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Pollinator attraction in a sexually deceptive orchid by means of unconventional chemicals

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Ophrys flowers mimic virgin females of their pollinators, and thereby attract males for pollination. Stimulated by scent, the males attempt to copulate with flower labella and thereby ensure pollination. Here, we show for the first time, to our knowledge, that pollinator attraction in sexually deceptive orchids may be based on a few specific chemical compounds. *Ophrys speculum* flowers produce many volatiles, including trace amounts of (ω-1)-hydroxy and (ω-1)-oxo acids, especially 9-hydroxydecanoic acid. These compounds, which are novel in plants, prove to be the major components of the female sex pheromone in the scoliid wasp *Campsoscolia ciliata*, and stimulate male copulatory behaviour in this pollinator species. The specificity of the signal depends primarily on the structure and enantiomeric composition of the oxygenated acids, which is the same in wasps and in the orchids. The overall composition of the blend differs significantly between the orchid and its pollinator and is of secondary importance. 9-Hydroxydecanoic acid is a rarely occurring compound that until now has been identified only in honeybees. Contrary to the standard hypothesis that *Ophrys* flowers produce only 'second-class attractivity compounds' and are neglected once the pollinator females are present, we show that flowers are more attractive to the males than are their own females.

Keywords: *Campsoscolia ciliata*; *Ophrys speculum*; pollination by sexual deception; chemical mimicry; oxygenated acids

1. INTRODUCTION

Ophrys orchids have evolved 'one of the most remarkable pollination mechanisms found in any plants' (Proctor *et al.* 1996, p. 205). They grow especially around the Mediterranean, and their pollinators are mostly bees (Andrenidae, Colletidae, Megachilidae and Apidae). Sphecid and scoliid wasps and beetles (Elateridae and Scarabaeidae) are involved in a few cases (Kullenberg 1961; Borg-Karlson 1990). *Ophrys* flowers mimic virgin females of their pollinators, and male insects are lured to the orchid by volatile semiochemicals and visual cues. At close range, chemical signals from the flowers elicit sexual behaviour in males, which try to copulate with the flower labellum and respond as if in the presence of female sex pheromones. Thereby the male touches the gynostemium, and the pollinia may become attached to his head or, in some species, to the tip of his abdomen. His copulatory attempts with another flower ensure that the pollinia are transferred to the flower's stigmatic surface and pollination is ensured. This phenomenon is termed 'Pouyannian mimicry' (Pasteur 1982), because Pouyanne was the first to describe the behaviour of males of the scoliid wasp *Campsoscolia ciliata* on *O. speculum* flowers as a 'pseudocopulation' (Correvon & Pouyanne 1916; Pouyanne 1917). Pseudocopulation was subsequently studied intensively by Kullenberg (1961) who suggested that *Ophrys* orchids mimic the females' sex pheromone of the pollinators to dupe males into visiting their flowers, and thereby pollinating them.

Ophrys flowers, and also females of their pollinator species, produce complex odour bouquets of more than 100 volatiles (Borg-Karlson 1987, 1990). Behavioural experiments using synthetic copies of the compounds produced by *Ophrys* flowers have shown that only certain volatiles are active in stimulating mating behaviour in the males (Kullenberg & Bergström 1976; Tengö 1979; Borg-Karlson 1990). Furthermore, these experiments demonstrated that those compounds identified only in insect glandular secretions had a greater capacity to attract males than did volatiles identified in flowers. Borg-Karlson (1990) therefore concluded that *Ophrys* flowers produce a set of 'second-class attractivity compounds', which attract only that part of the pollinator population with a low threshold for sexual stimuli in the absence of females. However, the synthetic compounds tested in these experiments rarely triggered highly intensive male mating behaviour (pseudocopulation) that would ensure pollination. A biotest comparing the attractiveness of floral fragrances produced by *Ophrys* flowers with the pheromonal secretions of the respective female insects should be preferably performed using those compounds that actually elicit copulatory behaviour.

Using coupled gas chromatography–electroantennographic detection (GC–EAD), gas chromatography–mass

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spectrometry (GC–MS) and behavioural field tests, we identified behaviour-mediating compounds in the orchid *O. speculum* and its pollinator, *C. ciliata*. Furthermore, we tested the hypothesis that *Ophrys* flowers produce only 'second-class attractivity compounds' (Borg-Karlson 1990) and are less attractive to males than are their own females.

