Chemical communication in the reproductive biology of *Ophrys sphegodes*

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Abstract: Chemische Kommunikation in der Bestäubungsbiologie von *Ophrys sphegodes* (Orchidaceae).
Orchideen der Gattung *Ophrys* werden hauptsächlich durch Bienenmännchen bestäubt. Duftstoffe, die von der Blüte abgegeben werden, stimulieren bei den Bestäubern, gleich wie die Sexualpheromone arteigener Weibchen, Paarungsverhalten. Ein wichtiger Punkt bei der Evolution reproduktiver Strategien ist der Befund, daß Bienenmännchen individuelle Duftbouquets ihrer Paarungspartner lernen. Einmal besuchte Weibchen werden vermieden und neue Paarungspartner bevorzugt. Um einen einmal getäuschten Besucher einer Pflanze auf möglichst mehrere Blüten eines Blütenstandes zu locken, sollten die Blüten unterschiedliche Duftbouqets produzieren. Das Ziel dieser Untersuchungen war der Nachweis von blütenspezifischen Duftbouquets bei der Spinnenragwurz *Ophrys sphegodes* mittels 1) Verhaltensexperimenten zum Lernverhalten der Männchen der Bestäuberart *Andrena nigroaenea* (Andrenidae) 2) Quantitativen chemischen Analysen von Headspaceproben einzelner Blüten. Weiterhin wurde untersucht, wie Selbstbestäubung oder die Bestäubung einer Nachbarblüte vermieden wird. Verhaltenstests zeigten, daß Männchen bei Paarungsversuchen die Duftbouquets von individuellen Blüten lernen und diese bei einer weiteren Konfrontation wiedererkennen. Weitere Kopulationsversuche mit diesen Blüten wurden vermieden, nicht jedoch mit weiteren Blüten derselben Pflanze oder mit den Blüten einer anderen Pflanze. Chemische Analysen ergaben unterschiedliche Duftbouquets nicht nur bei den Blüten verschiedener Pflanzen, sondern auch bei Blüten eines Blütenstandes. Diese Variation der Duftbouquets von einzelnen Blüten führte dazu, daß ca. 70% der Bienenmännchen, die die Pollinien einer besuchten Blüte entnommen hatten, zur selben Pflanze zurückkehrten und die Pollinien einer weiteren Blüte entnahmen. Letztendlich erhöht sich dadurch der reproduktive Erfolg der Pflanze. Entnommene Pollinien müssen, bevor sie in die Narbe einer anderen Blüte gelangen können, zunächst eine charakteristische Beugebewegung durchführen (Dauer ca. 1,5 Minuten). Da die Zeit, die ein Bestäubermännchen für den Besuch mehrerer nacheinander besuchter Blüten eines Blütenstandes benötigt, signifikant kürzer ist, kann eine Selbstbestäubung oder die Bestäubung einer Nachbarblüte ausgeschlossen oder minimiert werden.

Key words: *Ophrys sphegodes*, *Andrena nigroaenea*, chemical communication, semiochemicals, volatile bouquets, pollination, learning behaviour, reproductive success.

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Ophrys spp. orchids are mostly pollinated by male bees, which are lured to the orchid by visual cues and volatile semiochemicals (KULLENBERG 1961). At close range, chemical signals from the flowers elicit sexual behaviour in males, who act in a manner similar as they do to sex pheromones of females. The male bees try to copulate with the flower labellum and thereby transfer pollen from one flower to another. Our field observations show that the frequency of male pollinators visiting *Ophrys* plants is very low. In an *O. sphegodes* population near Illmitz (Austria), we recorded 1996 that 4.85 % of 887 plants had been visited by a pollinator (AYASSE & SCHIESTL, UNPUBLISHED), that either removed the pollinia of a flower or transferred pollen into the stigma or did both. Therefore, strategies that result in males visiting more than one flower of the same inflorescence should be expected to have evolved in plants in order to optimize the number of pollination events. Since male bees are able to learn the distinctive odour bouquets of individual females during mating attempts (DUTZLER & AYASSE 1996; SMITH & AYASSE 1987; SMITH 1993) and use this information to avoid females they have already mated with, one would expect the odour bouquets of *Ophrys* flowers to vary within populations and even within the same inflorescences (PAULUS 1988). The aim of our work has been to determine whether flower-specific olfactory recognition signals occur in the orchid *Ophrys sphegodes* by conducting: 1) Behavioural learning experiments with the pollinating male bees of *Andrena nigroaenea* (Andrenidae), 2) Quantitative chemical analyses of the head space odours from individual flowers , 3) Behavioural observations to show how self-pollination is prevented.

