Report

Orchids Mimic Green-Leaf Volatiles to Attract Prey-Hunting Wasps for Pollination

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Summary

An outstanding feature of orchids is the diversity of their pollination systems [1]. Most remarkable are those species that employ chemical deceit for the attraction of pollinators [2]. The orchid *Epipactis helleborine* is a typical wasp flower, exhibiting physiological and morphological adaptations for the attraction of pollinating social wasps [3]. As noted by Darwin [1], this species is almost entirely overlooked by other potential pollinators, despite a large nectar reward. Therefore, the mechanism for the attraction of pollinating social wasps was something of a mystery. By using a combination of behavioral experiments, electrophysiological investigations, and chemical analyses, we demonstrate for the first time that the flowers of *E. helleborine* and *E. purpurata* emit green-leaf volatiles (GLVs), which are attractive to foragers of the social wasps *Vespula germanica* and *V. vulgaris*. GLVs, emitted by damaged plant tissues, are known to guide parasitic wasps to their hosts [4]. Several *E. helleborine* GLVs that induced response in the antennae of wasps were also emitted by cabbage leaves infested with caterpillars (*Pieris brassicae*), which are common prey items for wasps [5]. This is the first example in which GLVs have been implicated in chemical mimicry for the attraction of pollinating insects.

Results and Discussion

Visual versus Olfactory Cues for Wasp Attraction

The orchid *Epipactis helleborine* (L.) Crantz is a prime example of a wasp flower; it is mainly pollinated by social wasps (Hymenoptera: Vespidae) like *Vespula vulgaris* and *V. germanica* [3]. Wasp flowers exhibit physiological and morphological adaptations for the attraction of pollinating social wasps. Although wasp-pollinated flowers have been the subject of a number of studies [3, 6–9], little is known about the floral signals that are responsible for the highly specific attraction of wasps.

Social wasps feed their larvae on insects like caterpillars [5], among them *Pieris rapae* [10]. In order to locate their prey, they use a combination of visual and olfactory cues [11]. Parasitic wasps use volatiles emitted by plants to locate insect prey [4, 12]. Social wasps may do likewise. Indeed, the ability of plants to induce resistance in response to herbivory has been reported for many species [13], and plants may even produce carnivore-attracting volatiles [4].

To investigate the relative importance of floral signals to foraging wasps, we compared the attractiveness of whole inflorescences, inflorescences covered with a quartz glass cylinder (visual cues), and natural scent of *E. helleborine* (olfactory cues), which were offered to workers of *V. germanica*. The results of our field bioassays showed that olfactory cues were significantly more attractive to wasps than visual cues (Mann-Whitney U test, U = 5.5, p < 0.001) and released the same number of approaches in the wasps as the whole inflorescences (Mann-Whitney U test, U = 65.5, p = 0.7, whole inflorescence: mean ± standard deviation [SD]: 4.83 ± 1.69, n = 12, scent: 5.41 ± 2.02, n = 12, visual cues: 1.8 ± 1.13, n = 10). *E. helleborine* grows in shaded areas, often in dark coniferous forests with a shortage of pollinators [14, 15]. Foraging wasps are obviously attracted from a distance by the flower's fragrance. This is evident by the optomotor-anemotaxis-mediated searching behavior of *V. germanica* workers who approach the flowers in a characteristic zigzag flight. The results of our behavioral experiments support the primacy of olfactory cues in the long-distance attraction of wasps. The floral scent offered without visual cues clearly attracted the wasps to visit flowers.

Do *Epipactis* Flowers Release Green-Leaf Volatiles?

Green-leaf volatiles (GLVs), mostly six-carbon aldehydes, alcohols, and acetates, are emitted by many plants infested by herbivores, e.g., caterpillars [16]. GLVs may attract predators or parasitoids of herbivorous insects [4, 17–19], and we suspected that *E. helleborine* flowers may produce GLVs in order to attract prey-hunting social wasps for pollination.

