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Temporal Pheromonal and Kairomonal Secretion in the Brood of Honeybees

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In a honeybee colony, brood care is ensured by adult bee behavior adapted to the different ages and castes of larvae. The brood is incubated, the larvae are fed, and their cells are capped [1 – 3]. In order to adopt the appropriate behavior, adult workers must be able to recognize the age and the caste of a larva. Like most social Hymenoptera, chemical signals play an essential role in brood recognition for the honeybee [4]. When the worker larvae are 9 days old (from the time of egg-laying), adult bees close the top of the brood cell with a wax cap [5]. This behavior can be artificially triggered by four of the ten methyl and ethyl fatty acid esters present on the surface of larvae: methyl palmitate, methyl oleate, methyl linoleate, and methyl linolenate (MP, MO, ML, MLN) [6]. The six other fatty acid esters identified in the larval cuticle are: ethyl palmitate (EP), methyl stearate (MS), ethyl stearate (ES), ethyl oleate (EO), ethyl linoleate (EL), and ethyl linolenate (ELN).

The parasitic mite, *Varroa jacobsoni,* reproduces on the brood of honeybees. It is attracted by worker and drone larvae just before their cells are capped, and reproduces in the sealed cell until the adult bee emerges at 21 days of age. Among the compounds present in cuticular larval extract, only three esters – methyl and ethyl palmitates, and methyl linolenate (MP, EP, MLN) – are attractive to *Varroa* [7]. Thus, two of these esters act both as pheromones and as kairomones.

It seems likely that the presence of these esters in the larval cuticle on the 9th day is responsible for both the entry of *Varroa* females into the cells and for the capping behavior of workers. This hypothesis is now supported by evidence that these compounds are present in the cuticle only a few hours before the cell is capped, and by the fact that they are released by the larvae themselves.

We have quantified esters in the cuticle of workers from one honeybee colony (*Apis mellifera ligustica*) at different ages in their development. According to most authors and to our observations, cell capping takes place on the 9th day of the preimaginal period [5]. From our data, 8.5-day-old larvae have almost reached their definitive weight and the cell has no evidence of capping. When the larvae are 8.75 days old workers begin to close the cells, and by 9 days they are totally capped and without any trace of the larval cocoon. At 9.5 days of age the larvae have begun to spin cocoons.

We therefore analyzed larvae at 6, 7, 8, 8.5, 8.75, 9, and 9.5 days of age; 11-day-old pre-pupae; and 12.5-day-old pupae at the white-eye stage. A queen was allowed to lay eggs during 1 day, thereafter, she was removed from the comb. Because the capping of the different cells of the comb occurred within 2 – 3 days, we backdated our samples by comparing them with similar control larvae (size, aspect of the cap, pigmentation) from the completion of capping. Two or three replicates of each age group were analyzed, each sample consisting of about 15 g of larvae or pupae. The insects were taken intact from their cells and their cuticles extracted twice with n-pentane and thereafter once with dichloromethane. For each sample, the combined extracts were fractionated by chromatography on a column of silica gel (Merck, 230 – 400 mesh, diameter 0.5 cm, length 20 cm). We first passed 6 ml of pentane through the column; thereafter a second fraction containing the esters was obtained using 6 ml of dichloromethane.

This second fraction of larval or pupal extract was purified by HPLC with a silica column (Interchrom 250, diameter 4.6 mm, intersphere 5 μm). The mobile phase was a mixture of n-heptane and ethyl acetate (95:5) and the flow rate was 1 ml/min. The pump (Waters 6000) was equipped with a Waters U6K injector and an Erma ERC 7510 refractometry detector.

The quantification was performed by GC on a capillary Carbowax column (SGE, length 50 m, internal diameter 0.32 mm, film thickness 0.5 μm). Hydrogen was used as carrier gas (inlet pressure 40 kPa). The temperature of the oven increased from 55 to 230 °C at a rate of 7 °C/min and the temperature was kept at 230 °C over 20 min. The oven was a Carlo Erba 6000 equipped with an on-column injector and flame ionization detector.

