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Report

Orchid Mimics Honey Bee Alarm Pheromone in Order to Attract Hornets for Pollination

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Summary

Approximately one-third of the world's estimated 30,000 orchid species are deceptive and do not reward their pollinators with nectar or pollen [1]. Most of these deceptive orchids imitate the scent of rewarding flowers or potential mates [2, 3]. In this study, we investigated the floral scent involved in pollinator attraction to the rewardless orchid *Dendrobium sinense*, a species endemic to the Chinese island Hainan that is pollinated by the hornet *Vespa bicolor*. Via chemical analyses and electrophysiological methods, we demonstrate that the flowers of *D. sinense* produce (Z)-11-eicosen-1-ol and that the pollinator can smell this compound. This is a major compound in the alarm pheromones of both Asian (*Apis cerana*) and European (*Apis mellifera*) honey bees [4, 5] and is also exploited by the European beewolf (*Philanthus triangulum*) to locate its prey [6]. This is the first time that (Z)-11-eicosen-1-ol has been identified as a floral volatile. In behavioral experiments, we demonstrate that the floral scent of *D. sinense* and synthetic (Z)-11-eicosen-1-ol are both attractive to hornets. Because hornets frequently capture honey bees to feed to their larvae, we suggest that the flowers of *D. sinense* mimic the alarm pheromone of honey bees in order to attract prey-hunting hornets for pollination.

Results and Discussion

The Orchid and Its Pollinator

In this study, we investigated the floral scent involved in pollinator attraction of the rewardless orchid *Dendrobium sinense*, a species endemic to the Chinese island Hainan [7]. The flowers of *D. sinense* are white with a red center (Figure 1A). The pollinator of this orchid was unknown prior to our studies of eight populations of *D. sinense* in the Bawangling National Nature Reserve in Hainan. During an observation period of 121 hours, we counted 35 visiting insects, 30 of which were identified as the hornet *Vespa bicolor* (Hymenoptera: Vespoidea). The other visitors were bees, wasps, and a butterfly. Rather than landing and pausing on the flowers as would be typical for most pollinators, the hornets instead pounced on the red center of the flower, much like their behavior when attacking prey. Contact with the flower during these pounces was typically less than one second. Of the visiting insects, only hornets were observed to effect pollination, with both pollinia deposition and pollinia removal on the pronotum of the insects observed in the field (Figure 1B). Removal or deposition of pollinia by *V. bicolor* was observed in 5 of 30 visits. Furthermore, during a time period of 30 minutes, we registered 277 nest-entering or nest-leaving wasps from three colonies. Thirty of the females carried pollinia.

During the flowering time of *D. sinense*, there are two other sympatrically occurring orchids in bloom, *Epigeneium fargesii* and *Coelogyne fimbriata*. Hornets are not interested in the flowers of *C. fimbriata* but occasionally visit the flowers of *E. fargesii*. However, we never observed them to remove pollinia (S.X.-q., unpublished data). Therefore, the pollinia that the hornets carry are definitely from *D. sinense*. This result supports that *V. bicolor* is the pollinator of *D. sinense*. Our observations led us to hypothesize that *V. bicolor* is the sole pollinator of the orchid *D. sinense*. This hypothesis is supported by a comparison of the pollinator and orchid flower size (Figure 1). The orchid flowers are assumed to have adapted morphologically to the visits of and pollination by *V. bicolor*. The mean height of the thorax of *V. bicolor* is 0.554 ± 0.022 cm standard deviation (SD) (n = 16) and the mean width is 0.538 ± 0.025 cm SD (n = 16), allowing the pollinator to fit optimally within the flower passage, which has a mean height of 0.567 ± 0.061 cm SD (n = 118) and a mean width of 0.544 ± 0.101 cm SD (n = 118). These morphological adaptations of the flowers maximize the chance that pollinia are removed by the hornets and ensure secure transfer of the pollinia to another flower. We assessed the fruit set of *D. sinense* in three different locations and found about 13% of the flowers (n = 703) to be pollinated, as can be expected for a nectarless orchid [8].

Hornets belong to the group of social wasps that feed their brood with meat nutriments, mainly insects [9]. Foraging hornets are known to capture honey bees often, either in the surroundings of a colony or while they forage for pollen and nectar on flowers [9, 10]. Behavioral experiments have shown that searching wasps use a combination of visual and olfactory cues to locate their prey [11]. However, thus far, the chemical structures of the corresponding volatile signals have remained unknown.

