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Palmitic Acid Released from Honeybee Worker Larvae Attracts the Parasitic Mite *Varroa jacobsoni* on a Servosphere

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Varroa jacobsoni Oud (Acari; Varroidae), an ectoparasitic mite originating in Asia on *Apis cerana* [1], is currently threatening colonies of honeybees, *Apis mellifera* L., worldwide. If infested honeybee colonies are left untreated, they collapse within 2 – 4 years due to reduced performance of individual worker bees [2], or fall prey to secondary bacterial or virus infections. By now it is clear that the parasite's life cycle is closely adapted to its host's development. Having finished maturation on a nurse bee [3], adult female *Varroa* enter brood cells of 8 – 9-day-old worker larvae or 9 – 10-day-old drone larvae to reproduce, i.e., 20 – 10 and 40 – 20 h, respectively, before operculation [4]. *Varroa* slips between the larval body and the wall to the bottom of the brood cell where it immerses in the food jelly until capping is completed by the worker bees [4]. The mite climbs onto the bee larva as it consumes the food [5]. A crucial step for *Varroa* is thus to determine the age of the larva in the brood cell. If the larva is too young, the chance of being detected by nursing bees is high, but soon afterwards the cell will be sealed with the wax cap. To select larvae of the correct age the mites apparently use chemical signals: Three fatty acid esters (methyl palmitate, ethyl palmitate, and methyl linolenate) present in increased amounts in larval cuticle 1 day before operculation [6] attract *Varroa* [7]. However, we could not detect these esters in larval volatiles, but report here on the attractivity for *Varroa* of palmitic acid identified in the headspace over 8-day-old worker larvae.

In order to establish the relative attractivity of different components of the bee hive we bioassayed humid air, wax, adult bees, and larvae. Odors were delivered to *Varroa* on a locomotion compensator [8], and the walking response recorded as follows: The mite walks on the apex of a sphere (50 cm diameter) with a retro-reflective foil (No. 7610, 3M, Switzerland) glued to its back (foil 10 – 20 % of *Varroa*'s body weight). Changes in the position of the mite are recorded by a light-emitting detector system which sends signals to two motors placed orthogonally on the equator of the sphere to compensate for the movement. In this way the mite will always walk on the north pole of the sphere, the location to which odors are delivered. Rotations of the sphere are registered by two incremental pulse generators at a resolution of 1 pulse/0.1 mm displacement. The x and y coordinates of the mite's position are thus sampled at 100-ms intervals and fed into a computer for track record analysis. As all our experiments were carried out in the dark, the visible portion of the light from the detector system was removed with an infrared filter (cut-off at 780 nm).

A chamber built around the servosphere and a treated airstream (ca. 0.05 m/s and 36 mm diameter) directed at its north pole maintained test conditions at 32 °C and 70 % r.h. Into these climatized conditions a glass capillary (0.75 mm i.d.) focused an airstream (0.2 m/s) on the place where the mite walked. The capillary bore, alternatively, one of two solenoïd-activated charcoal-filtered airflows: one passing through an empty 50-ml conical glass flask with charcoal-filtered air alone (control), the other passing through a similar flask containing the odor source. Between test runs we allowed headspace volatiles to build up in the flask for 5 min. Silicone tubing, which connected to the glass capillary, and all glassware were replaced between tests with different odor sources. The sphere was rotated between each run to prevent *Varroa* walking on an area already exposed to a stimulus, and the whole sphere was washed intermittently. Walking responses to the different test stimuli were recorded for 10 s following immediately on a control of the same duration. Bees seen to be carrying mites were collected from the hive and kept for no more than 2 – 5 days in the laboratory. Mites were removed from these bees immediately before testing. Almost all such mites walked at random in the control airstream on the sphere,

with only some showing a slight tendency to walk downstream. When a mite walked down- or crosswind, we switched to the stimulus airstream. The overall direction or vector angle, i.e., the angle between the wind direction (0°) and the straight line joining the start and finish points of a track, was calculated for each 10-s record. A positive response was defined as an upwind-turning mite whose vector angle shifted from a value greater than 60° on either side of due upwind during control to an angle smaller than 30° during test. The small proportion of mites (10 – 20 %) which walked upwind during the control period was eliminated. Responses to the different stimuli were compared pairwise (Fisher-Exact test). Larval odor proved to be the most attractive hive component to *Varroa* (Table 1), but bees also proved attractive.