Compounds were identified by their retention times and molecular fragmentation by GC-MS, and we compared the mass spectra of the compounds present in the extract with those of reference esters (Sigma). The GC-MS analysis was performed with a Varian 3400 oven, with the same column and temperature program, coupled with a mass spectrometer INCOS 50 (Finnigan).

In 6- and 7-day-old larvae, there is a small amount of the total esters present in the cuticle (extreme values: 3 – 30 ng/larva); at 8 days, this amount is 56 – 59 ng/larva. During the 9th day, the amount of total esters increases up to 500 – 590 ng/larva, while the amount of esters responsible for triggering capping (CE) is up to 230 – 360 ng/larva, and the amount of esters attractive to *Varroa* (VE) is up to 200 – 250 ng/larva (Fig. 1). For the five esters (MS, ES, EO, EL, ELN), which do not trigger these biological activities (NAE), the increase is six to eight times smaller. The variability observed for 8.5-day-old larvae indicates that the increase in secretion begins around that time.

After capping, the amount of esters decreases: Twelve hours after capping, when the larvae are 9.5 days old, CE is reduced to 90 – 165 and VE to 60 – 100 ng/larva. In 11-day-old pupae, these amounts drop to 20 – 30 and 4 – 7 ng/pupa, respectively, and the level of esters is lower than at the 8th

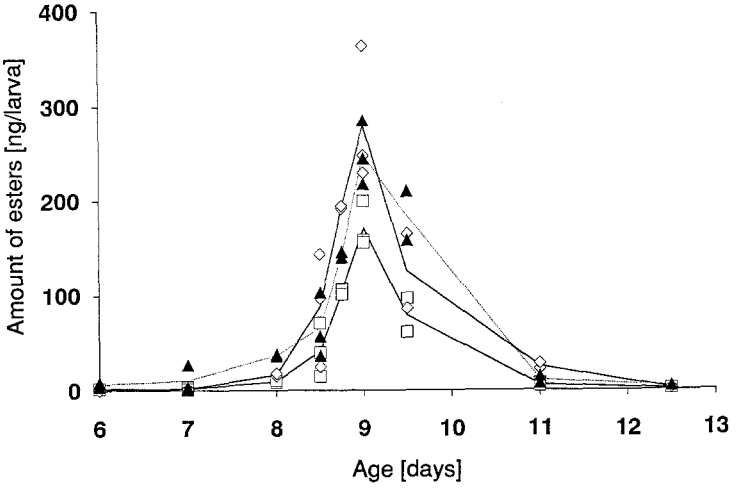

Fig. 1. Amount of esters in worker larvae cuticle as a function of age. The secretion of esters increases between the 8th and 9th day peaking at 9 days before decreasing to its previous level. ◇ (*solid line*) Amount of esters which trigger the capping behavior of the cell (CE), □ (*solid line*) amount of esters which are attractive to *Varroa* (VE), ▲ (*dotted line*) amount of esters which show no activity in capping behavior or in *Varroa* attraction. The curves follow the mean values of each day. At 8.75 days, *Varroa* enters the cell which is subsequently capped by adult workers. At 9 days, the cell is totally capped over

