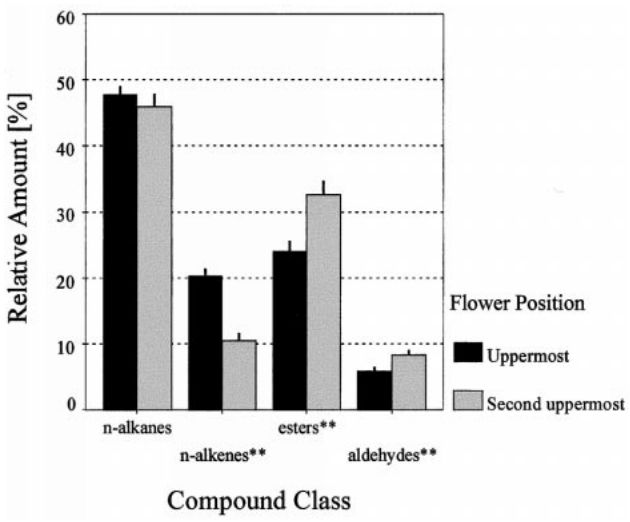


FIG. 4. Comparison of the relative proportions (mean + SEM) of the main chemical classes of compounds in labellum extracts of the uppermost and the second uppermost flowers of an inflorescence (**, significant difference, t-test, $P < 0.01$).

A univariate comparison of the relative proportions of the main chemical classes of compounds is shown in Figure 3. n-alkanes and aldehydes were relatively more abundant at Illmitz, n-alkenes were higher at Bisamberg, and nonterpenoic esters did not differ between populations.

Comparison of the Odor Bouquets Produced by Flowers of Different Stem Positions

The multivariate comparison performed with the factor scores of six PCs showed a significant difference between the uppermost and the second uppermost flowers of an inflorescence (DFA: $\chi^2 = 35.1$, df = 6, $P < 0.001$). Because the standardized canonical discriminant function coefficients were highest for PC1 and PC2, nonterpenoic esters, aldehydes (octadecanal, eicosanal), E,E-farnesol, nonanoic acid, and (Z)-9-heptacosene contributed most to the differences between flower positions. When comparing the relative amounts of the main chemical classes of compounds, we found that n-alkenes were relatively more abundant in the uppermost flowers, nonterpenoic esters and aldehydes were higher in the second uppermost flowers, and n-alkanes did not differ between flowers of different stem positions (Fig. 4). There was no significant difference in the total amount of odor in the labellum extracts from the uppermost (mean ± SEM = 11.59 ± 0.58 μg, n = 20) and second uppermost flowers (mean ± SEM = 12.90 ± 0.74 μg, n = 20) of an inflorescence (t-test, $t = -1.39$, df = 38, $P = 0.174$). A comparison of the amount of odor of single compounds, however, showed an increase in certain aldehydes and saturated esters (Table 1).

Similarities of Odor Bouquets Produced by Individual Flowers

We found the smallest mean Euclidean distance value when we compared the relative proportions of GC-EAD active compounds from the uppermost and second uppermost flower of the same inflorescence (mean ± SEM = 0.21 ± 0.03, n = 20, see Fig. 5), indicating a high similarity of odor bouquets. Within the Illmitz population, odor bouquets produced by (uppermost) flowers of different plants showed significantly larger distance values (mean ± SEM = 0.39 ± 0.02, n = 70; $t = -4.49$, df = 89, $P < 0.001$). When we used the relative proportions of GC-EAD nonactive compounds, the calculated mean Euclidean distance values were significantly larger as compared to those based on GC-EAD active compounds (Fig. 5).