We then collected volatiles by cold-trapping from larvae that were within 24 h of capping. Air filtered through activated charcoal was passed through a 50-ml conical glass flask containing 50 freshly harvested worker bee larvae at 32 °C and was sucked (100 ml/min) through a glass U-tube (5 mm i.d.) that was immersed to 15 cm in acetone/dry ice (– 70 °C) in a Dewar flask. A 2-h condensate was washed in the U-tube twice with 1.5 ml dichloromethane (Merck, analytical grade) and the recovered solution concentrated by evaporation at room temperature to 1 ml. The response of *Varroa* to this condensate of larval volatiles was tested by applying 100 µl of the extract to a filter-paper disk (5 cm diameter). After evaporation of the solvent, the filter paper was placed in a conical flask above a 20-mm bed of glass beads soaked to 15 mm in water. The latter was introduced to humidify the air to the same extent (60 – 70 % r.h.) as that from larvae. A similar flask plus 100 µl of the solvent alone applied to the filter paper served as control. The mites showed a similar reaction to 100 µl of headspace extracts as to larval odor (Table 1), but not to the solvent control.

The cold-trapped larval volatiles were analyzed by gas chromatography-linked mass spectrometry (GC-MS) with an HP 5890 Series II chromatograph coupled with an HP 5917A mass-selective detector (Hewlett-Packard). A high-resolution HP-1 capillary column (Hewlett-Packard, cross-linked methyl silicone gum, 12 m, 0.2 mm i.d., 0.33 µm film thickness), with helium as carrier gas and splitless injection at 280 °C was temperature-programmed after 2 min at 40 °C at 20 °C/min to 320 °C. Detector temperature was 190 °C operating in EI mode, and identification was based on comparison of spectral data and retention times of authentic compounds. GC-MS analysis of three cold-trap extracts indicated squalene as well as saturated and unsaturated C_{25}- and C_{27}-hydrocarbons as major components. Palmitic acid (PA) was also detected in each of the three replicates at a maximum of 0.6 ng/larva. None of these products was found in control extracts. Esters of fatty acids reported from the cuticle of 8-day-old larvae in amounts of 55 ng/individual or higher [6] were not found (detection threshold 5 ng on the liquid phase employed). This is surprising in view of the quantities previously detected and the volatility of the esters relative to the acid. However, the esters were reported from washes of the cuticle which may have served to liberate the products more readily than collection by cold-trapping as applied here.

We recorded responses of *Varroa* to dilutions of the major component of the headspace extracts, squalene, as well as to PA, the probable fatty acid precursor of the previously reported attractant, methyl palmitate (MP) [7]. The latter was also included for comparison. Synthetic compouds were dissolved in dichloromethane and applied to filter paper; air in test and control flasks was humidified as described above. A 2.5-µg source of PA was as attractive as the odor from larvae (Table 1). A similar dose of MP evoked a weaker response of *Varroa*, although the difference was not significant (p = 0.12). Squalene proved unattractive. These experiments show that *Varroa* is capable of perceiving volatiles of its host and a component thereof, PA. Most responding mites turned upwind within 5 s of stimulus onset.

In order to study the attraction of *Varroa* to PA in more detail, tracks of 10 mites were recorded for 60 s in a humidified airstream alone and in combination with PA (2.5-µg source). Since displacements constituting individual 100-ms vectors (highest resolution of the servosphere) were too small to ac-

Table 1. Responses of *Varroa* to air (70 % r.h.), to the odor of hive components (40 empty brood cells including cocoons of previous molts, i.e., wax, 20 adult worker bees taken from combs with 8-day-old worker brood, 20 8-day-old worker larvae), larval headspace extract (see text), and to squalene, palmitic acid, and methyl palmitate

Odor source	*Varroa* walking cross- or downwind during control	Percentage of upwind turning mites during test[a]
Humid air	34	35.3 cd
Wax	18	38.9 cd
20 live bees	28	67.9 ab
20 live larvae	31	87.1 ab
Solvent	19	26.3 cd
Extract larvae	20	85.0 ab
Squalene [µg]		
25	20	15.0 cd
250	17	41.2 cd
Palmitic acid [µg]		
0.025	18	44.4 cd
0.25	22	68.2 ab
2.5	17	88.2 ab
25.0	31	67.7 ab
Methyl palmitate [µg]		
0.025	37	45.9 cd
0.25	15	40.0 cd
2.5	16	62.5 b
25.0	16	62.5 b

[a] Letters following each treatment indicate significant difference (Fisher-Exact test, $p < 0.05$) from a = humid air, b = solvent, c = larvae, d = extract.

