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1. Reddy, G. R.: Can. J. Soil Sci. *44,* 254 (1964)
2. Zacherl, B., Amberger, A.: VDLUFA-Schriftenr. *11,* 55 (1984)
3. Amberger, A., Vilsmeier, K.: Z. Pflanzenernaehr. Bodenkd. *142,* 778 (1979)
4. Vilsmeier, K.: ibid. *143,* 113 (1980)
5. Vilsmeier, K.: Z. Wasser-Abwasser-Forsch. *21,* 140 (1988)
6. Rathsack, K.: Landw. Forsch. *6,* SH, 116 (1955)
7. Hauser, M., Haselwandter, K.: Soil Biol. Biochem. *22,* 113 (1990)

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Identification of a Brood Pheromone in Honeybees

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The ability of social insects to identify the brood, and more precisely its sex, caste, and developmental stage is essential for the workers in charge of the brood care. This identification implies the combination of a few signals, particularly mechanical and chemical signals which play a fundamental role for ants [1], wasps [2], and bees [3].

The existence of brood pheromones has been postulated often for the social Hymenopterae, but only two have been identified with certainty [4, 5].

The honeybee brood care is secured through a few behavioral sequences including the feeding of the larvae, the capping of the cells, and the thermoregulation of the brood area in the colony. A triglyceride, glyceryl-1,2-dioleate-3-palmitate, induces the attraction and the clustering of the worker bees on artificial queen cells impregnated with this component [5].

The capping of the cells is a collective behavior which is displayed by numerous workers closing the top of the cells with wax [6].

Chemical signals produced by the brood are detected by an acarian, *Varroa jacobsoni* Oudemans, which induces significant damage in the honeybee colonies all over the world. In order to reproduce, the mites penetrate the cells a few hours before the capping, when the larvae are 4 – 5 days old. We have recently identified ten fatty esters in the honeybee drone larvae analyzed just before capping [7]. These compounds are also present in smaller amounts in the worker larvae (unpublished results). Three of these substances, methyl palmitate, ethyl palmitate, and methyl linolenate are involved in the attraction of the mites by the larvae [7]. Because these compounds represent a kairomone for the parasite, we assumed that they could have a pheromonal role for the honeybees, in relation to the brood care, particularly the capping of the cells, and have confirmed this hypothesis.

The influence of these esters in triggering the capping of the cells by the worker bees was studied through topical applications on worker larvae of the same age. About 24 h before capping, 0.25 µl of the mixture of the ten esters in the natural proportions [7], which corresponds to 150 larvae equivalent (lar-eq), was applied, without solvent, on each larva. Worker bees capped the cells containing the treated larvae significantly faster than the control ones (Fig. 1).

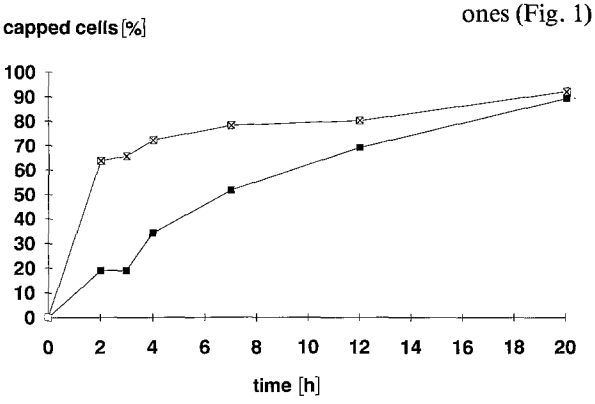

Fig. 1. Capping of the cells after topical applications of a mixture of the 10 esters on worker bee larvae of the same age. A queen was isolated on a comb for 24 h in order to obtain eggs of the same age. When larvae were 5 days old, 1 day before capping (last larval stage), they were individually located with a transparent film, and separated at random in two sets, each with 80 to 200 larvae. Topical applications were realized with a microsyringe, and 0.25 µl of the mixture was spread on each larva of one of the set chosen at random (⊠); the other set was used as control (■). Then the comb was replaced in the center of the colony and the capping of the cells by worker bees was observed as a function of time. The experiment was repeated 5 times with 5 different colonies, and a total of 687 treated and 1013 untreated (control) cells was observed. Percentages of capping were significantly different between the treated and the control cells (Mann-Whitney test, $p < 0.001$), at any time, except at 20 h. Amount of aliphatic esters in one drone larva [7]: methyl oleate (MO), 0.07 µg; ethyl oleate (EO), 0.03 µg; methyl linoleate (ML), 0.05 µg; ethyl linoleate (EL), 0.01 µg; methyl linolenate (MN), 0.59 µg; ethyl linolenate (EN), 0.18 µg; methyl palmitate (MP), 0.26 µg; ethyl palmitate (EP), 0.09 µg; methyl stearate (MS), 0.26 µg; ethyl stearate (ES), 0.08 µg