Learning Experiments with the Male Pollinators

Field learning experiments showed that *O. sphegodes* flowers fixed on insect pins were highly attractive to *A. nigroaenea* males, which tried to copulate with the flowers. A retest of the same flower in the same location triggered significantly fewer copulation attempts and pouncing events as compared to the first test (t = 2.64, df = 22, P = 0.02; Sokal and Rohlf 1995; see Fig. 6, left). When a flower was transferred to a second test location where different males patrolled, its at-

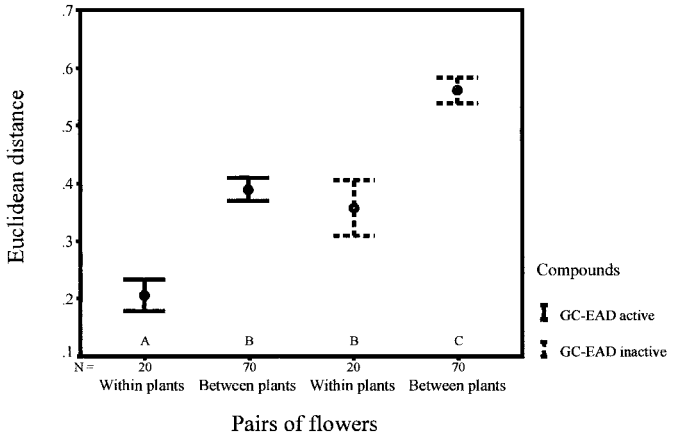


FIG. 5. Comparison of Euclidean distance values (mean ± SEM) of odor bouquets from flowers within and between plants (different letters indicate significant differences; t-test, $P < 0.05$).

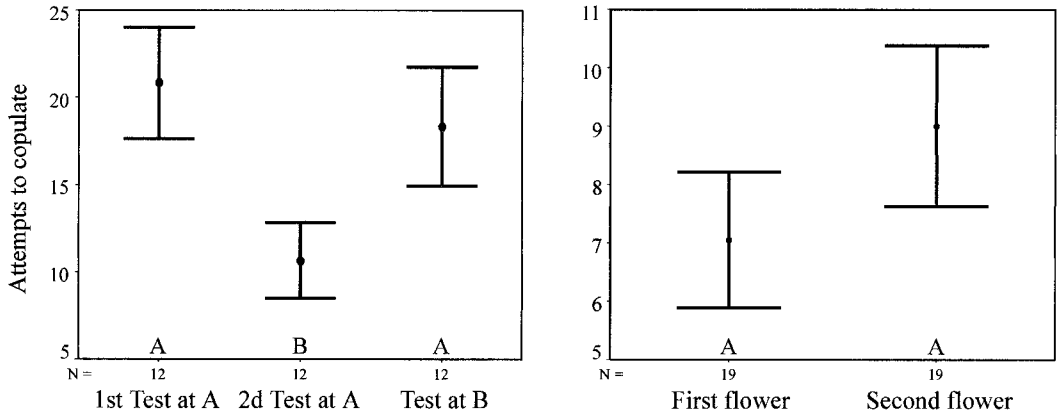


FIG. 6. Field studies on the learning of distinct odor bouquets from individual flowers by male *Andrena nigroaenea*. Flower attractiveness measured by the mean number of copulation attempts (mean ± SEM) that males performed on a flower. Left: A flower was offered in location A for 3 min. It was retested in the same location and in a second location (B) where other males patrolled. Right: Experiment to exclude both visual learning of a flower and odor marking by a pollinating male. Males were offered a dead, odorless female *A. nigroaenea* and a flower fixed on an insect pin upside down beneath the dummy bee, invisible and out of reach of the incoming male. After 2 min, the same dummy bee was retested with a flower of another plant (different letters indicate significant differences; *t*-test, $P < 0.05$).

tractiveness increased again ($t = 1.89$, df = 22, $P = 0.07$) and was not significantly different to the attractiveness in the first test ($t = 0.54$, df = 22, $P = 0.59$). To exclude both visual learning of a flower and odor marking by a pollinating male, we conducted retests on dead, odorless females in the same location using the same males that were stimulated to mate by the scent of a second flower of another plant. These experiments resulted in the same or even slightly higher attractiveness of the dead female ($t = -1.08$, df = 36, $P = 0.29$; see Fig. 6, right).

A retest in the same location using a second flower either from the same inflorescence or from another plant from the same or a different population showed that the attractiveness of the two flowers was the same (Table 2).