2. MATERIAL AND METHODS

(a) *Sample collection*

Campsoscolia ciliata females and *O. speculum* flower labella were collected at various locations near C'an Picaford (Mallorca, the Balearic Islands). Individual *Ophrys* labella were cut from flowers and extracted in 0.5 ml diethyl ether (Merk Uvasol) at 20 °C for 2 days. Virgin *C. ciliata* females attractive to patrolling males were killed by freezing. We checked for emptiness of the spermatheca by dissections and found all of the attractive females to be unmated. The following solvent extracts were obtained from individual females: cuticle extracts—single frozen females were extracted for 5 min in 400 µl diethyl ether (Merk Uvasol); and head extracts—after decapitation, heads were extracted in 200 µl diethyl ether for 24 h. Samples were stored in the freezer until chemical analyses or behavioural experiments were performed.

(b) *Electrophysiology*

To detect electrophysiologically active compounds in *C. ciliata* cuticle extracts or *O. speculum* labellum extracts, GC–EAD analyses were performed as described in Ayasse *et al.* (2000) and Schiestl *et al.* (2000).

(c) *Behavioural assays*

Our bioassays were conducted at the same locations near C'an Picaford as those where we also collected *Ophrys* and wasp samples. All behavioural experiments were performed between 10.00 and 14.00, when the flight activity of mate-searching male wasps was highest.

In a first experiment we tested the behavioural responses of *C. ciliata* males to individual female wasps, to *O. speculum* flowers, to odour samples of individual female wasps and *Ophrys* flowers, and to different mixtures of synthetic compounds that were found to be electrophysiologically active and occurred in both the wasp and the orchid odour samples.

Ten synthetic compounds were prepared according to the results of GC–MS analysis performed with cuticle extracts of females and *Ophrys* labellum extracts. The relative and absolute amounts of the compounds in all test solutions were checked by GC. Odourless *C. ciliata* females were used as dummies. These dummies had been Soxhlet-extracted in dichloromethane for 48 h, dried and fixed on insect pins. Odourless dummies were impregnated with various odour samples and offered to *C. ciliata* males. To avoid habituation, the tests were carried out at different spots within the male patrolling area; these sites were at least 10 m apart. Earlier marking experiments showed that the males do not move freely throughout the nesting aggregation (M. Ayasse, unpublished data), and, therefore, different males were present at the testing spots. Within a testing period of 3 min, two different male reactions were recorded (Ayasse *et al.* 1999): pouncing—a male contacts the dummy wasp with his head or antennae; and copulation—a male mounts the dummy wasp and tries to copulate. To combine the tests carried out on different days and at different localities where the numbers of patrolling males were not the same, the male reactions were standardized by measuring the flight activity. A 1 m long wire was mounted in the experimental area. The number of males crossing the wire over a period of 1 min was counted at least every 30 min. For each 3 min test, individual reactions of the males were divided by the mean male flight activity and multiplied by 100.

In a second set of experiments we tested the hypothesis that *Ophrys* flowers are less attractive to males than are their own females. In two behavioural tests we compared the attractiveness of female wasps and *Ophrys* flowers. In the first test frozen virgin female wasps were brought to the field in a container with ice. A randomly collected individual *Ophrys* flower and a dead female were fixed on insect pins and positioned 1.5 cm above the ground (the height at which *C. ciliata* males are patrolling and searching for females; Paulus & Gack 1980) and 5 cm apart. In a dual-choice experiment, copulation attempts with the female and the flower were recorded during a 3 min test period. Each couple of flower and female was tested only once in the same location.

To exclude the possibility that the use of dead females influenced the result of the first test, we conducted a second experiment using live females. Fresh flowers were collected at random. In addition, virgin *C. ciliata* females were collected, which were highly attractive to patrolling males. Dead Soxhlet-extracted female wasps were used as dummies and placed in a male patrolling area. In a 3 min test period the scent of either an attractive live wasp or an *Ophrys* flower confined in a glass vial was continuously blown over the dummy. The surface of the vial was previously chemically deactivated (Sylon CT, Supelco Inc.). In this experiment the visual cues were always the same and male behaviour was influenced only by the scent emitted by the flower or by the female wasp.