Table 1. Comparison of the capping of the cells after topical applications on honeybee larvae of the same age of 0.25 μl of a mixture of paraffin oil/10 esters (1/1) or of pure paraffin oil (control). Percentages of capping were significantly different between the treated and the control cells (Mann-Whitney test, $p < 0.001$), at any time, except at 20 h

	Time [h]			
	0	3	12	20
Paraffin oil/ esters	0	38.17	75.00	90.40
Paraffin oil	0	13.33	56.00	86.33

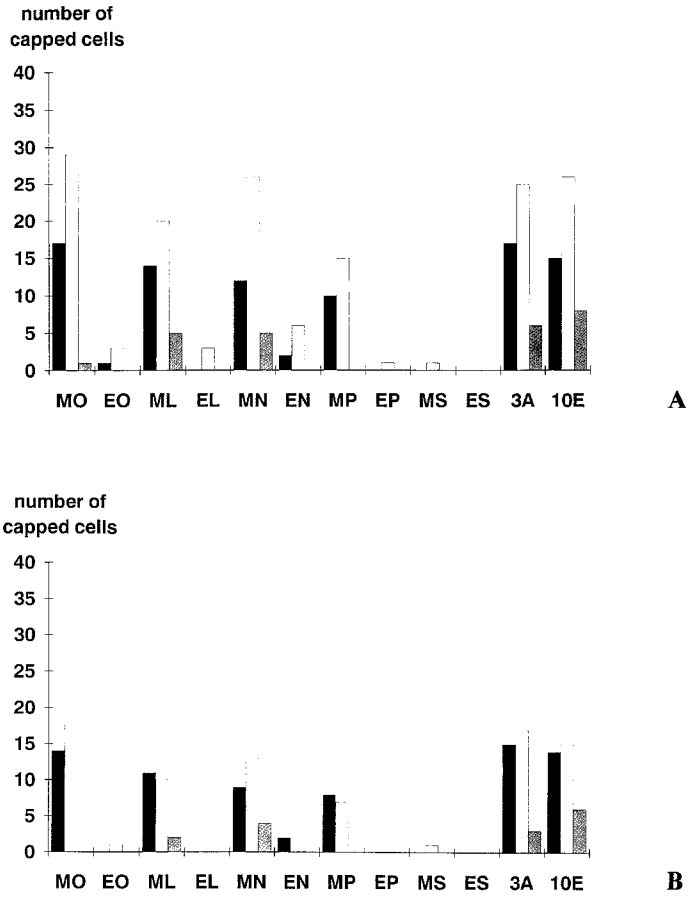

Fig. 2. Capping of the cells containing lures. Lures were prepared by mixing an ester or a mixture of esters with liquid paraffin (at 58–60 °C) at concentrations of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} (w/w), and 0 (control) of ester. The liquid mixture was molded by letting it flow into the empty cells of a comb. Once cold, the lures (0.05 to 0.1 g) were withdrawn from the cells to be used in the test. Four lures of larvae of each of the 10 esters and of the two mixtures (3A and 10E) were placed in the empty cells of a comb at the center of the brood of a colony. The capping of the cells was observed 12 h (A) and 36 h (B) later. The experiment was repeated 10 times with 3 different colonies, and 40 lures were tested for each ester and each mixture, and at different concentrations (*black bars* 10^{-2}, *white bars* 10^{-3}, *gray bars* 10^{-4}). Symbols see legend to Fig. 1

Thus, the application of these substances on the larvae could either 1) act directly on the worker bees and trigger the capping behavior, 2) or induce a behavioral or a physiological reaction of the larvae which could trigger the capping, 3) or prevent the diffusion, from the larvae, of some compounds inhibiting the capping behavior. To test these hypotheses, we treated the larvae with paraffin oil, which is chemically inert but of similar viscosity to the esters used. The capping of the cells containing the larvae treated with 0.25 μl paraffin oil was slower than that of the cells containing the larvae treated with 0.25 μl of a mixture (1/1) of the ten esters in paraffin oil (Table 1). Thus, it was the presence of the esters which triggered the capping behavior, and not the prevention of the diffusion of some inhibitory compounds by the larvae.