Gas chromatography coupled with an electroantennographic detector (GC-EAD) was used to identify those compounds in the complex flower scent perceived by the antennae of worker wasps, a technique we have found to be an effective method to identify volatile pollinator attractants in *Ophrys* flowers [2, 20]. In headspace samples collected from *E. helleborine* flowers, we found seven compounds inducing an electrophysiological response in antennae of workers of *V. germanica* and of *V. vulgaris* (Figure 1). By using gas chromatography coupled with mass spectrometry (GC-MS), we identified the aldehydes octanal, nonanal, and decanal, as well as benzaldehyde and the GLVs hexyl acetate, *Z*-3-hexenyl acetate, and *Z*-3-hexen-1-ol in inflorescences.

In parallel investigations, we found the same compounds to be present when caterpillars of *Pieris brassicae* infest cabbage (*Brassica oleracea gemifera*), a plant that is known to release GLVs upon herbivore attack [21–23]. The total amount of emitted volatiles was significantly higher in infested cabbage (mean 1.7 μg ± 0.17 standard error [SE] per cabbage) than in *E. helleborine* (mean 0.4 μg ± 0.08 SE per inflorescence) (Mann-Whitney U test, U = 5.0, p = 0.013, n = 14), and the

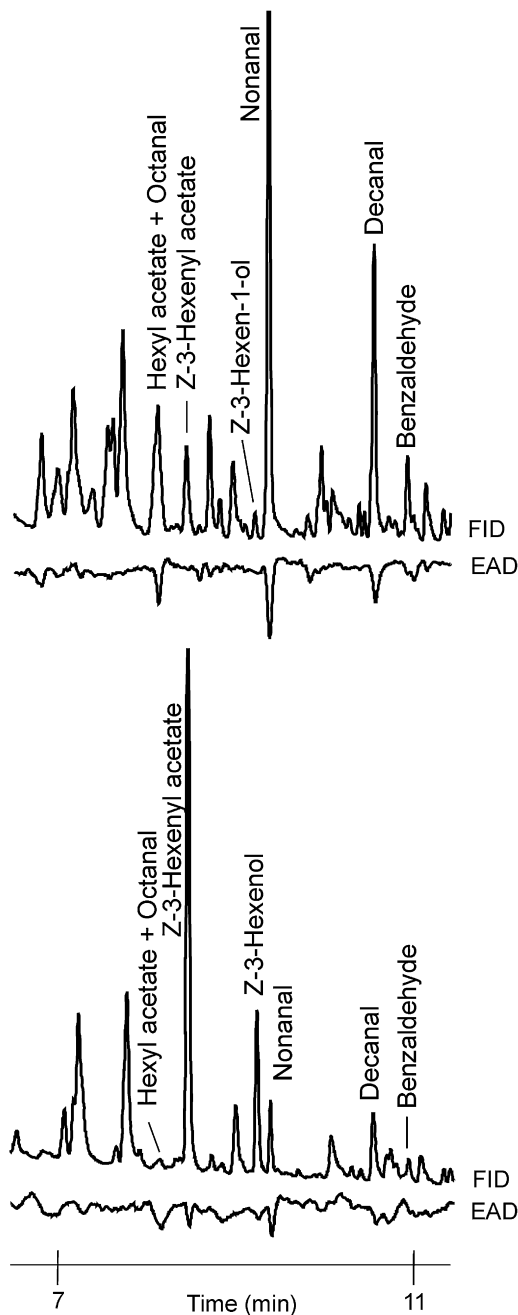*Correspondence: manfred.ayasse@uni-ulm.de

Figure 1. Electrophysiologically Active Compounds

Simultaneous recordings of GC (FID) and EAD signals obtained with head-space samples of *E. helleborine* flowers (above) and *Pieris* infested cabbage (below) with the antenna of a *V. vulgaris* worker. The GC analyses were performed on a polar DB-Wax capillary column. Hexyl acetate and octanal could not be separated with the GC parameters used. We found seven EAD-active compounds common to both plants that are present in quantitatively different compositions.

