# The role of pollinator attracting scent in the sexually deceptive orchids *Ophrys chestermanii, O. normanii* and *O. tenthredinifera*

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**Abstract:** Sexualtäuschorchideen der Gattung *Ophrys* (Orchidaceae) imitieren die Weibchen ihrer Bestäuber in Duft, Form und Farbe. Insektenmännchen versuchen mit dem Labellum der Blüte zu kopulieren und transportieren den Pollen von Blüte zu Blüte, wodurch die Orchidee bestäubt wird. In dieser Arbeit untersuchten wir die Bestäuber anlockenden Duftstoffe der beiden endemisch auf Sardinien vorkommenden Arten *O. normanii* und *O. chestermanii*, die beide von *Bombus vestalis* Männchen (Hymenoptera: Apidae) bestäubt werden und von *O. tenthredinifera*, die *Eucera nigrilabris* (Hymenoptera: Apidae) zur Bestäubung anlockt. *O. normanii* wurde von Wood (1983) als Primärhybride beschrieben. Nach Paulus und Gack (1995) handelt es sich um eine hybridogene Art oder um eine Art die durch Abspaltung von *O. tenthredinifera* entstanden ist. Das Ziel der Untersuchungen war die Identifizierung Männchen-anlockender Verbindungen. Die Attraktivität der drei Arten für *B. vestalis* Männchen sollte Hinweise auf den Artstatus von *O. normanii* geben.
In Biotests mit *B. vestalis*-Männchen lösten Blütenextrakte von *O. normanii* und *O. chestermanii* ebenso wie *B. vestalis*-Weibchen Kopulationsverhalten der Männchen aus, nicht jedoch Extrakte von *O. tenthredinifera*. Folglich handelt es sich bei *O. normanii* nicht um einen aktuellen Hybriden zwischen *O. chestermanii* und *O. tenthredinifera*. Ein Vergleich der GC-EAD-aktiven Duftbouquets mittels Diskriminanzanalyse ergab große Ähnlichkeiten zwischen *O. normanii* und *O. chestermanii* für die Substanzklassen der Ester, Alkohole und Fettsäuren, die daher vermutlich eine Schlüsselfunktion bei der Bestäuberanlockung haben.

**Key Words:** *Ophrys*, *Bombus vestalis*, pollination by sexual deception, floral scent

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Sexual deception of male bees is one of the most remarkable mechanisms of pollination (Ackermann 1986, Proctor & al. 1996). Flowers of the orchid genus *Ophrys* mimic females of their pollinator species, usually bees and wasps, to attract males, which try to copulate with the flowers. During this so-called "pseudocopulation" the male removes the pollinia and transfers them to another flower to ensure pollination. Apart from visual and tactile cues, floral scent was shown to be most important for eliciting mating behaviour in males (Kullenberg 1961, Schiestl & al. 1999, Ayasse & al. 2003). Pollination in *Ophrys* is highly specific and usually each *Ophrys* species attracts only one pollinator species (Paulus & Gack 1990). The high degree of specialization provides the means of reproductive isolation between the intercrossable *Ophrys*-species (Ehrendorfer 1980). The complex odour-bouquets released by the flowers are species-specific and often consist of more than 100 different chemical compounds (Borg-Karlson & al. 1985, Ayasse 2006). Speciation in *Ophrys*-orchids may be brought about by changes in the pollinator attracting floral scent. The attraction of a new pollinator may act as a pre-zygotic isolation barrier (Stebbins 1970, Paulus & Gack 1990, Soliva & al. 2001).

We investigated three sympatrically occuring *Ophrys*-species on Sardinia. *O. chestermanii* and *O. normanii* are endemic and are both pollinated by males of the bumblebee *B. vestalis*. *O. tenthredinifera*

is pollinated by *Eucera nigrilabris*. There are different opinions concerning the taxonomic status of *O. normanii*. It has been described as an actual hybrid between *O. chestermanii* and *O. tenthredinifera* (Wood 1983). Paulus & Gack (1995) suggested that it is an own species, that either has developed from a hybrid between *O. chestermanii* and *O. normanii* or that has evolved by radiation from *O. tenthredinifera*. By conducting behavioural-tests with *B. vestalis* males, performing gas chromatographic analyses and electrophysiological studies we wanted to identify pollinator attracting scent and to clarify the taxonomic status of *O. normanii*.