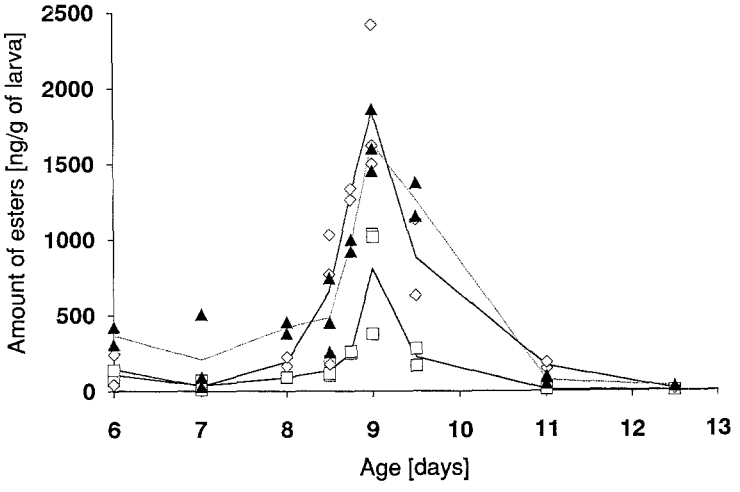

Fig. 2. Amount of esters in worker larvae cuticle per gram of larva or pupa as a function of age. ◇ (*solid line*) Amount of esters which trigger the capping behavior of the cell (CE), □ (*solid line*) amount of esters which are attractive to *Varroa* (VE), ▲ (*dotted line*) amount of esters which show no activity in capping behavior or in *Varroa* attraction. The increase in ester secretion does not depend only on the growth of larvae. The curves follow the mean values of the different age groups

day. This decrease in secretion appears to be synchronous with the spinning of the larval cocoon.

The change in the amount of esters is not due to a variation in weight of the larva, but is due to a large increase in secretion between the ages of 8 and 9 days, followed by a reduction on the 10th day (Fig. 2). The total amount of esters is 600 – 750 ng/g in 8-day-old larvae increasing to 3300 – 4000 ng/g at 9 days.

During the hours before capping, the increase in secretion does not affect each ester identically. Relative proportions of the constituents in 8- and 9-day-old larvae are shown in Fig. 3. The greatest change occurs with the active compound methyl linolenate (MLN) which is present in low amounts on the 8th day and increases to become the most abundant compound by the 9th day. The reverse occurs with ethyl oleate (EO). Methyl stearate (MS) is the only inactive methyl ester in capping behavior. During this period, VE increases from 15 – 17 % of the total esters to 30 – 34 % and CE from 28 – 30 to 43 – 60 %. Thus, the secretion of the active compounds increased more than that of inactive ones. The amount of methylic esters shows a higher increase than the ethylic esters. Methylic esters constitute 39 – 42 % of total esters in 8-day-old larvae and 59 – 84 % in 9-day-old larvae.

In a previous study [6], we showed that capping behavior did not occur when paraffin lures were mixed with about 500 – 1000 ng of CE, whereas it did occur when the lures contained about 5000 – 10 000 ng. Unfortunately, we cannot compare these amounts with our present results (15 – 18 ng of CE in 8-day-old larvae and 230 – 360 ng in 9-day-old larvae) because we could not measure precisely the amount of esters present on the surface of the lures. However, our results for VE (200 – 250 ng in 9-day-old larvae) are comparable to the 1000 ng used in our bioassay [7].

To localize the source of the secretion, we determined the amounts of esters in different parts of 9-day-old larvae. The internal part of a larva contains 7 % of the total esters, compared with 93 % in the cuticle; 76 % being in the anterior cuticle (before the third abdominal segment) and 24 % in the posterior part. The secretion site could be localized on the anterior part of larvae from where esters eventually diffuse to the rear. We do not know in which part of the cuticle or epiderm the esters are located. We are currently working to find the exact site.

To check whether the compounds were truly secreted by the larvae and not the result of contamination of the cuticle during feeding by adult worker bees, we isolated 8-day-old larvae from adult workers for 1 day. In this case, the amount of esters detected on larvae the next day, at 9 days of age, was the same as when the larvae were in contact with adult workers. In addition, the larval food was analyzed and no esters found. The esters are therefore secreted by the larvae themselves.