relative proportions of volatiles differed, too (Figure 1). However, several studies have shown that plant species emit different patterns of volatiles when attacked by herbivorous insects, and different species of *Pieris* caterpillars induce varying amounts of emitted volatiles [21–23]. Therefore, differences in the scents of cabbage infested with *P. brassicae* caterpillars and of *E. helleborine* flowers were not surprising. Because
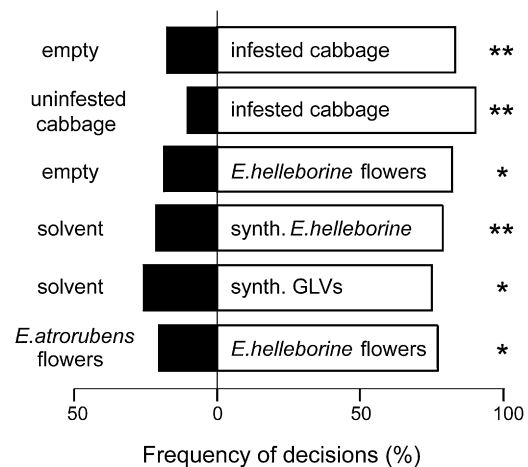

Figure 2. Attraction of Wasps to Various Odor Samples

Comparison of the attractiveness of the odor from *Pieris*-infested cabbage, *E. helleborine* and *E. atrorubens* flowers, a synthetic mixture of GC-EAD active substances of *E. helleborine*, and a synthetic mixture of GLVs to social wasps in a Y tube olfactometer (Sign test *$p \le 0.05$, **$p \le 0.01$).

prey-hunting wasps search for insects that feed on many different plant species that produce different bouquets of volatiles, we expected the wasps to react instantly to certain key compounds, even if quantitative volatile compositions were not identical. Our finding that octanal, hexyl acetate, *Z*-3-hexenyl acetate, and *Z*-3-hexen-1-ol were produced in higher amounts in cabbage plants damaged by *Pieris* caterpillars [21] is consistent with our hypothesis that *E. helleborine* flowers produce GLVs in order to attract prey-hunting social wasps for pollination.

In behavioral experiments, we tested the attractiveness of various odors to *V. vulgaris* and *V. germanica* workers. In a Y tube olfactometer, the wasps significantly preferred the odor of *Pieris*-infested cabbage compared to the empty control (Sign test, $p < 0.001$, n = 24) and compared to uninfested cabbage (Sign test, $p < 0.001$, n = 43) (Figure 2), indicating that insect-hunting wasps find their prey by using GLVs. In further tests, we could show that floral volatiles emitted by *E. helleborine* flowers (Sign test, $p < 0.001$, n = 34)—as well as a synthetic mixture of all EAD-active compounds identified in *E. helleborine* (Sign test, $p < 0.01$, n = 24) and a mixture consisting of the three GLVs hexyl acetate, *Z*-3-hexenyl acetate, and *Z*-3-hexen-1-ol (Sign test, $p < 0.02$, n = 28), which are produced by *E. helleborine* flowers—were significantly more attractive than the empty control. In addition, the attractiveness of a synthetic mixture consisting of all of the electrophysiologically active *E. helleborine* compounds that were found to co-occur in damaged cabbage was confirmed as attractive by a choice experiment in a flight cage in the field (Sign test, $p = 0.01$, n = 170). Over 60% of the foraging wasps selected the flowers impregnated with a synthetic blend of *Epipactis* volatiles.

