Smells like aphids: orchid flowers mimic aphid alarm pheromones to attract hoverflies for pollination

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Most insects are dependent on chemical communication for activities such as mate finding or host location. Several plants, and especially orchids, mimic insect semiochemicals to attract insects for unrewarded pollination. Here, we present a new case of pheromone mimicry found in the terrestrial orchid Epipactis veratrifolia. Flowers are visited and pollinated by several species of aphidophagous hoverflies, the females of which also often lay eggs in the flowers. The oviposition behaviour of these hoverflies is mainly guided by aphid-derived kairomones. We show that the flowers produce α- and β-pinene, β-myrcene and β-phellandrene, and that these compounds attract and induce oviposition behaviour in female hoverflies. This floral odour profile is remarkably similar to the alarm pheromone released by several aphid species, such as Megoura vicieae. We therefore suggest that E. veratrifolia mimics aphid alarm pheromones to attract hoverflies for pollination; this is the first time, to our knowledge, that such a case of mimicry has been demonstrated.

Keywords: deceptive pollination; chemical mimicry; pheromone

1. INTRODUCTION

The huge diversity of pollination systems in orchids has fascinated evolutionary biologists from the very beginning [1]. One main factor behind this fascination was the fact that one-third of the approximately 30,000 orchid species described have evolved a deceptive pollination mechanism [2,3]. Deceptive flowers do not offer a reward (e.g. nectar or pollen) to the pollinators but attract them by visual mimicry, olfactory mimicry or both. The deceptive mechanisms include food deception, brood-site mimicry, sexual deception or prey mimicry [4–6]. With approximately 11 per cent of orchid genera using such a case of mimicry has been demonstrated.

Hoverflies (Syrphidae) are important pollinators of flowering plants throughout the world [9] and, furthermore, some species are very efficient predators of aphids [10]. Episyrphus balteatus De Geer 1776 (Diptera, Syrphidae) is the most frequently encountered syrphid species at aphid-infested sites in temperate regions of the Northern Hemisphere [11]. While the larvae are aphidophagous (feed on aphids), adults feed on nectar and pollen from flowers [12]. Syrphid larvae are unable to disperse far [13], which makes female choice of oviposition site a crucial factor in offspring survival. Host search and oviposition behaviour in E. balteatus have been intensively studied in the past decade (reviewed in Almohamad et al. [14]): the search for oviposition sites is mainly guided by plant- and aphid-derived semiochemicals (chemicals used for communication), used as kairomones (chemical signals that benefit the receiver) by the flies. Females of E. balteatus are able to locate plant and aphid species, discriminate between them and adjust their oviposition behaviour according to the performance of their larvae on different host species [15] and aphid colony size [16]. Furthermore, aphid honeydew and aphid alarm pheromone compounds (e.g. (E)-β-farnesene, α- and β-pinene) elicit oviposition in E. balteatus females [17,18]. Visual cues are important for the search for host plants and flowers, but do not trigger oviposition behaviour [14].

The genus Epipactis (Orchidaceae) contains 25–59 species with a predominantly Eurasian distribution [19,20]. Epipactis veratrifolia Don. (syn. E. consimilis) is found throughout the Middle East as well as eastwards to the Himalayas and southwards to Somalia and Ethiopia [21]. The plant consists of separate stems growing from a common rhizome. The stems reach a height of 1.5–2 m and produce 20–40 flowers. The pollination biology of E. veratrifolia has been studied by Ivri & Dafni [22]: E. veratrifolia is exclusively pollinated by five species of aphidophagous hoverflies (Syrphidae), namely Sphaerophoria rupestris Wiedemann 1830, Sphaerophoria scripta (L. 1758), Ischiodes acutipennis Wiedemann 1830, Eupeodes corollae (F. 1794) and E. balteatus. Flies of the genus Paragus also visit the flowers, but are too small to carry the pollinia.
and effectively pollinate the flowers. The flowers produce small amounts of nectar that is presented freely on the label-

lum. Flowers are not autogamous, and the natural pollination rate is about 15 per cent [22].