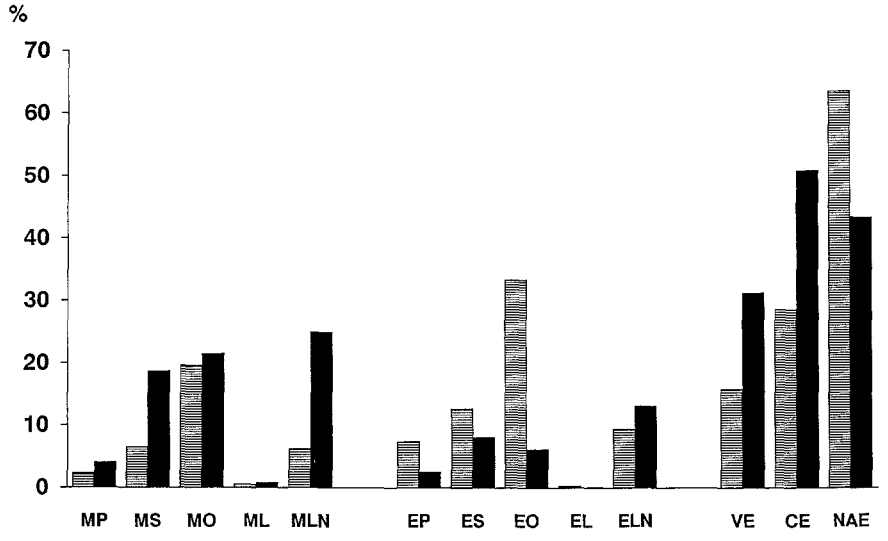

Fig. 3. Comparative percentages of the different esters present in the cuticle of 8-day-old worker larvae (*striped bars*) and 9-day-old worker larvae (*black bars*). *MP* methyl palmitate, *MS* methyl stearate, *MO* methyl oleate, *ML* methyl linoleate, *MLN* methyl linolenate, *EP* ethyl palmitate, *ES* ethyl stearate, *EO* ethyl oleate, *EL* ethyl linoleate, *ELN* ethyl linolenate, *CE* esters eliciting the capping behavior, *VE* esters attractive to *Varroa, NAE* esters which do not elicit either activity. For each fatty acid, the ratio between methyl and ethyl esters is reversed between 8- and 9-day-old larvae

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1. Free, J. B., in: Pheromones of Social Bees, p. 69. London: Chapmann and Hall 1987
2. Koeniger, N., Veith, H. J.: Apidologie *14,* 59 (1983)
3. Wilson, E. O., in: The Insect Societies, p. 306. Cambridge, Mass.: Belknap Press of Harvard University Press 1971
4. Free, J. B., Winder, M. E.: Behavior *31,* 539 (1983)
5. Jay, S. C.: J. Apic. Res. *2,* 117 (1963)
6. Le Conte, Y., Arnold, G., Trouiller, J., Masson, C., Chappe, B.: Naturwissenschaften *77,* 334 (1990)
7. Le Conte, Y., Arnold, G., Trouiller, J., Masson, C., Chappe, B., Ourisson, G.: Science *245,* 638 (1989)

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Homing Strategies of Pigeons Investigated by Clock Shift and Flight Path Reconstruction

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Pigeons rely on a time-compensated sun compass to select the home direction, which they have determined as the first step of their map-and-compass mechanism [1]. Clock-shifted birds consistently show a deflected initial orientation [2], even over familiar areas [3,4]. Even so, they are still able to reach home in many cases. Before the present study, observations on clock-shifted pigeons had been restricted to their initial orientation, homing speed, and success, which made a full understanding of their behavior impossible.

The present paper reports the results of the first application of a recently developed direction recorder [5], which is carried by birds in flight. It has allowed reconstruction of the flight paths of twelve clock-shifted and seven control pigeons released over familiar areas, 12 – 23 km from the loft. We used 12- to 17-month-old homing pigeons from a loft near Pisa. To increase the birds' motivation to fly home, the experiments were performed with pigeons released in pairs or small flocks; one bird carried the direction recorder (DR), while the other (one to three) birds carried a dummy DR. As an exception, the DR-carrying bird was only released in flights E7 and E9.

The DR consists of a traditional compass with a transducer, which converts the angle between magnetic north and the main axis of the bird's body into electrical resistance values; these entrain the frequency of an oscillator. At regular time intervals the current value of the oscillator frequency is stored in a digital 2-KB memory (see [5] for further details). In the present experiments, the periods between successive recordings of oscillator frequency values were 2.4, 10, and 20 s in different experiments. The stored data were processed by a program which gave a reconstruction of the path. The releases were performed in calm weather, so that the effect of wind drift could be disregarded in reconstructing the birds' paths. The arrival point of the birds, resulting from the reconstructed path, was never found to be more than 4.8 km distant from the real loft. Since the values stored in the memory showed noise due to oscillations of the mobile part of the compass during flight,