We tested the hypothesis that the males learn flower-specific patterns of esters and aldehydes by first offering the uppermost flower of an inflorescence to males in one location and then by retesting the same flower after impregnation with esters and aldehydes. In a retest the attractiveness of the flower was not diminished (Table 3). However, when the flower in the retest was impregnated with solvent only (control), it elicited significantly fewer copulation attempts than in the first test (Table 3).

Behavioral Observations and Experiments to Prove How Self-Pollination Is Prevented

From 30 males that visited one flower of an inflorescence, 20 males (67%) returned to visit a second flower of the same inflorescence. Some males even visited a third flower of an inflorescence. The time from the beginning until the end of the bending mechanism, which amounts to the time needed by a removed pollinium to bend into a correct position for fitting into the stigma of another flower, is longer than the total time for visiting the first and second flower of an inflorescence, including the transit time between flowers (Fig. 7).

DISCUSSION

Intraspecific Variation of Flower Scent

Orchids of the genus *Ophrys* produce a huge number of volatiles (Borg-Karlson 1990). By GC-MS analyses and coinjection technique we identified a complex mixture of compounds in the labellum extracts of *O. sphegodes*. However, behavioral experiments and EAD analyses have shown that only some of these compounds are active in stimulating mat-

TABLE 2. Field studies of learning of individual flowers' distinct odor bouquets by male *Andrena nigroaenea*. In all test groups, the attractiveness (mean number of copulation attempts) of both flowers tested one after another in the same location was the same.

Test groups (*n*)	Attractiveness: First flower Mean ± SEM	Attractiveness: Second flower Mean ± SEM	*t*-test *P*
Flowers of the same inflorescence (24)	8.83 ± 1.59	10.33 ± 1.44	0.49
Flowers of different plants (31)	7.68 ± 1.47	8.87 ± 1.72	0.60
Flowers of different populations (20)	14.65 ± 2.55	16.70 ± 2.46	0.57

TABLE 3. Learning experiments to test hypothesis that males learn individual flowers of an inflorescence by the patterns of aldehydes and esters. The uppermost flower of an inflorescence was offered in a location for 3 min and retested after impregnation with (1) aldehydes and esters; or (2) pentane (control). Attractiveness was measured by the mean number of copulation attempts.

Test groups (*n*)	Attractiveness: First test Mean ± SEM	Attractiveness: Second test Mean ± SEM	*t*-test *P*
Flowers impregnated with aldehydes and saturated esters (15)	8.33 ± 1.71	8.47 ± 1.49	0.95
Flowers impregnated with pentane (10)	7.00 ± 0.92	4.00 ± 0.60	0.01

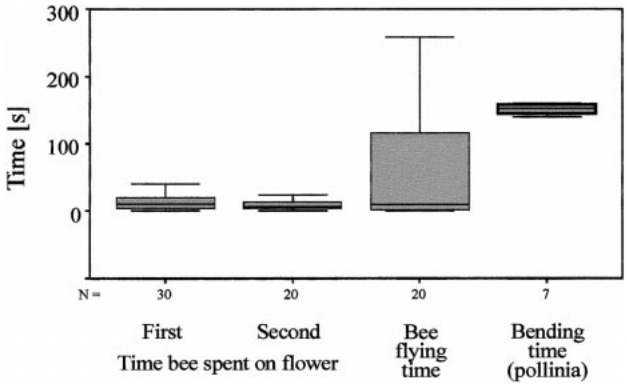


FIG. 7. Behavioral experiments to test how self-pollination is prevented. We recorded and compared the time (median) spent on any visited flower, bee flying time between visiting flowers of the same inflorescence, and time needed by a removed pollinium to bend into a correct position for fitting into the stigma of another flower. Boxes represent interquartile ranges and are divided at the median. Stems represent smallest or largest observations within 1.5 interquartile ranges of the bottom or the top of the boxes.