In a recent study, flowers of *Epipactis helleborine*, another wasp-pollinated orchid, were shown to emit green-leaf volatiles (GLVs), which are attractive to the foraging social wasps *Vespula germanica* and *V. vulgaris* [12]. GLVs are emitted by plant tissues upon damage by herbivorous insects—for example, by cabbage leaves infested with caterpillars (*Pieris brassicae*), which are common prey items for wasps [9]. Therefore, we hypothesized that the flowers of *D. sinense* are mimicking a signal of hunting hornets' prey in order to attract the hornets for pollination.

Is the Floral Scent of the Orchid Attractive to Hornets?

To investigate the relative importance of floral signals to foraging hornets, we compared the attractiveness of single

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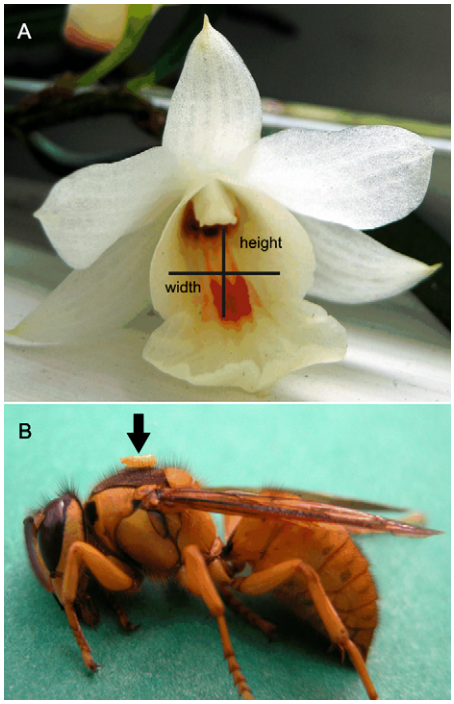

Figure 1. *Dendrobium sinense* Flower and *Vespa bicolor* Forager

D. sinense flower (A) and *V. bicolor* forager with pollinia stuck onto the thorax (B). (Photographs by J.B. and S.X.-q.)

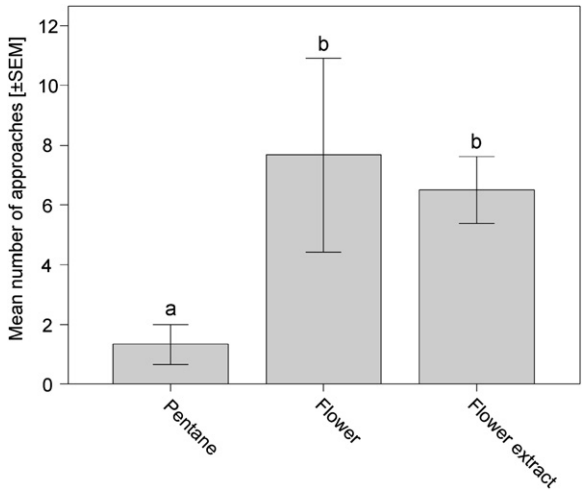

Figure 2. Approaches of Hornets to Various Odor Samples Tested in a Flight Cage

Comparison of the attractiveness of the scent from an orchid flower and honey bee dummies impregnated with flower extract or pentane solvent in a flight cage (Mann-Whitney U test + Benjamini-Hochberg correction [28], $p \leq 0.05$, n = 6). Bars represent median number of approaches ± standard error of the mean. Different letters indicate significance differences between the test groups.

flowers and of odorless European honey bee dummies impregnated with floral scent to hornets in a flight cage. Hornets approached dummies impregnated with the flower extract and whole flowers significantly more often in comparison with the control consisting of an odorless honey bee dummy (Figure 2). Pentane extracts of flowers elicited in the hornets the same number of approaches as intact flowers did (Mann-Whitney U test, U = 17.5, p = 0.935; whole flower: mean 7.67 ± 3.983 standard error of the mean [SEM], n = 6; flower extract: mean 6.50 ± 1.378 SEM, n = 6). Therefore, we concluded that these extracts contained the most important compounds used by the hornets while searching for food. In interactions with flowers or scent-impregnated dummies, the hornets showed a behavior similar to that observed in interactions with flowers in the field: they pounced on the flowers or the dummies impregnated with floral scent. This implies that the scent of the flowers plays an important role for hornets searching for prey.

Do Orchid Flowers Mimic Honey Bees in Scent?