**Material and Methods**

**Sample collection:** Flower labella of *O. chestermanii, O. normanii* and *O. tenthredinifera* were collected at various locations near Domus Novas and Tertenia. Individual labella were cut from the flowers and extracted in 1ml pentane (Sigma-Aldrich, HPLC grade) at 20°C for 24h. Resulting extracts were concentrated to 100µl/flower equivalent in a water bath (40°C). For chemical analyses C16 (hexadecane) was added as internal standard.

**Behavioural tests:** In series of behavioural tests we investigated the attractiveness of labellum extracts of the three orchid-species and of shock-frozen virgin *B. vestalis* females for *B. vestalis* males. The behavioural experiments were carried out in two flight-cages (60x60x70cm) in a glass house of the Botanical Garden Ulm. Dead *B. vestalis*-females, which were Soxhlet-extracted with dichloromethane for 24 h, dried at 20°C and fixed on insect pins, were used as dummies. Virgin *B. vestalis* females, were killed in liquid nitrogen by shock-freezing for 5-10 sec. In each test a single dummy was treated with 50µl (1/2 flower-equivalent) of a test sample or pentane (solvent control) and offered to 30 males simultaneously. Before the treated or control dummy was fixed in the flight-cage the solvent was allowed to evaporate for 30s. Shock-frozen females were kept 10 min at room-temperature and fixed on insect-pins before they were offered to the males. The copulation attempts of the males towards the dummies and towards shock-frozen females were recorded for 10 min. To avoid habituation the position of the dummy in the flight-cage was changed after each test and males were renewed every second day. Various test-groups and the two cages were tested alternatively in order to ensure comparable test conditions. *B. vestalis* males were caught in the Botanical Garden Ulm.

**Chemical analyses:** Chemical analyses of electrophysiologically active compounds of flower-extracts were performed by gas chromatography, gas chromatography – mass spectrometry. For details see Ayasse & al. 2003. The assignment of the peaks was made by coinjection with synthetic mixtures of the identified compounds.

**Statistics:** Differences in the number of copulatory events of all test groups in comparison with the control were examined with a Mann-Whitney-U-Test. We used Benjamini and Hochberg-correction (1995) for multiple testing (Verhoeven & al. 2005). To determine differences and similarities between the three species we used relative proportions of GC-EAD-active compounds of the three species. Statistical analyses were performed with four data sets, namely esters, alcohols, fatty acids, hydrocarbons and aldehydes. To reduce the number of variables a principle component analysis (PCA; varimax rotation) was performed. Resulting principle components (PCs) with an eigenvalue above one were used for discriminant function analysis (DFA). All statistics were performed with SPSS 13.0.

**Results**

**Behavioural-tests:** In our behavioural tests labellum extracts of *O. normanii* and *O. chestermanii* stimulated significantly more copulation attempts than the control (Fig. 1). Males never tried to copulate with dummies impregnated with natural extracts of *O. tenthredinifera*. Shock-frozen females induced the highest number of copulation attempts.

**Chemical analyses:** In a PCA performed with all GC-EAD active esters two PCs with an eigenvalue above one explained 85.5% of the total matrix variation. A DFA with the factor scores of the PCs for esters could categorize 68.3% (67.3% at cross-validation) of all cases to the correct species (Fig. 2).

The PCA based on all GC-EAD active alcohols generated two PCs with an eigenvalue above one explaining 77.4% of variance. A DFA (DFA 1, $\chi^2$=30.493, df=4, P<0.001, DFA 2, $\chi^2$=8.972, df=0.399 P=0.527) could classify 51.5% (47.5% at c. v.) to the correct species. There is a separation of *O. tenthredinifera* from the other two species, whereas *O. chestermanii* and *O. normanii* are overlapping.

In a PCA performed with all GC-EAD active fatty acids two PCs with an eigenvalue above one explained 87,5% of the total matrix variance. When the acids were included to perform a DFA (DFA 1, $\chi^2$= 48.983, df=4, P<0,001, DFA 2, $\chi^2$= 0.026, df=1, P=0.871) 65.3% (61.4% at c. v.) of all cases were classified to the correct species. *O. normanii* and *O. chestermanii* are overlapping whereas *O. tenthredinifera* is separated from these two species. All of the variance was explained by discriminant funcion 1 (DF 1).

In the electrophysiologically active hydrocarbons (alkenes/alkanes) three PCs with an eigenvalue above one explained 86% of the total variance. A comparison with a DFA showed overlaps between *O. normanii* and *O. chestermanii* and between *O. normanii* and *O. tenthredinifera* (DFA 1, $\chi^2$=33.573, df=6, P<0.001, DFA 2, $\chi^2$=9.648, P<0,05). 74.3% (73.3% at c. v.) of all cases were classified to the correct species.