In former investigations, it was shown that hunting wasps can use several different kinds of cues to find their prey, including frass odors [24]. Our results show for the first time that prey-hunting foragers of social wasps use GLVs to find herbivorous insects. Until now, this was only known in parasitic wasps [4]. We do not exclude the possibility that visual cues have an additional function, e.g., in close-range orientation. Like other wasp flowers, *E. helleborine* is characterized by a dull coloration that may play an additional role in the
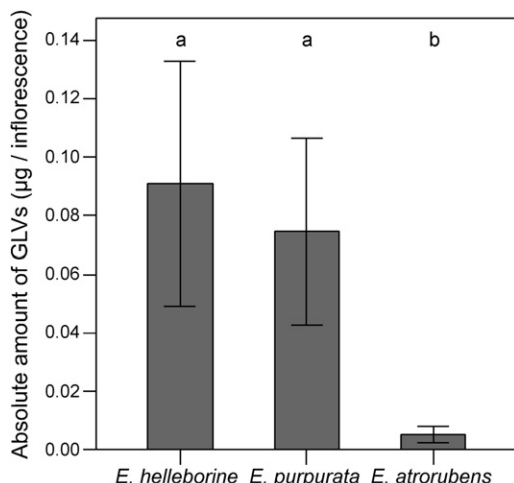
Figure 3. GLVs in Wasp-Pollinated and Bee-Pollinated Species of *Epipactis*

Comparison of absolute amounts (mean ± 2 SE) of GLVs in *E. helleborine* and *E. purpurata*, both wasp pollinated, as well as in *E. atrorubens*, a species that is mainly visited by various bee species. Different letters indicate significant differences (Mann-Whitney U test with a Benjamini-Hochberg correction [31], p < 0.05).

attraction of wasps and that may even prevent visual attraction of insects that are morphologically unsuitable as vectors for the pollinaria. Some of the compounds that are particularly attractive to foraging wasps may even repel other potential visitors [9]. It is likely that learning of the odor and association with visual cues of prey may optimize foraging activities of the wasps [25, 26].

The Importance of GLVs for Wasp Attraction

Within the genus *Epipactis* certain species are pollinated by social wasps, whereas others attract bees [27]. We expected the GLVs found in *E. helleborine* to also occur in other wasp-pollinated species of *Epipactis*. Therefore, we looked for the presence of the GLVs hexyl acetate, *Z*-3-hexenyl acetate, and *Z*-3-hexen-1-ol in two wasp-pollinated species of *Epipactis*—*E. helleborine* and *E. purpurata*—and in *E. atrorubens*, a species that is visited by a broad spectrum of pollinators, mainly bumblebees [27]. Our results clearly show that both wasp-pollinated species produce significantly higher amounts of GLVs than does *E. atrorubens* (Figure 3). In a comparative olfactometer test, the wasp flower *E. helleborine* was significantly more attractive to wasps than was the bumblebee-pollinated species *E. atrorubens* (Sign test, p = 0.05, n = 28) (Figure 2). The two wasp-pollinated species *E. helleborine* and *E. purpurata* emit significantly higher amounts of GLVs than does *E. atrorubens*, and these GLVs definitely have a key function in wasp attraction.

The fact that other insect species rarely visit *E. helleborine* and *E. purpurata* may primarily be a consequence of the habitat specificity. These wasp-pollinated *Epipactis* species, *E. helleborine* and *E. purpurata*, mainly grow in dark forest understory, where other insect pollinators like honey bees, solitary bees, butterflies, etc. are rare or absent [14]. In addition, quality and quantity of nectar of *E. helleborine* and other wasp-pollinated species could be different from that of flowers of species that are visited by other insect pollinators. Baker and Baker [28] found that wasp-pollinated species seem to be rather rich in sucrose, whereas many flowers pollinated by bees, butterflies, and other insects produce higher amounts

of glucose. Whether nectar collected by *Vespula* females is inappropriate for honey bees or bumble bees or whether nonsugar components are present that repel other insects is unknown so far.

To test our prediction that the scent of *E. helleborine* flowers does not attract other potential pollinators like honeybees, we also performed electrophysiological investigations and behavioral experiments with workers of the honeybee *Apis mellifera*. We found that antennae of *A. mellifera* workers respond to the same compounds as *V. germanica* and *V. vulgaris* in the electrophysiological investigations but were not attracted by the synthetic mixture of *E. helleborine* flowers or by the mixture of the GLVs in the Y tube experiment (Sign test, p > 0.5, n = 20).