Gas chromatography coupled with an electroantennographic detector (GC-EAD) in combination with gas chromatography coupled with mass spectrometry (GC-MS) was used to identify the compounds in the complex flower scent that are perceived by the antennae of worker hornets, a technique that we previously found to be effective for the identification of volatile pollinator attractants in the wasp-pollinated orchid *Epipactis helleborine* [12]. In pentane extracts collected from *D. sinense* flowers, we detected five compounds inducing an electrophysiological response in the antennae of *V. bicolor* workers (Figure 3). Via GC-MS, we identified not only benzyl acetate and benzyl alcohol, two compounds that are among the most common components of floral scent [13], but also octadecan-1-ol, eicosan-1-ol, and (Z)-11-eicosen-1-ol. The occurrence of octadecan-1-ol, eicosan-1-ol, and especially (Z)-11-eicosen-1-ol in the floral scent of *D. sinense* supports our hypothesis that flowers mimic odor cues emitted by honey bees. The lower electrophysiological response that we found for (Z)-11-eicosen-1-ol in comparison to benzyl acetate and benzyl alcohol is probably a result of the lower volatility of the compound. All of these electrophysiologically active compounds have previously been described in the stinging apparatus of the Asian honey bee *Apis cerana* [4, 14]. We also identified them in body surface extracts of *A. cerana* workers by chemical analyses (Figure 3B). The total amounts of octadecan-1-ol and (Z)-11-eicosen-1-ol were nearly the same (octadecan-1-ol: *A. cerana*: 1.1 ± 0.4 SEM μg/sample, n = 6; *D. sinense*: 1.22 ± 0.12 SEM μg/sample, n = 13; (Z)-11-eicosen-1-ol: *A. cerana*: 17.3 ± 5.3 SEM μg/sample, n = 6; *D. sinense*: 10.7 ± 2.2 SEM μg/sample, n = 13) in the bees and in the orchids. In contrast, amounts of eicosan-1-ol (*A. cerana*: 0.2 ± 0.05 SEM μg/sample, n = 6; *D. sinense*: 22.2 ± 3.7 SEM μg/sample, n = 13) varied between honey bee and flower surface. (Z)-11-eicosen-1-ol is known to be a major compound in the alarm pheromones of both the Asian (*Apis cerana*) and the European (*Apis mellifera*) honey bee [4, 5]. It is highly attractive to honey bee foragers, and in behavioral experiments performed at the hive entrance of honey bees, it elicits aggressive behavior as well as stinging [5]. *A. cerana* is assumed to mark floral or other resources with (Z)-11-eicosen-1-ol to attract other foragers [5]. It has also been described as a major component in the secretion of the Dufour's gland in the neotropical stingless bee *Frieseomelitta varia* [15] and in the thoracic glands of male *Xylocopa micheneri* carpenter bees [16]. Males of the solitary European beewolf (wasp) species *Philanthus triangulum* produce (Z)-11-eicosen-1-ol in the secretion of a cephalic gland [17, 18] to attract females, which exclusively hunt *A. mellifera* as provisions for their larvae. *P. triangulum* females use olfactory cues to find and identify honey bees on flowers [6]. Although (Z)-11-eicosen-1-ol is only a minor component