ing behavior in males (Schiestl et al. 1999). Similar information also exists for other *Ophrys* species (Priesner 1973; Kullenberg and Bergström 1976; Borg-Karlson 1990). In *O. lutea,* a blend of synthetic compounds consisting of aliphatic 1-alcohols, 2-alcohols, and terpenes elicited the same level of excitation in *Andrena fuscipes* males as did whole labellum extracts (Borg-Karlson and Tengö 1986). Why do *Ophrys* flowers produce compounds that are not behaviorally active? With the exception of n-alkenes, all other compounds we identified in *O. sphegodes* labellum extracts are abundant in cuticular waxes of plants and are said to have the primary function to protect the plant from dehydration (Eigenbrode and Espelie 1995). Many of the compounds we identified in *O. sphegodes* labellum extracts can also be found in other *Ophrys* species (Borg-Karlson 1990) and could therefore have such a function. Compounds that are not used to attract pollinators may furthermore act as a solvent for the male-attracting volatiles or they may repell other bee species (Francke 1986). Another selective pressure for the production of a wide array of compounds in *Ophrys* orchids might have been a frequent adaptation to new pollinator species. Over longer terms, it may be a more economic evolutionary strategy to use a broad spectrum of components and to alter the pattern of compounds that already exist than to synthesize new compounds that may involve major changes in the biosynthetic pathways. Which of these reasons is valid in explaining the large number of compounds in *Ophrys* orchids remains unanswered.

In *O. sphegodes,* we found a smaller intraspecific variation of the behaviorally active compounds as compared to the nonactive compounds. Why do behaviorally active compounds show a smaller flower-specific variation? One explanation for the smaller intraspecific variation of the behavior-modulating compounds may be a higher selective pressure on the pollinator-attracting communication signal. Sex pheromones of female insects consist of highly species-specific mixtures of compounds with a fairly low degree of variability (Baker 1989; Löfstedt et al. 1991). Therefore, the male-attracting volatiles of *Ophrys* flowers should also show a fairly low degree of variability to match the signal that males are able to perceive.

Floral scents can influence pollinators in several ways: they attract from a distance, trigger searching behavior, and act as cues for alighting or probing by the pollinators (Faegri and van der Pijl 1979). In both rewarding and sexually deceptive orchids, variability of the scent of individual flowers may influence the frequency of visits and thereby the overall fitness of individual plants under conditions of a low pollinator visitation rate (Moya and Ackerman 1993). Our hypothesis that *O. sphegodes* flowers from different plants and even from the same inflorescence produce different pollinator-attracting olfactory recognition signals was confirmed by the results of our chemical analyses. The floral volatile bouquets differed between as well as within populations. Based on the statistical analyses, mainly n-alkanes and n-alkenes showed a characteristic population-specific odor pattern, whereas esters varied only in dependence of the stem position of a flower. Furthermore, our data showed for the first time intraspecific floral scent variation of ''behaviorally active compounds'' in a sexually deceptive orchid.

Differences in the scent of three forms of *O. insectifera,* another sexually deceptive orchid, have been described by Borg-Karlson et al. (1993). However, because all three forms attracted males of different pollinator species, the forms may represent separate species (Paulus and Gack 1990; van der Cingel 1995). Considerable quantitative and qualitative scent variations also exist within and among plants of food-deceptive orchids (Tollsten and Bergström 1989; Knudsen and Tollsten 1993; Moya and Ackerman 1993). In *Epidendrum ciliare,* floral fragrances vary according to the age of flowers, their stem positions, and their genotypes (Moya and Ackerman 1993). Scents produced by individual *O. sphegodes* flowers may vary due to that plant individual's genotypic makeup or within an inflorescence as a consequence of interactions of the genotype with the environment. Post-pollination changes in the production of scent were shown to guide pollinators of *O. sphegodes* to the unpollinated flowers of an inflorescence (Schiestl et al. 1997), thereby increasing the number of pollinated flowers. The changes involved a decrease of the total amount of scent produced as well as an alteration of the odor bouquet. E, E-farnesyl hexanoate is the major component of the Dufour's gland secretion in breeding (mated) females of the pollinator bee, *A. nigroaenea,* and is produced by *O. sphegodes* flowers after pollination. Behavioral tests showed that E, E-farnesyl hexanoate has a key function as a repellent signal of pollinated flowers (Schiestl and Ayasse 2001).