(d) *Chemical analyses*

Samples of volatiles were analysed using a gas chromatograph HP 5890 (Hewlett–Packard) equipped with a DB5 capillary column (30 m × 0.32 mm inside diameter). The GC was operated splitless at 120 °C for 30 s, before a programmed increase to 280 °C at 4 °C min^{-1}. For quantitative analysis, n-octadecane was added as an internal standard. Response factors were determined by single level calibration. Structure elucidation of individual compounds was based on GC–MS analysis (external ionization, 70 eV; double focusing instrument VG70/250 SE, Vacuum Generators, Manchester, UK, linked to a HP 5890; gas chromatographic condition as mentioned above); identifications were carried out by comparing the mass spectra of natural compounds with literature data (McLafferty & Stauffer 1989). For structure confirmation, synthetic samples were used to check mass spectra and gas chromatographic retention times by co-injection. Gas-chromatography separation of the enantiomers was carried out on a non-polar DB5 capillary after transformation to the diastereomeric mixture of methylesters of the corresponding acetyl-L-lactic derivatives.

(e) *Statistics*

Differences in the reactions recorded in the choice experiments for females and flowers were examined with a Wilcoxon signed-ranks test. Differences in the numbers of pounces and copulatory events of all testing solutions in comparison with the control (diethyl ether blank) were examined with a Mann–Whitney *U*-test, which was also used to compare the number of copulatory events elicited by live wasps and *Ophrys* flowers. We used Bonferroni correction for multiple testing and set the sig-

nificance level p to 0.005. The relative amounts of single compounds in wasps and flowers were compared by a Mann–Whitney *U*-test. All statistical analyses were carried out using the statistical package SPSS (Norušis 1993).

3. RESULTS AND DISCUSSION

(a) *Location and chemistry of the male-attracting compounds*

Intact, freeze-killed, virgin and odourless females (virgins that were Soxhlet-extracted and dried), *O. speculum* flowers, and various samples of volatiles obtained from virgin females and flowers were tested for their attractiveness to male wasps in field trials. Odourless females rarely elicited mating behaviour, whereas a high number of pouncing and copulatory events were observed with freeze-killed virgin females and intact *O. speculum* flowers (figure 1*a*). Therefore, volatile compounds proved to be essential in stimulating mating behaviour in *C. ciliata* males, as already stated by Kullenberg (1961). Our bioassays showed the behaviour-inducing volatiles to be located on the surface of the cuticle of attractive females (figure 1*a*), while head extracts were significantly less attractive. Labellum extracts obtained from *O. speculum* flowers likewise elicited copulation behaviour in male *C. ciliata*. There is increasing evidence that, in many hymenopterans, sex pheromones are located on the surface of the cuticle (reviewed in Ayasse *et al.* 2001), and copulation-behaviour-eliciting semiochemicals in other *Ophrys* species are also located on the surface of the flowers (Schiestl *et al.* 2000).

Although more than 130 compounds have been identified from solvent extracts of wasps and orchids (Erdmann 1996), GC–EAD analyses showed that male antennae respond to only 10 compounds (figure 2). This represents less than 2.7% of the total amount of volatiles on the cuticle surface of virgin *C. ciliata* females (mean ± s.e.m. = 26.21 ± 2.33 μg). These trace compounds were identified by GC–MS as saturated (ω-1)-hydroxy and (ω-1)-oxo acids, aldehydes and ethyl esters. The relative proportions of most of the GC–EAD-active compounds differed significantly between the orchid and its pollinator (figure 3). 9-Hydroxydecanoic acid, however, a major component of the active compounds in the wasps, was also a major component of the active volatiles in the orchid flowers. Furthermore, the enantiomeric compositions of the 9-hydroxydecanoic and 7-hydroxyoctanoic acids were identical in wasps and orchids (absolute configurations R (right) : S (left) = 6 : 4).

In field tests, the number of copulatory attempts by males to one wasp or flower equivalent of synthetic blends did not differ from those to labellum extracts or cuticular extracts (Mann–Whitney *U*-test, $p > 0.05$). Complete mixtures of GC–EAD-active compounds were most attractive (figure 1*b*), although quantitative compositions of blends in wasps and orchids differed significantly (figure 3). All test mixtures containing (ω-1)-hydroxy acids in naturally occurring enantiomeric proportions elicited copulatory attempts in males. Blends of pure aldehydes and ethyl esters and those with a racemic mixture of (ω-1)-hydroxy acids did not stimulate significantly more copulatory attempts than odourless dummies (figure 1*b*). Therefore, the presence of the highly specific (ω-1)-hydroxy acids in their naturally occurring enantiomeric proportions proved to be essential to elicit copulatory behaviour in *C. ciliata* males.