Conclusions

The refinement of adaptations for insect pollination has led to a high morphological diversity within the Orchidaceae. There are approximately 10,000 pollinator-deceit species, among them food deceptive orchids that mimic the floral structures of food-providing species and that represent the most numerous group of cheaters [29]. The pollination system that we found in the GLV-producing *E. helleborine* has not been described so far and represents a new form of chemical mimicry. By constitutively emitting volatiles that are usually emitted transiently by wounded plants infested by herbivores and, thus, deceptively indicating the presence of prey, the flowers are capable of attracting their pollinators. After reaching a flower, wasps most likely associate the odor of the orchid with its nectar reward and visit further flowers of the same species, assuring a highly specific and effective pollination system. This is the first time that GLVs have been found to be involved in chemical mimicry for the attraction of pollinating insects. We are presently investigating other wasp-pollinated species in order to see whether there are common chemical principles responsible for wasp attraction by plant volatiles.

equipped with a fused silica column (FFAP, 50 m × 0.25 mm, operated at an initial temperature of 60°C and programmed to 220°C at a rate of 5°C/min). Structural assignments were based on comparison of analytical data obtained with natural products, data reported in the literature [30], and those of synthetic reference compounds. Structures of candidate active compounds were verified by coinjection. For quantitative analyses, defined amounts of n-octadecane (Sigma-Aldrich) served as internal standard.

Electrophysiology

Electrophysiological analyses of headspace samples from *E. helleborine* flowers and cabbage infested by *P. brassicae* caterpillars were performed on a HP 6890 gas chromatograph (Agilent Technologies) equipped with an FID and an EAD setup (Syntech, Hilversum, Netherlands). Antennae from workers of *V. germanica* and *V. vulgaris*, caught from two nests in the surrounding of the campus of the University of Ulm, were tested. *Apis mellifera* workers were captured in the field and used for GC-EAD analyses with headspace samples from *E. helleborine* flowers. For each EAD, the tip of an excised antenna was cut off and the antenna was mounted between two glass-capillary electrodes filled with insect Ringer solution. The electrode at the antenna's base was grounded via an Ag-AgCl wire, and the recording electrode at the tip of the antenna was connected via an amplifier to a signal interface board (Syntech, Hilversum, Netherlands) of a PC. The gas chromatograph was operated splitless at 50°C for 1 min, followed by opening of the split and programming to 240°C at 10°C/min. The effluent was split and 30 ml/min of make-up gas (nitrogen) was added (variable outlet splitter [SGE, Darmstadt, Germany]; split ratio FID:EAD = 1:3). The outlet for the EAD was placed in a cleaned and humidified airflow that was directed over the female wasp's antenna. Natural samples (collected scent) and synthetic compounds (identified upon GC-MS-analyses) were run under the same conditions.

Behavioral Experiments

Bioassays were performed in July and August 2001 at the Institute of Zoology (Vienna) and in July and August 2007 at the Institute of Experimental Ecology in Ulm. In the first bioassay, the importance of visual versus olfactory cues of *E. helleborine* flowers was examined in a field experiment. All tests were made under sunny conditions and temperatures of about 26°C–29°C at the terrace of the Institute of Zoology where the abundance of *V. germanica* workers was high. A plant covered with an ultraviolet (UV)-permeable quartz glass cylinder with two holes for incoming and outgoing air (enriched with scent of the flowers) allowed for testing of the importance of optical versus olfactory cues of *E. helleborine* flowers. With this setup, we performed three test series: (1) So that combination of visual and olfactory cues could be tested, the whole plant was presented in the cylinder. (2) For testing of the olfactory cues only, the cylinder was covered with an additional cardboard cylinder so that the wasps could not see but could smell the flowers. In these tests, the inflowing air stream (200 ml/min) was cleaned from atmospheric pollutants by a charcoal filter (activated charcoal, Supelco, Orbo 32 large), passed the flowers, and left through the second hole. (3) So that the importance of visual cues alone could be tested, the holes of the cylinder were closed. Each test lasted 20 min and was performed at least 10 times in the field.