Does Scent Variation Influence the Behavior of the Pollinators?

Our behavioral tests indicate that male bees learn the odor bouquets of individual flowers during mating attempts in the same manner as when interacting with their own females (Smith and Ayasse 1987; Smith 1993; Dutzler and Ayasse 1996). Because females of most bee species are monandrous and mate soon after emergence (Eickwort and Ginsberg 1980), selection should proximately reward males capable of

precisely recognizing promising mates, which should ultimately lead to a mating behavior wherein males spend no time chasing unreceptive females (Ayasse et al. 1999). However, the diminished attractiveness of previously visited flowers in subsequent encounters can be interpreted with several hypotheses: (1) males learn the location where they visited a nonrewarding flower; (2) males mark flowers with repellents (''antiaphrodisiac,'' Kukuk 1985) that deter other males from copulatory attempts; (3) males learn visual cues of visited flowers; (4) flower odor dissipates during the first test of a flower resulting in a diminished attractiveness of a retested flower; or (5) males learn that flowers do not copulate and during subsequent encounters they do not respond in the same intensity to those learned odors (motivational decay).

The learning of precise locations was shown in wasps (Evans 1966). However, our finding that a retest in the same location with new flowers did not diminish attractiveness (Table 2) argues against this hypothesis and also against motivational decay. Our data indicate that males do not learn flowers by visual cues and furthermore do not mark flowers with repellents (antiaphrodisiacs) that deter other males from copulation attempts: Because the same dead, odorless female was retested in the same location (with the same males that were stimulated to mate by the scent of an *Ophrys* flower), marking of the female with a repellent or learning by visual cues should have decreased the attractiveness during the retest of the female (Fig. 6, right). Visual cues might play a more important role in other *Ophrys* species, in which optical cues are more pronounced or where the overall similarity of the *Ophrys* flower to the pollinator female is obvious like in *O. vernixia* (Paulus and Gack 1994). When a tested flower was transferred to a new test location with other patrolling males, its attractiveness increased again (Fig. 6, left). This leads us to exclude flower odor dissipation as a reason for the diminished attractiveness of flowers tested a second time in the same test location.

Our data clearly show that experience with a flower odor decreases the flower's attractiveness for pollinators and that this habituation is based on the learning of chemically distinct odor signals in individual flowers. In the Australian sexually deceptive orchid genera *Chiloglottis, Caladenia,* and *Drakea,* behavioral experiments with male pollinators (thynnine wasps) showed comparable results. Following an initial encounter, males avoided the orchid flowers (Peakall 1990; Peakall and Handel 1993; Peakall and Beattie 1996). Peakall (1990) discussed a ''site-specific refractory response'' of the males or alternatively learning of a recognition pheromone. Because conclusive experiments with changed labella tested in different locations were not performed, however, he could not rule out one of both explanations.

Does Flower-Specific Scent Variation Lead Pollinators to Revisit a Plant?

Our behavioral experiments where two *O. sphegodes* flowers were offered successively in the same male location indicate that individual flowers from different plants or within an inflorescence smell differently. These experiments are therefore consistent with our chemical analyses. Learning the scent of the first flower of an inflorescence offered to the males, along with an identical or similar scent of the second flower offered, should have resulted in a diminished attractiveness.