Male hoverflies are often found in the vicinity of the orchids. Males occupy a territory comprising a few plants, in which they try to copulate with females approaching the flowers. Males occasionally visit the flowers in search of nectar and thereby also pollinate them. Hoverfly females approach the flowers in hovering flight, land on the labellum, may lick the exposed nectar droplets and lay an egg on the labellum or in other parts of the flower. During nectar feeding and egg laying, they pollinate the flowers [22].

The fact that females lay eggs on the flowers of the orchid is very interesting as aphidophagous hoverflies normally lay their eggs exclusively in places where aphids are present, because the larvae feed on aphids [23]. Based on this fact and on the aphid-like dark warts in *E. veratrifolia* flowers, Ivri & Dafni [22] suggested that the flower mimics the shape and colour of aphids to attract syrphid flies for pollination. However, as volatiles seem to play a key role in host location and oviposition behaviour of syrphid flies [14], we reasoned that the flowers would also have to mimic aphid volatiles if they were to achieve pollination by attracting female hoverflies searching for oviposition sites. Mimicry of aphid volatiles has so far not been demonstrated in any pollination system. To lay eggs, they pollinate the flowers [22].

The population of *E. balteatus* comprises approximately 100–200 plants.

(b) **Nectar collection**

The amount of nectar produced by the flowers of *E. veratrifolia* was measured in the morning (09.00–10.00 h) and at noon (12.00–13.00 h) using 0.5 μl microcapillary tubes (Hirschmann, Germany).

(c) **Volatile collections**

For collection of headspace samples of *E. veratrifolia*, stems bearing several flowers or only buds were enclosed in a poly-
ethylene terephthalate oven bag (Toppsit, Germany) without cutting the stem. A headspace filter (50 mg Super-Q, Analytical Research Systems, USA) was put inside the bag and connected to a hand-held air-sampling pump (Casella, USA) using a silicone tube. Air was pumped out of the bag at a rate of 0.8 l min \(^{-1}\) for 4 h. Filters were eluted with 500 μl pentane (Sigma–Aldrich, Germany) concentrated to 100 μl and samples were stored at −20 °C until further analy-

sis. To avoid contamination of plant volatiles, we carefully checked the plants to ensure that the stem, flowers and buds were free of aphids or syrphid larvae before headspace collection.

Volatile samples of the aphid *Megoura viciae* Buckton 1876, a common species in the Middle East, were collected by extracting approximately 20 individuals of mixed stages for 120 s in 1 ml pentane. The extract was concentrated under a gentle stream of nitrogen and stored at −20 °C until analysis.

(d) **Chemical analysis**

Headspace samples were analysed by gas chromatography–mass spectrometry (GC–MS) (Agilent 7890GC and 5975c MS). The gas chromatograph (GC) was equipped with a DBS-MS column (30 m long, 0.25 mm internal diameter, 25 μm film thickness; Agilent) with helium as a carrier gas (18.5 μl s \(^{-1}\) constant flow). The inlet temperature was set to 250 °C. The temperature of the GC oven was held at 40 °C for 180 s and then increased by 5 °C per 60 s to 280 °C. The final temperature was maintained for 600 s. The MS transfer line was held at 300 °C, the MS source at 230 °C and the MS quad at 150 °C. Mass spectra were taken in electron ionization mode (at 70 eV) in the range of 33–350 m z \(^{-1}\) with a scanning rate of 4.42 scan s \(^{-1}\). To evaluate the enantio-
meric composition of α- and β-pinene, selected samples and authentic standards were also analysed on a chiral column (β-dex, 30 m long, 0.25 mm internal diameter, 25 μm film thickness; Agilent) under the same analytical conditions.

GC–MS data were processed with the MDS-ChemStation software (Agilent). Compounds were provisionally identified with the NIST 2.0 mass spectra database using the NIST algor-
ithm. Identification was confirmed by comparison with synthetic standards or in the case of β-phellandrene, by compar-
ison of the Kovats retention index with the published data.