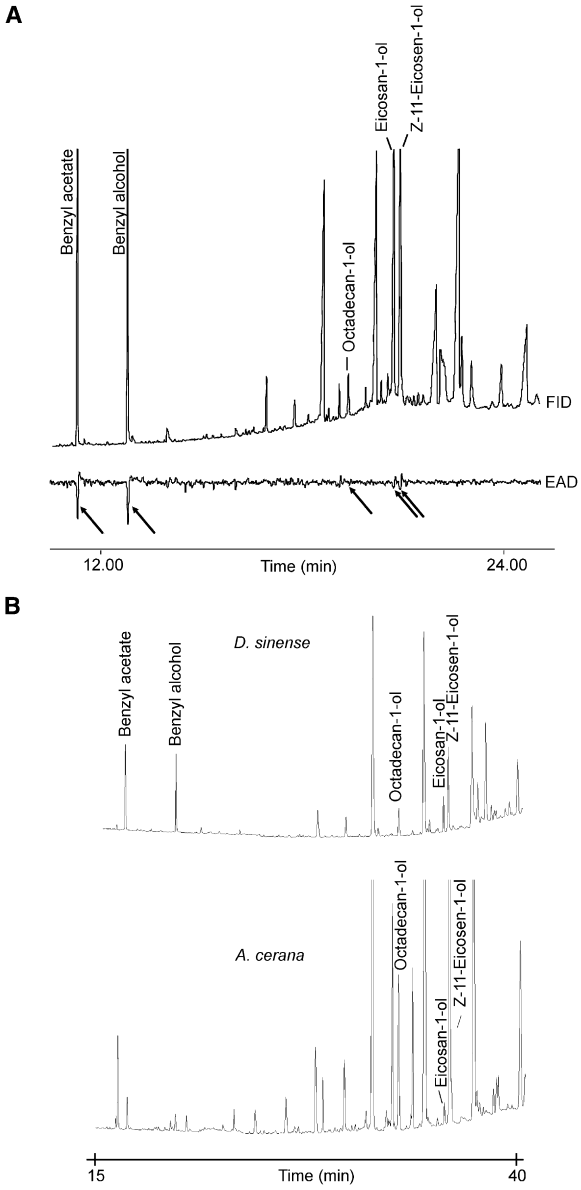

Figure 3. Electrophysiologically Active Compounds

Electrophysiologically active compounds in the flower extract of *D. sinense* (A) and comparison of the electrophysiologically active compounds of a flower extract of *D. sinense* and the body surface extract of an *Apis cerana* forager (B). Simultaneous recordings of gas chromatography (flame ionization detector [FID]) and electroantennographic detector (EAD) signals obtained with flower extracts of *D. sinense* via the antenna of a *V. bicolor* worker were performed on a polar DB-WAX capillary column. Benzyl acetate, benzyl alcohol, octadecan-1-ol, eicosan-1-ol, and (Z)-11-eicosen-1-ol were electrophysiologically active in the flower extract; octadecan-1-ol, eicosan-1-ol, and (Z)-11-eicosen-1-ol also were detected in the *A. cerana* body surface extract.

among the cuticle volatiles in honey bees, it is used as an essential component for prey recognition by hunting female wasps [6]. However, (Z)-11-eicosen-1-ol has hitherto not been reported in nonhymenopteran insects or in flowers.

Mimicry of the Alarm Pheromone

In order to test our hypothesis that (Z)-11-eicosen-1-ol is produced by the orchid in order to mimic the alarm pheromone

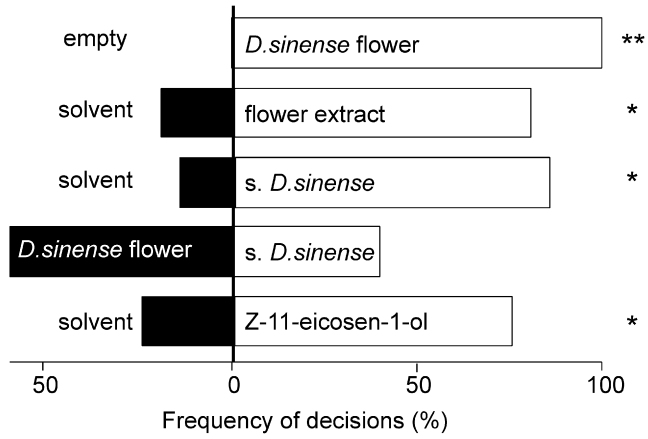

Figure 4. Attraction of Hornets to Various Odor Samples

Comparison of the attractiveness of the odor from an orchid flower, the flower extract, the synthetic mixture of the EAD-active compounds of the flower extract (s. *D. sinense*), and (Z)-11-eicosen-1-ol in a Y tube olfactometer (sign test, *$p \leq 0.05$, **$p \leq 0.001$, n = 20 for each test).

of honey bees and to attract hunting hornets to pollinate flowers, we performed further behavioral experiments. In a Y tube olfactometer experiment, the hornets significantly preferred a synthetic mixture of all EAD active compounds identified in *D. sinense* (sign test, p = 0.01, n = 20) and (Z)-11-eicosen-1-ol alone (sign test, p = 0.05, n = 20) (Figure 4) compared to the empty control. *D. sinense* flowers (sign test, p = 0.001, n = 20) and solvent extracts of the flowers (sign test, p = 0.02, n = 20) were significantly more attractive than an empty control or a solvent control (Figure 4). However, in a Y tube olfactometer experiment that gave the hornets the choice between the flower scent and the synthetic mixture, they showed no preference (sign test, p > 0.05, n = 20), implying that the synthetic mixture contains all essential components for pollinator attraction, including (Z)-11-eicosen-1-ol. The attractiveness of a synthetic mixture consisting of all of the electrophysiologically active *D. sinense* compounds found also to be present in the honey bee *A. cerana* was confirmed by a field experiment in a flight cage. The mixture attracted the same number of hornets as natural flowers (Mann-Whitney U test, U = 3, p > 0.05, n = 11).