In *O. sphegodes,* previous studies showed that hydrocarbons are responsible for eliciting male copulatory behavior (Schiestl et al. 1999), whereas this was not achieved by synthetic blends of esters and aldehydes (F. P. Schiestl, unpubl. data). Here we demonstrate that, by varying the relative proportions of saturated esters and aldehydes between flowers of different stem positions, a plant may take advantage of the learning abilities of the pollinators and influence flower visitation behavior. Contrary to the idea that habituation of males to the scent of a visited *Ophrys* flower would moderate their response to all flowers of an inflorescence (Paulus 1988; Nilsson 1992), we found in *O. sphegodes* that 67% of the males that visited one flower of an inflorescence returned to visit a second flower of the same inflorescence. Data on the frequency of repeated visits to various flowers of an inflorescence by a single pollinator are rare in sex-deceptive orchids. In the wasp-pollinated orchid *Drakea glyptodon,* most pollinator males immediately leave the area after they visit a flower and will not visit nearby flowers (Peakall 1990). Such behavior produces long-distance pollen flow and outcrossing. However, plants in *D. glyptodon* normally bear one flower only. Therefore, revisiting a plant may cause fitness disadvantage by self-pollination and does not increase reproductive success in the same range as in *O. sphegodes.* Pollinator movement between flowers on an inflorescence may also lead to geitonogamous pollination, like in the sexually deceptive orchid *Prasophyllum fimbria,* where geitonogamous transfer of pollen was found in 22% of all pollinations (Peakall 1989). The genetic consequence of self-pollination is inbreeding depression (Proctor et al. 1996). Therefore, in orchids mechanisms have evolved to prevent autogamy and geitonogamy (van der Cingel 1995).

How is Autogamy and Geitonogamy Prevented in Ophrys sphegodes?

In *O. sphegodes,* we found that the time needed by a removed pollinium to bend into a correct position to fit into the stigma of another flower is longer than the total time for males to visit the first, second, and third flower of an inflorescence and in addition to move between these flowers. The likelihood that geitonogamy takes place is therefore reduced in *O. sphegodes.* Similar data exist for other orchids. In *Platantera blephariglottis,* pollinators visited three to four flowers per inflorescence and the average time spent on an inflorescence was 34 sec, whereas complete bending of the pollen stalk requires about 1 min (Cole and Firmage 1984).

Evolution of Reproductive Strategies in Ophrys sphegodes

According to Proctor et al. (1996) *Ophrys* orchids have evolved ''one of the most remarkable pollination mechanisms found in any plants.'' A strong specialization of *O. sphegodes* in attracting only males of one pollinator species plays an important role in preventing hybridization and pollen loss. As a consequence of this and of the highly sophisticated learning capabilities of bees (Smith and Ayasse 1987; Smith 1993), however, the pollinator visiting rate is low (Ayasse

et al. 1997), as in other deceit-pollinated orchids (Fritz and Nilsson 1996), resulting in a low number of fruits produced. Mechanisms have evolved in *O. sphegodes* to increase reproductive success. Characteristic variation in the relative proportions of the chemical compounds of flowers within an inflorescence uses the learning abilities of the male pollinators, raising the chance of more than one flower being visited by the same male bee. Assuming that a flower-visiting pollinator is carrying pollinia packed up from flowers of another plant and would visit at least two flowers on an inflorescence, male and female reproductive success of a plant would increase. First, the pollinator could remove pollinia of the visited flowers and carry them to the flowers of another plant; second, at least two flowers of the visited inflorescence could receive pollen and produce seeds.

Outcrossing and a wide dispersal of pollen is mechanically prevented (see above) and by a high degree of odor variation between individual plants, which is considerably greater than the variation among flowers on single plants. By minimizing learned avoidance of the flowers, the likelihood that a given pollinator would visit several to many different plants within a population is therefore increased (Neiland and Wilcock 1995). Density-dependent soft selection or, less likely, group selection may be responsible for the evolution of variability in scent in individual plants.

We are currently investigating the pollination biology of additional *Ophrys* species. We do have indications for flower-specific scent variation in other *Ophrys* species as well (pers. obs.). Nonetheless, based on different ecological and ethological constraints including the mating strategies and male mating behavior of pollinators, we also expect to find reproductive strategies that differ from the one described here for *O. sphegodes.* Our aim is to reveal the chemical communication processes involved in the pollinator attraction to obtain information on the evolution of reproductive strategies in orchids of the genus *Ophrys.*

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