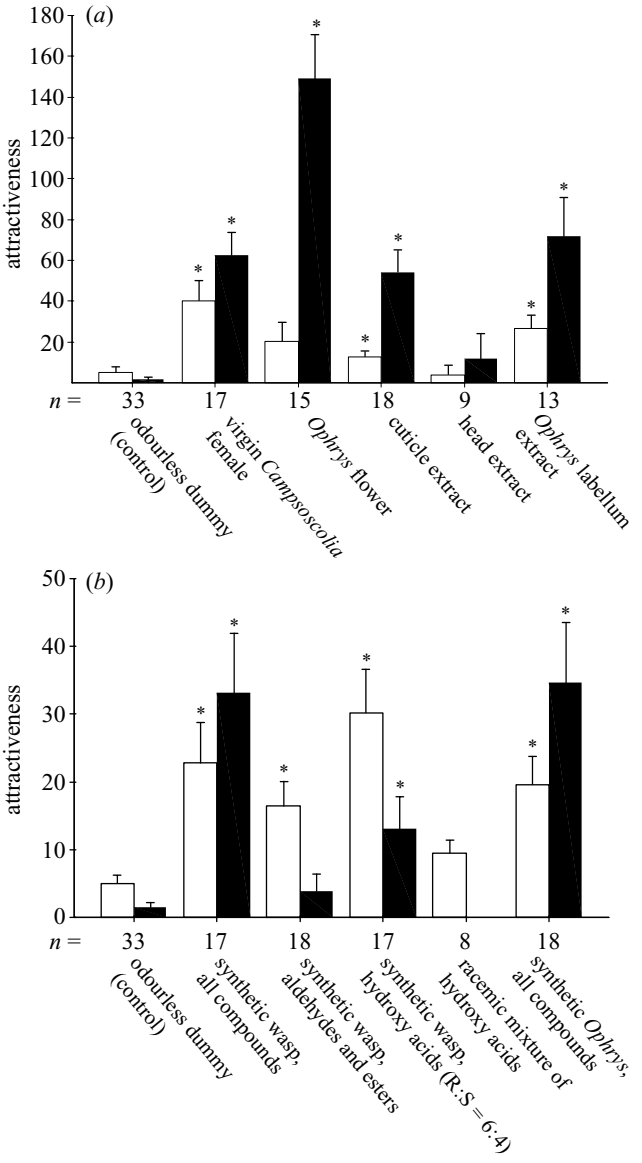

Figure 1. Attractiveness (mean ± s.e.m.) of (*a*) intact freeze-killed virgin females, *Ophrys* flowers, various odour samples obtained from virgin females and *Ophrys* flowers, and (*b*) synthetic mixtures to *C. ciliata* males. Odourless dummies (Soxhlet-extracted dried *C. ciliata* females) impregnated with odour samples were offered to the males. Assays were carried out for 3 min, and two different male reactions were recorded: pouncing on the dummy (open bars) and attempts to copulate with the dummy (filled bars). In each assay individual reactions of males were divided by the number of patrolling males crossing a 1 m long wire and multiplied by 100. Columns marked with an asterisk differ significantly from the odourless dummy (Mann–Whitney *U*-test, $p < 0.005$).

Fatty acids, oxygenated at the (ω-1) position, have been found in many phylogenetically distinct organisms, and, interestingly, several have been reported in the Hymenoptera. These include 9-oxo-(E)-2-decenoic acid, the so-called 'queen substance' of honeybees (Barbier & Lederer 1960; Callow & Johnston 1960), which modulates many activities including mating and retinue behaviour (Free