Conclusions

Orchids show a remarkable variation of floral forms and a high diversity in pollination systems. Nonrewarding flowers are widespread among Orchidaceae [19], and deceptive orchid species are well known for their specific pollination systems in which only one or a few animal species are attracted [20]. Orchid species pollinated by social wasps are rare, and most of them offer edible rewards [12, 21] or are assumed to mimic food [22, 23]. The pollination system that we have found in the orchid *D. sinense* represents another fascinating example of chemical mimicry in deceptive pollination. By emitting volatiles indicating the presence of prey, the flower is capable of attracting its pollinator, the foraging social wasp *V. bicolor*. Moreover, to the best of our knowledge, this is the first time that (Z)-11-eicosen-1-ol has been identified as a floral volatile. We are presently investigating pollinator attraction in another wasp-pollinated deceptive orchid, *Steveniella satyrioides*, in order to determine whether the principles that we have found

in *D. sinense*, i.e., the mimicking of the scent of prey, are common in wasp-pollinated deceptive orchids.

Various species of *Vespa* are problematic for beekeepers because they plunder hives. Besides this, their ravages of fruit crops make hornets a serious pest to man [24, 25]. Our results may be the first step toward developing an environmentally responsible system for trapping pest hornets.

Experimental Procedures

Pollination System

Insects visiting *D. sinense* flowers were observed for a total 121 hr in the moss forest of Bawangling National Nature Reserve on the island of Hainan in South China. The fruit set data were collected from 703 flowers at three locations in 2003 and 2004. For size measurements of *V. bicolor* foragers, the mean height and mean width of the thorax were measured (n = 16). For *D. sinense*, we measured the height of the flower passage (the distance between the column as the dorsal part of the flower opening and the labellum as the ventral part of the flower opening) and the width of the passage (the distance between the two side lobes of the labellum) (n = 118).

In three colonies of *V. bicolor*, we registered during a time period of 30 min the number of nest-entering or nest-leaving wasps that carried pollinia.

Hornets

Workers of the hornet species *V. bicolor*, which were needed for behavioral experiments and for electrophysiological analyses, were collected in the moss forest of Bawangling National Nature Reserve near three nests from August 20 to September 11, 2007 and from September 1 to September 11, 2008.

Collection of Volatiles

Samples of *D. sinense* were collected in the moss forest of Bawangling National Nature Reserve in 2007. For collection of odor samples, individual freshly opened, unpollinated flowers were cut off from plants and extracted in 10 ml pentane (99%, for high-pressure liquid chromatography; Chromasolv, Sigma-Aldrich) at room temperature for 24 hr. The flowers were then removed, and the samples were stored at −20°C.

Surface body extracts from foraging *A. cerana* honey bees were collected in 2008. Worker honey bees were killed by freezing and extracted for 30 s in 1 ml of hexane solvent. The samples were subsequently frozen until use for chemical analyses.

Chemical Analyses

Flower extracts of the orchid *D. sinense* (n = 13) and surface extracts of the honey bee *A. cerana* (n = 6) were analyzed on a Thermo Trace gas chromatograph (Thermo Electron) equipped with a polar DB-WAX capillary column (Agilent J&W, 30 m × 0.25 mm) and a flame ionization detector (FID). Hydrogen (2 ml/min constant flow) was used as the carrier gas. The sample (1 μl) was injected splitless at 40°C. After a 1 min delay, the splitter was opened, and the oven temperature was increased at a rate of 5°C/min to 240°C.

GC-MS was performed with a double-focusing VG70/250 SE mass spectrometer (Vacuum Generators Ltd.) linked to an HP 5890 gas chromatograph (Hewlett-Packard) equipped with a fused silica column (FFAP, 50 m × 0.25 mm, operated at an initial temperature of 60°C and programmed to reach 220°C at a rate of 5°C/min). Structural assignments were based on a comparison of the analytical data obtained with natural products, data reported in the literature [26], and data of synthetic reference compounds. The structures of candidate active compounds were verified by coinjection. For quantitative analyses, defined amounts of n-octadecane (Sigma-Aldrich) served as an internal standard.

