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

Johannes Stökl\textsuperscript{1,}*,†, Jennifer Brodmann\textsuperscript{2,}†, Amots Dafni\textsuperscript{3}, Manfred Ayasse\textsuperscript{2} and Bill S. Hansson\textsuperscript{1}

\textsuperscript{1}Department of Evolutionary Neuroethology, Max-Planck-Institute for Chemical Ecology, Hans-Knoll-Strasse 8, 07745 Jena, Germany
\textsuperscript{2}Institute of Experimental Ecology, University of Ulm, Albert-Einstein-Allee 11, 89069 Ulm, Germany
\textsuperscript{3}Institute of Evolution, Haifa University, Haifa 31905, Israel

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 \textit{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 \(\alpha\)– and \(\beta\)-pinene, \(\beta\)-myrcene and \(\beta\)-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 \textit{Megoura vicinae}. We therefore suggest that \textit{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 creation, brood-site mimicry, sexual deception or prey mimicry [4–6]. With approximately 11 per cent of orchid genera using brood-site mimicry, this is one of the most widespread deceptive pollination mechanisms in the Orchidaceae [7,8].

Hoverflies (Syrphidae) are important pollinators of flowering plants throughout the world [9] and, furthermore, some species are very efficient predators of aphids [10]. \textit{Epiyrus 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 \textit{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 \textit{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. (\(E\))-\(\beta\)-farnesene, \(\alpha\)- and \(\beta\)-pinene) elicit oviposition in \textit{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 \textit{Epipactis} (Orchidaceae) contains 25–59 species with a predominantly Eurasian distribution [19,20]. \textit{Epipactis veratrifolia} Don. (syn. \textit{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 \textit{E. veratrifolia} has been studied by Ivri & Dafni [22]: \textit{E. veratrifolia} is exclusively pollinated by five species of aphidophagous hoverflies (Syrphidae), namely \textit{Sphaerophoria rueppelli} Wiedemann 1830, \textit{Sphaerophoria scripta} (L. 1758), \textit{Ischiodon aegyptius} Wiedemann 1830, \textit{Eupodes corollae} (F. 1794) and \textit{E. balteatus}. Flies of the genus \textit{Paragus} also visit the flowers, but are too small to carry the pollinia.

* Author and address for correspondence: Chemical Ecology Group, University of Regensburg, Universitätsstrasse 31, 93053 Regensburg, Germany (johannes.stoekl@biologie.uni-regensburg.de).
† These authors contributed equally to this study.
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 nor-

2. MATERIAL AND METHODS

(a) Study site
Samples were collected in March 2009 in the Enot Tsukim 
Nature Reserve, Israel. The nature reserve is a small wetland 
on the Dead Sea coast, characterized by dense grass and reed 
vegetation. The population of E. veratrifolia comprises 
approximately 100–200 plants.

(b) Nectar collection
The amount of nectar produced by the flowers of E. veratri-
folia was measured in the morning (09.00–10.00 h) and at 
onoon (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 (Toppits, 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 0.1 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 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 
DB5-MS column (30 m long, 0.25 mm internal diameter, 
25 μm film thickness; Agilent) with helium as a carrier gas 
(18.3 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 NISTalg-
orithm. Identification was confirmed by comparison with 
synthetic standards or in the case of β-phellandrene, by com-
parison 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 mg ml⁻¹. Discs of filter 
paper (100 mm²) were impregnated with 20 μl solution (con-
aining 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. Differ-
ences 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
E. veratrifolia \((n = 10)\), as well as the synthetic mixture of compounds identified in the headspace samples \((n = 13)\). The dispenser guarantees a constant emission of the tested volatiles. We used the solvent pentane as a negative control. For each experiment, two \(V. faba\) plants \((0.3–0.4 \text{ m tall})\), each with a dispenser containing either the odour sample or the solvent only, were placed in a flight cage \((0.6 \times 0.6 \times 0.6 \text{ m})\) together with four gravid Episyrphus females. After 24 h, we counted the eggs laid on the plants. Experiments were conducted in a climate-controlled room at 24°C and 60–70% humidity. For the tests, we used \(E. balteatus\) females aged two to five weeks, mated and gravid. Two days before we tested the females they were allowed to lay eggs on a \(V. faba\) plant infested with \(Aphis fabae\), as females with oviposition experience were more active in the behavioural test and laid a higher number of eggs. The synthetic mixture used consisted of 51 ng \(\mu l^{-1}\) \((\pm)\)-\(\alpha\)-pinene; 41 ng \(\mu l^{-1}\) \((\pm)\)-\(\beta\)-pinene and 4 ng \(\mu l^{-1}\) \(\beta\)-myrcene (all Sigma–Aldrich, Germany). The qualitative and quantitative composition of the synthetic mixture was the same as that found in the flowers as verified by GC analysis using the same parameters as for the chemical analysis of headspace volatiles.

3. RESULTS

(a) Nectar collection

We collected nectar from 13 flowers. In the morning \((09.00–10.00 \text{ h})\), 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 \((51 \pm 1.0\%\), mean \(\pm\) s.e.), \(\beta\)-pinene \((41.7 \pm 0.6\%\), \(\beta\)-myrcene \((3.7 \pm 0.4\%\) and \(\beta\)-phellandrene \((3.0 \pm 0.3\%\); figure 1a). By contrast, in the leaves and buds of \(E. veratrifolia\), we found only trace amounts of \(\alpha\)-pinene and \(\beta\)-pinene. Epipactis

![Figure 1. GC–MS traces of (a) Epipactis veratrifolia flower headspace volatiles and (b) Megoura viciae aphid surface extract.](image)

\(E. veratrifolia\) produces both enantiomers of \(\alpha\)- and \(\beta\)-pinene with a slight dominance of the \((-)\)-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 \((\pm)\)-\(\alpha\)-pinene (mean 2.4, s.e. 0.15 mV), \((\pm)\)-\(\beta\)-pinene (mean 1.9, s.e. 0.14 mV) and \(\beta\)-myrcene (mean 2.1, s.e. 0.16 mV). The \((\pm)\)-form of \(\alpha\)-pinene released only a weak response (mean 0.9, s.e. 0.08 mV) as did \((-)\)-\(\beta\)-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

Episyrphus 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 a- and b-pinene, and b-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)-b-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 a- and b-pinene, and b-myrcene [25] and shows a striking similarity to the floral odour of *E. veratrifolia* (figure 1). Five more aphid species are known to produce a/b-pinene, three of them as the major pheromone component [26]. a/b-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 a- and b-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.

**Figure 2.** Antennal (EAG) responses of *E. balteatus* females and males to *E. veratrifolia* flower headspace compounds. Asterisks (*), significant difference from control (DCM) *p* < 0.001 (paired sample *t*-test, sequential Bonferroni correction for ties). Filled bars, females; white bars, males.

**Figure 3.** Effect of flower odour on oviposition of *E. balteatus* females (mean number of eggs ± s.e.). (a) Comparison of the headspace sample of the orchid *E. veratrifolia* and (b) the synthetic mixture against the solvent pentane (paired samples *t*-test, *n* = 10/13, *t* = 6.8/4.8, *p* < 0.001).
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 odour 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 will 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 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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