A PCA based on all GC-EAD active aldehydes generated four PCs with an eigenvalue above one explaining 83.1% of the total matrix variance. A DFA showed significant differences between all species (DFA 1, $\chi^2$=168.875, df=8, P<0.001, DFA 2, $\chi^2$=38.732, df=3; P<0.001). Of all cases 86.1% (86.1% at c. v.) could be classified to the correct species.

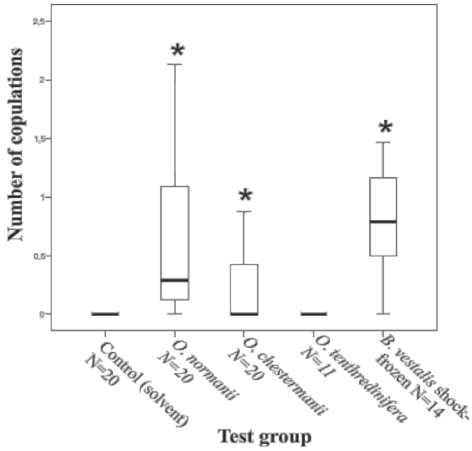


Fig. 1: Attractiveness (mediane, 1st and 3rd quartile, lowest and highest value) of various flower extracts and shock-frozen *B. vestalis*-females for *B. vestalis* males. We recorded the number of copulations within 10 min. In the control test 50µl pentane was used. To normalize data, the number of copulation attempts was divided by the mean number of males flying around. Columns marked with an asterisk differ significantly from the control (Mann-Whitney U-test, p≤0.05, Benjamini and Hochberg correction).

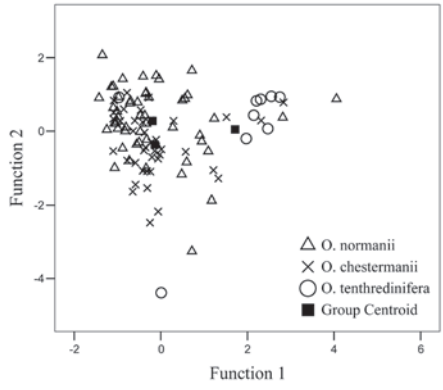


Fig. 2: Comparison of the relative proportions of GC-EAD active esters of the flower-extracts of the three *Ophrys*-species by means of a DFA. There is a significant separation of *O. tenthredinifera* from the other two species, whereas *O. chestermanii* and *O. normanii* are overlapping (DFA 1, $\chi^2$=34.385, df=4, P<0.001, DFA 2, $\chi^2$=8.972, df=1, P<0.05).

**Discussion**

While dummies impregnated with flower-extracts of *O. normanii* and *O. chestermanii* and shock-frozen *B. vestalis* females elicited significantly more copulatory events than control dummies, *O. tenthredinifera*-extracts stimulated no intensive mating behaviour. This findings support the hypothesis of Paulus & Gack (1990) that *O. normanii* is not an actual hybrid between *O. chestermanii* and *O. normanii*, but an own species. However, it still remains unclear whether *O. normanii* has an hybridogenic origin.

In former investigations it was shown that sympatrically and allopatrically occurring *Ophrys*-species with the same pollinator use the same volatiles in very similar proportions for pollinator attraction (Stökl & al 2005). The DFAs we performed with GC-EAD active compound from different classes of compounds showed a high similarity between *O. chestermanii* and *O. normanii* in the odour bouquets of esters, alcohols and fatty acids. *O. tenthredinifera* flowers produce identical compounds, but in different relative proportions. Consequently these compound classes may have a key function in attracting *B. vestalis* males and eliciting mating behaviour.

Borg-Karlson & Tengö (1986) demonstrated that aliphatic primary alcohols, methylcarbinols and several terpenes, both occurring in bees and flowers, play a major role in pollinator attraction of *O. lutea*. Our findings indicate that *O. chestermanii* and *O. normanii* produce the same volatiles, however, only a subset of all electrophysiologically active compounds probably play a role in pollinator attraction as was also shown in other *Ophrys* species (Schiestl & al. 2000).

Molecular analyses have to show whether the similar attractiveness between *O. normanii* and *O. chestermanii* is a result of speciation by hybridisation or of a convergent evolution of the pollinator attracting scent in *O. normanii* and *O. chestermanii*. In future studies we will perform behavioural assays with synthetic copies of the different substance-classes to improve the importance of certain of the pollinator attracting substances.

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