The olfactometer experiment involved a Y tube olfactometer (length 22 cm, diameter 0.8 cm), horizontally fixed in a polystyrene box (18 × 18 × 16 cm). So that visual disturbance could be avoided, the only light resource was a cold light lamp (Schott KL 1500 LCD, 2950K) placed above the center of the Y tube. The test plants were put into glass cylinders (length 25 cm, diameter 15 cm), which were connected with Teflon or silicon tubing to the Y tube. Both glass cylinders were connected by equally long Teflon tubes to a motor pump (Volcraft, Laboratory Power Supply, PS-302A). Air forced into each glass chamber (50 ml/min) through a single inlet was filtered and cleaned from atmospheric pollutants by a cylindrical borosilicate glass cartridge packed with activated charcoal (Orbo-32, Supelco). After having passed the glass chamber containing the test plants or a blank control, the air streams were directed into the shanks of the Y tube. To test synthetic volatiles, 10 μl (representing five plant equivalents [PE]) of the test mixtures (the composition is given below) or of the pure solvent was applied on a piece of filter paper (3 × 0.5 cm) and placed at each end of the shorter Y tube arms. In all tests, an insect (wasp or honeybee) was released into the long arm of the Y tube, and its choice was registered. A site was counted as chosen if the insect touched the filter paper baring it at the end of the tube. For each test, a new wasp (honeybee), a new Y tube, and new filter papers were used. So that preference of the insects for one side

of the Y tube could be avoided, the positions of shanks for treatment and blank control were shifted after every run.

The following samples were used in the Y tube tests: (1) infested and noninfested cabbage plants, (2) five flowers of either *E. helleborine* or *E. atrorubens* put in each shank of the Y tube (for direct comparison), (3) synthetic test mixture of EAD active compounds of *E. helleborine* consisting of 0.03 μg hexyl acetate, 0.09 μg octanal, 0.04 μg *Z*-3-hexenyl acetate, 0.01 μg *Z*-3-hexenol, 1.28 μg nonanal, 0.22 μg decanal, and 0.37 μg benzaldehyde dissolved in pentane, and (4) synthetic mixture of the GLVs consisting of 0.03 μg hexyl acetate, 0.04 μg *Z*-3-hexenyl acetate, and 0.01 μg cis-3-hexenol dissolved in pentane. The qualitative and quantitative composition of the synthetic mixtures was the same as natural samples, as verified by GC analyses. Synthetic compounds were obtained from Sigma-Aldrich; purity ranged from 95%–99%.

In addition to the Y tube experiments, we performed a further choice experiment under semifield conditions in a flight cage (3 × 4 × 3 m). A table was placed in the center of the flight cage that was used as a foraging area for the wasps. Four dishes (diameter 3 cm) containing a 50% sugar solution of API-Invert (72.7% glucose; Südzucker AG, Germany; 1 g citric acid and 3 g potassium sorbate were added per liter API-Invert solution for preservation) and each with an artificial paper flower (radially symmetric flower shape, yellow, diameter 4.5 cm) were placed on top of the table. Two of the artificial flowers were impregnated with 10 μl (five PE) of the synthetic mixtures, and the other two with solvent only (control). Every 5 min, the artificial flowers were replaced with a new impregnated artificial paper flower. During a test period of 60 min, numbers of visiting wasps for each of the four dishes were counted.

Data Analysis

We compared the total number of approaches in the field experiment by a Mann-Whitney U test. For the statistical analysis of the Y tube experiments and the choice experiments in the flight cage, we used the Sign test. In the flight-cage test, the registered numbers of behavioral events for the two flowers impregnated with the same samples (solvent or test mixtures) were pooled. Comparison of the total amount of GLVs released by *E. helleborine*, *E. purpurata*, and *E. atrorubens* and infested cabbage was done with the Mann-Whitney U Test with a Benjamini-Hochberg correction [31].

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