Material and Methods

Behavioural tests on the learning of distinctive odour bouquets of individual flowers during pollination were conduced in Oberweiden (eastern Austria) in the month of May 1994-1996. On the same day the behavioural experiments were performed, individual flowers of plants were sampled at random from two populations (Bisamberg and Illmitz), fixed on insect pins and offered to the males. Contacts (pouncing) and copulation attempts were recorded during a 3 min test period. For further methodological details, see Fig. 1.

Gas chromatographic analyses of the head space odours from individual flowers were performed as described in SCHIESTL et al. (1997A, B). Relative amounts of single compounds were tested for significance of differences between the volatile bouquets of the uppermost and the second uppermost flourishing flowers of an inflorescence by means of a discriminant function analysis (SOKAL & ROHLF 1995).

Behavioural experiments to prove self-pollination were performed by recording the number of individually marked males that visited one flower of an inflorescence and returned to visit another flower of the same inflorescence. We recorded and 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 (transit time). Furthermore, we recorded the time needed by a removed pollinium to bend into a correct position for fitting into the stigma of another flower and pollinate it.

Results and Discussion

Our data from the behavioural tests indicate that male bees learn the odour bouquets of individual flowers during mating attempts in the same manner as if interacting with their own females (Smith 1993). Male bees recognise previously visited flowers in further encounters since they avoid trying to mate with them (Fig. 1, top left). A retest in the same location with a flower of the same inflorescence (Fig. 1, bottom left) or a flower of another plant (Fig. 1, bottom right) showed the same attractiveness of the second flower relative to the first. Therefore, the two flowers, even the ones of the same inflorescence, obviously smell different. When a flower is removed from the location in which it was tested two times and placed into an area, where other males are patrolling, its attraction value increases again (Fig. 1, top left). Therefore, we can exclude a conceivable flower odour dissipation. The results of the control experiment (Fig. 1, top right) indicate that males do not learn mates (flowers) by visual cues and furthermore do not mark flowers with repellents (antiaphrodisiacs) that deter other males from copulation attempts (KUKUK 1985). Since in this experiment the same dead odourless female was retested in the same location (with the same males), marking of the female or learning by visual cues should have resulted in a decrease of the attractiveness during the retest of the female. The same (or even a little bit higher) attractiveness of the odourless dead female during the retest is obviously based on the different odour bouquet of the two flowers offered to the males.

Our hypothesis that flowers of different plants and even of the same inflorescence produce different flower-specific olfactory recognition signals is furthermore confirmed by the results of the chemical analyses. Gas chromatographic analyses of individual flower odours showed that the floral volatile bouquets between the uppermost and the second uppermost flowers of an inflorescence differed (discriminant function analysis, $Chi^2 = 31.4$, $DF = 4$, $P < 0.001$, $n=82$). We assume that variation in the odour bouquets of individual flowers of an inflorescence raises the chance of more than one flower being visited by the same male and thereby influences plant pollination success and individual fitness. Actually, by behavioural observations we could show that 67 % of the males that visited one flower of an inflorescence returned to visit a second flower of the same inflorescence. Some males even visited a third flourishing flower of an inflorescence. Since the time needed by a removed pollinium to bend into a correct position for fitting into the stigma of another flower (median = 152s, n=7) is longer than the total time for visiting the first (median = 10s, n=30), second (median = 6s, n=20) and third flower (median = 3s, n=13) of an inflorescence and in addition the transit time between visiting these flowers (median = 6s, n=33; transit time between visiting two flowers of an inflorescence), likelihood of pollination of a neighbouring flower can be excluded.