(e) **Electrophysiology**

We used electroantennograms (EAGs) to test whether *E. balteatus* was able to perceive the floral compounds of *E. veratrifolia*. For an EAG, the head of a fly was cut off and pinned onto a glass electrode filled with insect saline. A second glass electrode was brought into contact with the tip of one of the antennae. The electrodes were connected by Ag–AgCl wires through a pre-amplifier to an amplifier (Syntech, Germany). Signals from the antenna were recorded on a PC running EAG recordings software (all Syntech, Germany). Test compounds were diluted in dichloromethane (DCM) with a concentration of 10 μg ml \(^{-1}\). Disks of filter paper (100 mm²) were impregnated with 20 μl solution (containing 200 μg of compound) and put into Pasteur pipettes. The solvent was allowed to evaporate before the experiment. Pipettes were used to puff odour compounds into a filtered and humidified airstream (3.33 ml s \(^{-1}\), which was directed over the antenna. Each compound was tested three times on each one of 18 female and 10 male flies. EAG reactions to single compounds were averaged for each individual. Differences in the EAG reactions to odour compounds and the control (DCM) were tested with a paired sample t-test and sequential Bonferroni corrections for multiple comparisons [24]. All tests were carried out using SigmaStat 3.5.

(f) **Oviposition experiments**

Bean plants (*Vicia faba*) were grown in the Botanical Garden of the University of Ulm. Hoverfly pupae (*E. balteatus*) were ordered from a commercial supplier (Katz Biotech, Germany), reared in a flight cage (0.6 × 0.6 × 0.6 m) in a climate-controlled room (13 L : 11 D cycle, 60–70% relative humidity, 24 °C), and fed with sugar water, pollen and water ad libitum.

For the experiments, a membrane dispenser (Wilhelm Biological Plant Protection, Germany) was used to test the behavioural activity of the natural headspace samples from
of nectar. Less than 1 (09.00–10.00 h), the flowers produced small amounts. We collected nectar from 13 flowers. In the morning, we found four compounds: \( \alpha \)-pinene (51 ± 1.0%, mean ± s.e.), \( \beta \)-pinene (41.7 ± 0.6%), \( \beta \)-myrcene (3.7 ± 0.4%) and \( \beta \)-phellandrene (3.0 ± 0.3%; figure 1a). By contrast, in the leaves and buds of \( E. \) veratrifolia, we found only trace amounts of \( \alpha \)-pinene and \( \beta \)-pinene. \( E. \) veratrifolia produces both enantiomers of \( \alpha \)- and \( \beta \)-pinene with a slight dominance of the (\( \alpha \))-forms in both compounds. The surface extracts of the aphid \( M. \) viciae (all stages) contained \( \alpha \)-pinene (12%), \( \beta \)-pinene (80%), \( \beta \)-myrcene (3%) and one unidentified compound (4%; figure 1b).

### 3. RESULTS

#### (a) Nectar collection

We collected nectar from 13 flowers. In the morning (09.00–10.00 h), the flowers produced small amounts of nectar. Less than 1 \( \mu \)l was collected per flower. Because of high ambient temperatures at the field site, the nectar became too viscous at noon to be collected with microcapillary tubes.

#### (b) Volatile collections

In the headspace collections of \( E. \) veratrifolia flowers, we found four compounds: \( \alpha \)-pinene (51 ± 1.0%, mean ± s.e.), \( \beta \)-pinene (41.7 ± 0.6%), \( \beta \)-myrcene (3.7 ± 0.4%) and \( \beta \)-phellandrene (3.0 ± 0.3%; figure 1a). By contrast, in the leaves and buds of \( E. \) veratrifolia, we found only trace amounts of \( \alpha \)-pinene and \( \beta \)-pinene. \( E. \) veratrifolia produces both enantiomers of \( \alpha \)- and \( \beta \)-pinene with a slight dominance of the (\( \alpha \))-forms in both compounds. The surface extracts of the aphid \( M. \) viciae (all stages) contained \( \alpha \)-pinene (12%), \( \beta \)-pinene (80%), \( \beta \)-myrcene (3%) and one unidentified compound (4%; figure 1b).