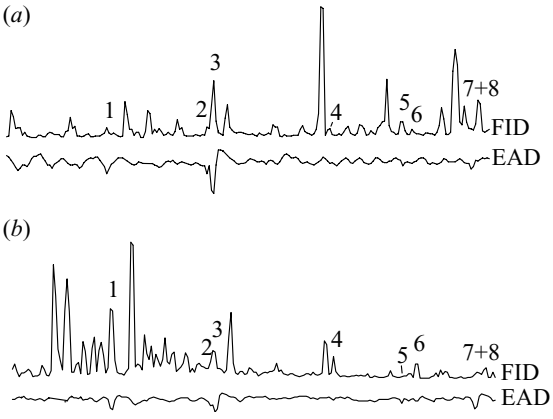

Figure 2. Simultaneous flame ionization/detection (FID) and electroantennographic-detection (EAD) analyses performed with (*a*) a cuticle extract of a *C. ciliata* female and (*b*) an *O. speculum* labellum extract. Numbered peaks correspond to compounds that elicit electroantennographic responses: 1, 7-hydroxyoctanoic acid; 2, 9-oxodecanoic acid; 3, 9-hydroxydecanoic acid; 4, hexadecanal; 5, (z)-9-octadecenal; 6, octadecanal; 7, ethyl linoleate; 8, ethyl oleate. In order to detect all 'physiologically active' compounds and to discriminate electrophysiological responses from noise we performed five to 10 GC–EAD runs for each type of sample.

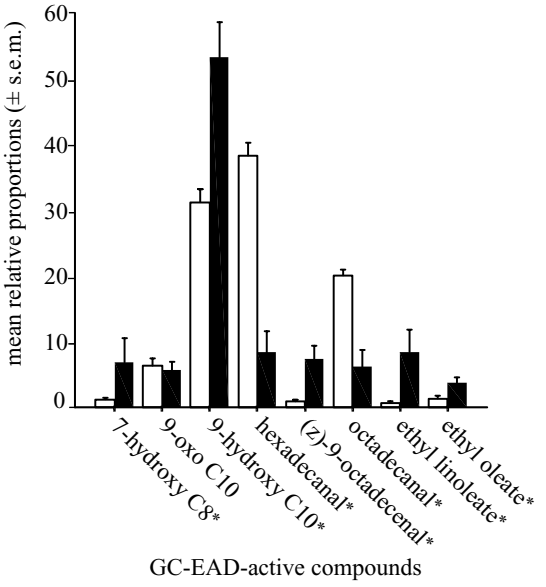

Figure 3. Comparison of relative proportions (mean ± s.e.m.) of electrophysiologically active compounds in cuticle extracts of virgin *C. ciliata* females (filled bars) ($n = 4$) and labellum extracts of *O. speculum* flowers (open bars) ($n = 16$). Asterisks indicate significant difference, Mann–Whitney *U*-test, $p < 0.01$. Three to five individuals were pooled for each of the four *C. ciliata* samples because of the small amounts of the target compounds. The total number of females investigated was 15. For statistical analyses the values equivalent to single females were used.

1987). As far as we know, 9-oxodecanoic acid, 9-hydroxydecanoic acid and 7-hydroxyoctanoic acid have been identified only in the mandibular glands of honeybees (Callow *et al.* 1964; Slessor *et al.* 1988; Plettner *et al.* 1996), and the latter two substances were detected in royal jelly (Lerker *et al.* 1981). Our results are, to our knowledge, the first identifications of these compounds in plants. Recently, (ω-1)-hydroxy acids and some related compounds with as yet unknown functions were identified in the tarsal glands of female bumble-bees, *Bombus terrestris* (Hefetz *et al.* 1996). Interestingly, the enantiomeric composition of 9-oxo-(E)-2-decenoic acid in honeybee queens (Slessor *et al.* 1990) (R : S = 7 : 3 varying with age) is similar to that of 9-hydroxydecanoic acid in the *C. ciliata–O. speculum* pair (see above). This suggests that similar enzymatic systems are involved (at least in the insects), either as a conserved ancestral trait or as a case of convergent evolution.

(b) *Attractiveness of females versus orchids*

Contrary to the hypothesis that *Ophrys* flowers produce only second-class attractivity compounds and are neglected once the pollinator females are present (Borg-Karlson 1990), we showed in a dual-choice experiment that *Ophrys* flowers were significantly more attractive to *C. ciliata* males than were their own females (mean ± s.e.m.; flowers: 49.64 ± 6.17; wasps: 26.25 ± 4.89; Wilcoxon: $p < 0.001$). This finding was confirmed by further tests: odourless dummies presented to the males with the scent of an *Ophrys* flower elicited significantly more copulatory events than dummies offered with the odour of attractive living wasps (mean ± s.e.m.; flowers: 82.66 ± 17.38; wasps: 29.94 ± 9.42; Mann–Whitney *U*-test: $p < 0.01$). Former observations on the same species also argue against the hypothesis that *Ophrys* flowers are neglected once the pollinator females are present: Göltz & Reinhard (1977) observed that *Campsoscolia* males continue to be deceived by flowers of *O. speculum* even though real copulations take place in the same period.