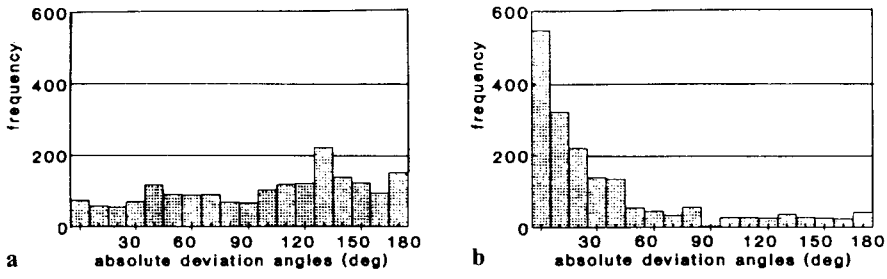

Fig. 1. Frequency distributions of 300-ms vector deviations from due upwind (0°) of 10 *Varroa* responding over 60 s to humidified air (a) and to the same bearing palmitic acid (b). For further explanation, see text

curately reflect the angles described, track sampling was decreased to 300-ms intervals. Vector length, deviation angle from wind direction, and the turn angle (difference in walking direction between successive segments of the track) were calculated for each 0.3-s segment. The frequency distributions of these track parameters in humidified air alone were compared with those in the presence of PA. Means and standard deviations of angular values were calculated as proposed in [9].

The most striking difference occurs in the deviation angles: The mean shifts from 102° (± 49) in humidified air to 40° (± 47) in the presence of PA. When *Varroa* is stimulated with PA, more than 50% of the deviation angles are located between 0 and 30°, whereas in humidified air alone the mites show a more even distribution of deviation angles with even a slight tendency to angles greater than 90° (distributions compared by Kolmogorov-Smirnov test, $p < 0.001$; Fig. 1). The results prove that *Varroa* is capable of closely following an airstream with the stimulus (Fig. 2). This is at least true for a limited period of time, for after some 35 s of upwind walking in the presence of PA we recorded a conspicuous increase in deviation angles with some individuals. Two explanations can be offered: *Varroa* either loses "interest" due to adaptation after some time to the unchanging stimulus conditions, or the concentration of PA in the headspace of our delivery flask dropped too quickly. Turn angles shift significantly from a mean of 23° (± 23) in humidified air to 18° (± 18) during stimulation with 2.5 µg PA (Mann-Whitney, $p < 0.001$). This arises from the fact that the animals walk straighter in the presence of PA. Indeed, path straightness (expressed as the length of the straight line between start and end of a track divided by the total length walked) increased from 0.36 (± 0.14) in humidified air to 0.70 (± 0.20) with PA (Mann-Whitney, $p < 0.01$). Simultaneous video-recording shows that *Varroa* move upwind with a typical zigzag behavior in the presence of PA, i.e., regular shifts of the body axis to the left and the right of the track. This behavior might be responsible for the lower speed of *Varroa* in the presence of PA (2.05 mm/s, ± 1.23) than in humidified air (2.50 mm/s, ± 1.42; Mann-Whitney, $p < 0.001$).

Palmitic acid has been identified in adult honeybee cuticle [10] and, in addition, is suggested to be of use for chemical camouflage by the bee-colony-invading moth *Acherontia atropos* [11]. PA functions as a phagostimulant for the hide beetle, *Dermestes maculatus*, as well as for other insects ([12] and references therein).

According to one hypothesis [13] concerning optimal search strategies in the absence of odors, crosswind walking is expected under constant wind direction, and upwind or downwind walking

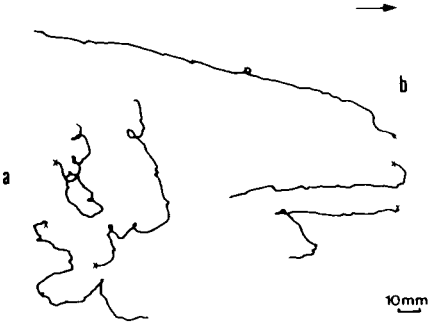

Fig. 2. Track records (60 s) of three *Varroa* a) in a humidified airstream, b) the same bearing PA (2.5-µg source). *Arrow*: direction of airflow; *x*: starting point of path

under variable wind directions. In the absence of an air current, *Varroa* walks randomly on the sphere, whereas the mite shows a slight downwind tendency in a constant airstream of 0.2 m/s (Fig. 1 a). This may be explained by the fact that *Varroa* searching in a bee colony for 8-day-old larvae is permanently surrounded by several potential wind sources, i.e., ventilating bees. Therefore, no other strategy might be necessary than walking randomly until larval odor is perceived and then moving toward the source.

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