#### (c) Electrophysiology

Synthetic copies of the volatiles found in the \( E. \) veratrifolia flower headspace (\( \alpha \)-pinene, \( \beta \)-pinene and \( \beta \)-myrcene) released EAG responses in the antennae of \( E. \) balteatus males (\( n = 10 \)) and females (\( n = 18 \); figure 2). Synthetic \( \beta \)-phellandrene could not be obtained and was therefore not tested. The antennae gave the highest responses to (\( \alpha \))-\( \alpha \)-pinene (mean 2.4, s.e. 0.15 mV), (\( \alpha \))-\( \beta \)-pinene (mean 1.9, s.e. 0.14 mV) and \( \beta \)-myrcene (mean 2.1, s.e. 0.16 mV). The (\( \alpha \))-form of \( \alpha \)-pinene released only a weak response (mean 0.9, s.e. 0.08 mV) as did (\( \alpha \))-\( \beta \)-pinene (mean 1.4, s.e. 0.12 mV). These values were, however, still significantly higher than the control (mean 0.6, s.e. 0.07 mV, \( p < 0.001 \) paired sample \( t \)-test, sequential Bonferroni correction for ties). There was no difference in the antennal reactions of males and females.

#### (d) Oviposition experiments

\( E. \) balteatus females laid significantly more eggs on plants combined with the headspace sample (mean 19.6, s.e. 2.1 eggs) than on plants with the solvent only (mean 7.9, s.e. 2.2; paired sample \( t \)-test, \( n = 10, t = 6.8, p < 0.001 \); figure 3a). The females also laid significantly more eggs on plants combined with the synthetic mixture (mean 14.6, s.e. 2.9 eggs) than on control plants (mean 3.3, s.e. 0.9 eggs; \( n = 13, t = 4.8, p < 0.001 \); figure 3b).

### 4. DISCUSSION

#### (a) Do orchid flowers mimic hoverfly alarm pheromone?

Our results provide evidence that \( E. \) veratrifolia flowers produce the same compounds as are found in the alarm
pheromone of some aphid species, e.g. M. viciae, namely α- and β-pinene, and β-myrcene [25,26]. The flowers, therefore, appear to mimic the alarm pheromone of aphids, thus attracting hoverflies for pollination. This is supported by our behavioural experiments, in which we show that the flowers’ scent, as well as a synthetic copy of it, induces oviposition behaviour in females of the hoverfly E. balteatus.

Aphids not only release alarm pheromone when under attack, but continuously release small amounts [27]. Predators can thus use aphid pheromones as faithful cues for locating aphid colonies. Although (E)-β-farnesene is the most commonly used aphid alarm pheromone, several other terpenoids are produced by aphids and also function as alarm pheromones [25,26]. The aphid M. viciae is a common species in the Middle East; its pheromone mainly consists of α- and β-pinene, and β-myrcene [25] and shows a striking similarity to the floral odour of E. veratrifolia (figure 1). Five more aphid species are known to produce α/β-pinene, three of them as the major pheromone component [26]. α/β-pinene and myrcene might, in fact, be more frequent components of aphid chemical communication than is currently recognized because, as yet, the alarm volatiles of only approximately 1 per cent of all aphid species have been analysed.

Our data suggest that E. veratrifolia does not mimic one aphid species specifically, as the orchid does not produce the same compounds in exactly the same proportions as a specific aphid species. The flower instead produces α- and β-pinene to mimic volatiles associated with a certain group of aphids, namely those using these compounds in their pheromone communication. This generalized mimicry makes sense as all five pollinating hoverfly species (i.e. their larvae) are not specialized in one certain aphid species, but can feed on a large number of hosts (I. aegyptus: 23 host species, S. ruepellii: 37, S. scripta: 79, E. corollae: 111, E. balteatus: 187; [28]). Megoura viciae is used as a host by three of these hoverflies (S. scripta, E. corollae and E. balteatus), and all but I. aegyptus use at least one additional pinene-producing aphid species as their host [28]. It is therefore plausible that most of the pollinating hoverflies do not react to the pheromone of only one aphid species, but use several aphid- and plant-derived volatiles for host location and as oviposition cues, as has been demonstrated for E. balteatus [18].

The pollination mechanism of E. veratrifolia is thus similar to those found in other plant species mimicking oviposition sites. Many Araceae, for example, mimic dung or faeces, the oviposition sites for coprophilic flies.
These species also produce odours typical for such substrates, but do not mimic the odour profile of a specific kind of faeces perfectly [29,30]. The genus *Epipactis* shows a huge variety in pollination systems, with most species showing specialist pollination systems, e.g. wasp-pollinated or bumble-bee-pollinated [31]. Floral odours have been investigated only in wasp-pollinated *Epipactis* species; those produce a completely different floral odor than *E. veratrifolia* [32]. This strengthens our hypothesis that *E. veratrifolia* specifically mimics aphid volatiles.