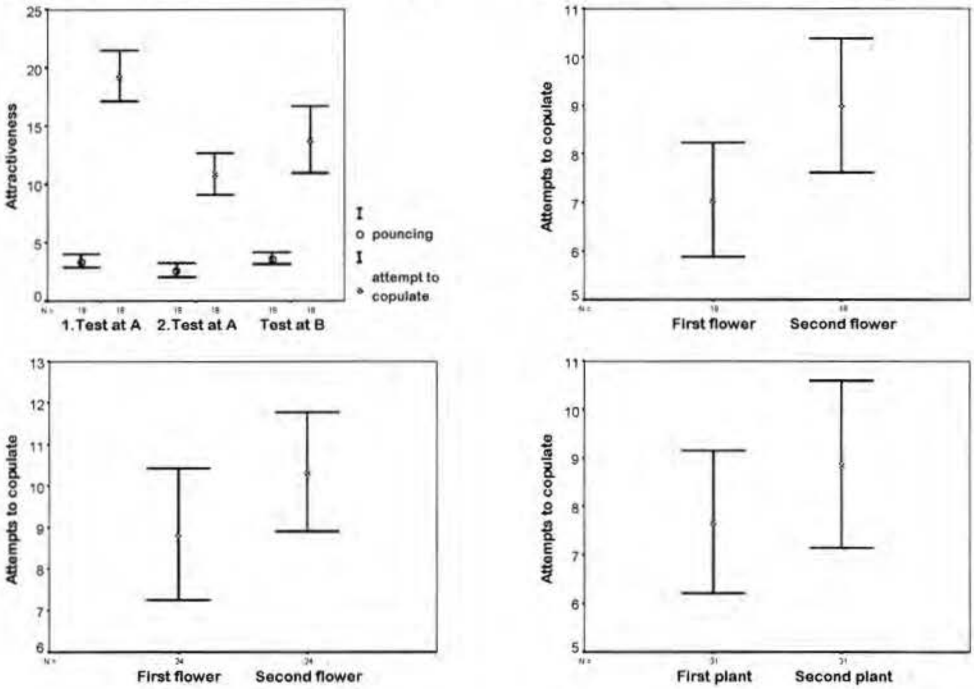

Fig. 1: Field studies of learning of distinct odour bouquets of individual flowers by male *Andrena nigroaenea*. Attractiveness measured by the mean number of pouncings or copulation attempts (±SE) that males performed on a flower.
Top left: A flower was offered in location A for 3 min before removal. It was retested in the same location and in a location (B) of other males patrolling.
Top right: To exclude both visual learning of a flower and odour marking by a pollinating male, a control experiment was performed. Males were offered a flower and a dead odourless female of *A. nigroaenea* fixed on an insect pin. The flower was fixed on the insect pin upside down beneath the dummy bee invisible and out of touch to the incoming male. After 2 min, the same dummy bee was retested with a second flower of another plant.
Bottom: A flower was tested and replaced by another flower of the same inflorescence (left) or by a flower of another plant (right).

When the same flower was retested in the same location (A) 2 min later, significantly fewer copulation attempts were observed than in the first test (T-test, $P<0.05$, SOKAL & ROHLF 1995). When a flower was placed into the test location B where other males patrolled, its attractiveness increased, but not to its original value. The attractiveness of two different flowers offered successively together with an odourless dummy bee was the same (T-test, $P>0.05$, SOKAL & ROHLF 1995). A retest in the same location with a flower of the same inflorescence or a flower of another plant showed the same attractiveness of the second flower relative to the first (T-test, $P > 0.05$, SOKAL & ROHLF 1995).

Conclusions

1. Males learn and recognize odour bouquets of individual flowers
2. Males avoid trying to mate with flowers they previously visited but not with other flowers of the same inflorescence or of a different plant
3. Odour bouquets of individual flowers of an inflorescence are different
4. In the early spider orchid *O. sphegodes* strategies evolved to optimize pollination events by directing pollinating males, that return to a plant, to not yet visited flowers of the same inflorescence

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