Electrophysiology

Electrophysiological analyses of *D. sinense* flower extracts were performed on an HP 6890 gas chromatograph (Agilent) equipped with an FID and an EAD setup (Syntech). Antennae of *V. bicolor* workers imported from China were tested. 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 base of the antenna 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) of a personal computer. The gas chromatograph was operated splitless at 50°C for 1 min, followed by opening of the split and programming to 240°C at 5°C/min. The effluent was split, and 30 ml/min of makeup gas (nitrogen) was added (variable outlet splitter [SGE]; FID:EAD split ratio = 1:3). The outlet for the EAD was placed in a cleaned and humidified airflow that was directed over the antenna of the female hornet. We considered a substance to be EAD active when it proved to be active in a minimum of 15 replicates.

Behavioral Experiments

Bioassays were performed in August and September 2007 and September 2008 in the moss forest of Bawangling National Nature Reserve.

We performed single-choice experiments in a flight cage (60 × 60 × 60 cm). The hornets were tested in the flight cage under natural climatic and humidity conditions in the field with an orchid flower or an odorless honey bee dummy. We used a honey bee as the dummy because they are common prey items for hornets. We extracted honey bee foragers for 24 hr in dichloromethane via Soxhlet extraction. The odorless Soxhlet-extracted honey bee dummy was impregnated with 10 μl of the flower extract (equivalent to five flowers), the synthetic mixture of the EAD-active components of the flower extract, or pentane solvent as a control. In each case, a single hornet was placed into the flight cage, and the behavior of the hornet was observed for a period of 10 min. We counted approaches (short contact) to the odor source. Each test with one odor sample was repeated with six different hornets.

The olfactometer experiment involved a Y tube olfactometer (length 22 cm, diameter 0.8 cm). To avoid visual disturbance, the Y tube was horizontally fixed in a box covered with a red foil. A glass cylinder (length 10 cm, diameter 2.5 cm) containing an orchid flower and an empty control glass cylinder were connected with silicone tubing to the Y tube. Both glass cylinders were connected by equally long silicone tubes to a motor pump (Laboratory Power Supply, PS-302A, Volcraft). Air forced into each glass chamber (50 ml/min) through a single inlet was filtered and cleaned of atmospheric pollutants by a cylindrical borosilicate glass cartridge packed with activated charcoal (ORBO-32, Supelco). After having passed the glass chamber containing the orchid flower or the blank control, the air streams were directed into the arms of the Y tube. To test synthetic volatiles, 10 μl (equivalent to one flower) of the test mixtures (composition given below) or of pure solvent was applied to a piece of filter paper (3 × 0.5 cm), which was placed at each end of the shorter Y tube arms. In all tests, a hornet was released into the long arm of the Y tube, and its choice of tube arm was registered. A site was counted as "chosen" when the insect touched the filter paper at the end of the tube. For each test, a new hornet, a new Y tube, and new filter papers were used. To avoid preference of the insects for one side of the Y tube, the positions of the shanks for treatment and blank control were changed after every run. Each test series was repeated 20 times.

The following samples were used in the Y tube tests: (1) a *D. sinense* flower; (2) a flower extract in pentane (one-flower equivalent); and (3) one-flower equivalent of a synthetic test mixture of EAD-active *D. sinense* compounds consisting of 2.8 μg benzyl acetate, 9.1 μg benzyl alcohol, 1.2 μg octadecan-1-ol, 22.2 μg eicosan-1-ol, and 10.7 μg (Z)-11-eicosen-1-ol dissolved in pentane. The qualitative and quantitative composition of the synthetic mixture was the same as those found in the flowers, as verified by GC analysis. Synthetic compounds were obtained from Sigma-Aldrich; purity ranged from 95% to 99%, except for (Z)-11-eicosen-1-ol, which was synthesized from commercially available methyl (Z)-11-eicosenoate upon reduction with lithium aluminum tetrahydride according to standard methods [27].

Data Analysis

Comparison of the total amount of the five identical compounds of *D. sinense* flowers and the honey bee surface was performed via Mann-Whitney U test.

For the dummy experiments in the flight cage, we compared the number of approaches made to the *D. sinense* flower, the flower extract, and pentane solvent on a honey bee dummy via Mann-Whitney U test with a Benjamini-Hochberg correction [28]. For statistical analysis of the Y tube experiments, we used the sign test.

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