Opinions diverge on whether such a generalized or non-model mimicry, which lacks a certain model (-species), should be referred to as mimicry sensu Bates [33] or perceptual exploitation [34]. As mimicry implies a very exact copy of the original, we would in the present case favour the generalized mimicry or perceptual exploitation terminology.

(b) Does *Epipactis veratrifolia* reward or harm its pollinator? *Epipactis veratrifolia* does provide some nectar in its flowers (although very little) and might thus not be a truly deceptive species. On the other hand, the floral signal that attracts the pollinators advertises a different reward (aphids) than actually provided (nectar). Thus, *E. veratrifolia* has to be considered deceptive, at least in terms of pollinator attraction. A similar case has been found in the wasp-pollinated *Epipactis helleborine* and *Epipactis purpurata*, where the flowers mimic volatiles associated with the wasps’ prey. But instead of the prey, the wasps get rewarded with nectar [32].

An important question that remains unanswered is the cost to the pollinators when they deposit eggs that cannot develop in the flowers. First-instar larvae of *E. balteatus* are limited in their dispersal [13]. If the larvae do not find a suitable host and consequently die, *E. veratrifolia* does harm its pollinators by reducing their fitness. Harming the pollinator is thought to be evolutionarily unstable and, therefore, very rare [35]. However, detailed data on the survival rate of hoverfly larvae on *E. veratrifolia* are needed to support this hypothesis.

(c) Deceptive pollination by syrphids Hoverflies are frequent and well-known pollinators of rewarding flowers and, as such, have been thoroughly studied. By contrast, deceptive pollination systems involving hoverflies are rare and have so far received little attention. The Japanese *Epipactis thunbergii* may exhibit a similar pollination strategy to *E. veratrifolia*: it is also pollinated by several syrphids, which also deposit eggs in the flower [36]. The slipper orchids *Paphiopedilum dianthum* and *Paphiopedilum rothschildianum* are also exclusively pollinated by hoverflies, predominantly females, which also lay eggs in the flowers [37,38]. Unfortunately, no data on the floral chemistry are available for all these species. A third slipper orchid, *Paphiopedilum barbigerum*, is also pollinated by hoverflies, but in this case no eggs are deposited by the flies and as no floral odour could be detected, it is assumed that the orchid rather exploits an innate colour preference of the flies [39].

(d) Evolution of floral odours—a case of pre-adaptation? Pre-adaptation (the acquisition of a new function by the pre-existing traits) is thought to play a major role in the evolution of specialized and deceptive pollination systems [40]. A prime example is the tropical genus *Dalechampia*. Here, resins produced as floral defence compounds appear to have been a pre-adaptation for the evolution of a resin-reward pollination system [41]. The use of aphid pheromones for herbivore defence is known from the wild potato *Solanum berthaultii* [42]. Its leaves produce the aphid alarm pheromone compound (E)-β-farnesene to deter aphids. α- and β-pinene are very common floral compounds (found in at least 350 species of Orchidaceae) [43,44]. We speculate that the initial function of pinene in flowers may have been to deter aphids away from the precious reproductive organs. Subsequently, α- and β-pinene may have gained importance in pollinator attraction, as the function switched from plant defence to pollinator attraction. It is of interest that the flowers of *E. veratrifolia* are mostly aphid free, even though the green parts of the flower are regularly infested [22]). However, one function does not exclude the other. Further studies are required to fully test the hypothesis that hoverfly pollination has evolved as a consequence of switching from aphid deterrence to pollinator attraction in this system.

5. CONCLUSIONS We provide new evidence that *E. veratrifolia* attracts aphidophagous hoverflies by generalized mimicry of alarm pheromone, adding a new pollination strategy to the long list of diverse and intriguing pollination strategies that have evolved in the Orchidaceae. There is an ongoing discussion among evolutionary biologists as to whether species richness in Orchidaceae is a cause or a consequence of specialized (and deceptive) pollination systems [45–48]. Compared with the high number of species, orchids are still under-represented in scientific investigations of pollination biology and, more generally, of reproductive isolation and speciation mechanisms [49]. The present results add another piece to the puzzle of the complex evolution of orchids.

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