Because we have shown that *O. speculum* flowers and *C. ciliata* females attract the males with the same compounds (see § 3a), the higher attractiveness of *Ophrys* flowers can be explained by a significantly higher total amount of male-attracting scent (GC–EAD-active compounds) produced by flowers (2.71 ± 0.21 μg) than by wasps (0.71 ± 0.34 μg) (Mann–Whitney *U*-test: $p < 0.01$). Generally, calling sexually active hymenopteran females often produce minute quantities of sex attractant (Ayasse *et al.* 2001), presumably detectable over only short distances. The risk of attracting predators and brood parasites by producing long-distance sex pheromones may be great for a female calling close to her nesting or oviposition site. Orchid flowers are exempt from this risk and can produce larger amounts of male-attracting scent limited only by physiological constraints.

(c) *Specificity of the male-attracting signal and reproductive isolation of wasps and orchids*

In insects, the specificity of a chemical signal in a 'noisy chemical background' may be achieved either by specific blends of simple and often ubiquitous compounds, or by the presence of unique compounds, including specific compositions of stereoisomers (Blum *et al.* 1959; Roelofs 1995; Francke & Schulz 1999). Recently, we reported a case of the first scenario, where *O. sphegodes* flowers and virgin *Andrena nigroaenea* females use almost identical bouquets of common saturated and unsaturated hydrocarbons to elicit mating behaviour in the males (Schiestl *et al.* 1999). Interestingly, the same hydrocarbons are also major volatiles in *O. speculum* flowers and *C. ciliata*

females, making up almost identical blends with respect to both chain length and double-bond positions (Erdmann 1996) but these neither stimulate *C. ciliata* male antennae nor induce mating behaviour. The *O. speculum–C. ciliata* interaction, however, represents the second possibility, with both flower and pollinator producing highly specific oxygenated carboxylic acids as the signal. In this case, the various hydrocarbons could potentially act as solvents for the male-attracting compounds, serve as tactile cues or, given their physico-chemical properties, have other functions as surface lipids (Eigenbrode & Espelie 1995).

The existence of two very different plant–pollinator chemical communication systems in various *Ophrys* species raises the following question: why do *C. ciliata* females and mimicking *O. speculum* flowers use complex and unusual compounds rather than a species-specific blend of common compounds, especially when the orchid possesses the same suite of common cuticular hydrocarbons used by sympatric *Ophrys* species to attract pollinators (Schiestl *et al.* 1999)? Ecological and phylogenetic factors and physiological constraints play a fundamental role in the evolution of systems of chemical communication. Many *Andrena* species live sympatrically with closely related species (Westrich 1989). Heterospecific mating is avoided by the use of species-specific blends of the same hydrocarbons (Schiestl & Ayasse 2002) and by pattern recognition. The same principle of species-specific mate attraction exists in various species of moth (Roelofs 1995). Among closely related, sympatrically distributed species, specificities of blends caused by varying the relative proportions of common compounds may be easily achieved by modulating existing systems that account for the biosynthesis and perception of the components.

By contrast, *C. ciliata* neither temporally nor spatially overlaps with other scoliid wasps (Osten 2000), and the male-attracting scent consists of a few specific compounds, which perhaps have arisen by intraspecific sexual selection, and which impart great species specificity. The biosynthesis of such unique compounds and the development of corresponding receptors may generate a less flexible system, but one that allows a particularly fixed relationship.

Ophrys orchids are interfertile (Ehrendorfer 1980), and reproductive isolation is linked to pre-pollination mechanisms, i.e. attraction of and pollination by males of only one pollinator species (Kullenberg 1961). Therefore, it is an advantage if an *Ophrys* species selects a pollinator species that is reproductively isolated from other sympatrically occurring species, such as *A. nigroaenea* (Schiestl & Ayasse 2002) or *C. ciliata* (Osten 2000), and does not visit flowers of sympatrically occurring *Ophrys* species. Mimicking the sex pheromone of a pollinator species that is reproductively isolated may prevent hybridization and pollen loss and as a consequence may lead to an increase in reproductive success. This is particularly important in sexually deceptive orchids, because they have a low rate of pollinator visitation and because the pollen, as in most orchids, is packed in pollinia (Ayasse *et al.* 2000).

The discovery of the behaviour-mediating capacities of (ω-1)-hydroxy acids produced by an orchid and by female wasps to compete for pollinators and for mates, respectively, adds a new facet to the fascinating field of insect–plant interactions.

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