Therefore, we tried to trigger the capping of the cells in the absence of honeybee larvae, but in the presence of lures of larvae. Different lures of paraffin, containing either one of the ten esters, the mixture of these esters (10E), or the mixture of the three esters attractive (3A) to the varroa mites (MP, EP, MN) [7] at a concentration varying from 10^{-1} to 10^{-5} (w/w), were introduced into the empty cells of a comb which was replaced at the center of the brood area in a honeybee colony.

The control lures (without esters) and the lures at the concentration of 10^{-1} and 10^{-5} (w/w) were removed by the worker bees. In contrast, after 12 h, the cells containing lures with methyl esters at a concentration of 10^{-2}, 10^{-3}, and 10^{-4} (w/w) were capped in the proportions given in Fig. 2, confirming the direct effect of each of these compounds on the capping behavior of the worker bees. After 36 h, the number of capped cells increased for the lures with methyl esters (except MS) and the mixture 10E at the concentration of 10^{-3} (w/w) (X^2 test, $p < 0.05$), but did not significantly differ for the lures at concentrations of 10^{-2} and 10^{-4} (w/w) (Fig. 2).

The most favorable ester concentration for the capping is 10^{-3} (w/w). At this concentration, the amount of esters contained in a lure of 0.05 to 0.1 g represents 30 to 60 lar-eq. However, during the 12- or 36-h experiment, only part of these esters was released on the lure surface, most of it remaining in the paraffin.

The response of the worker bees to the lures with a concentration of 10^{-4} ML, MN, MO and of the mixtures 3A and 10E, represented 3 to 6 lar-eq distributed in the lure as a whole, and showed the high sensitivity of the workers to these substances. With similar concentrations, responses obtained from the mixtures 3A and 10E were not more important than the responses from ML, MN, MO, or MP. Thus, these methyl esters can be considered as individually active components of the pheromonal blend produced by larvae and as triggering the capping of the cells by worker bees. The other compounds, MS, EL, EN, EO, EP, triggered weaker responses or no response (ES).

The demonstration of the role of these semiochemicals in the capping behavior

does not rule out the involvement of other signals, for instance mechanical ones, under natural conditions.

It is important to point out that two compounds, MP and MN, appear to act at the same time as a pheromone for the honeybee and as a kairomone for the parasitic mite *Varroa jacobsoni*. These compounds, emitted by honeybee larvae, first attract the mites, and then the honeybee workers for the capping of the cells. The analysis of the composition of the pheromone blend and of the kairomone blend, in relation to the age of the larvae, is in progress.

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1. Morel, L., Van der Meer, R. K.: Ann. Entomol. Soc. Am. *81,* 705 (1988)
2. Koeniger, N., in: Insect Communication, p. 267 (ed. Lewis, T.). London: Academic Press 1984
3. Free, J. B., Winder, M. E.: Anim. Behav. *31,* 539 (1983)
4. Veith, H. J., Koeniger, N.: Naturwissenschaften *65,* 263 (1978)
5. Koeniger, N., Veith, H. J.: Apidologie *14,* 59 (1983)
6. Lindauer, M.: Bee World *34,* 63 (1953)
7. Le Conte, Y., Arnold, G., Trouiller, J., Masson, C., Chappe, B., Ourisson, G.: Science *245,* 638 (1989)

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Carrier-Mediated Uptake of Digoxin by Larvae of the Cardenolide Sequestering Moth, *Syntomeida epilais*

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Syntomeida epilais (Ctenuchidae) is a neotropical moth. Its larvae feed on leaves of oleander (*Nerium oleander*) and sequester the cardenolides (cardiac glycosides, CG) of this plant [1, 2]. *S. epilais* has bright orange larvae with long bundles of black hair and metallic blue-red imagines. These aposematic forms are likely to be protected from predators by their CG content, similar to the situation in CG-storing monarch (danaine) butterflies [3] and milkweed bugs *Oncopeltus* [4].

Cardiac glycosides have a carbohydrate side chain, which provides them with a polar and hydrophilic moiety. Since these qualities reduce the free permeation of CG through biomembranes, we were interested in studying the intestinal mechanisms of CG uptake from the plant diet. We recently analyzed an analogous problem in larvae of the arctiid moth, *Creatonotos transiens*, which appeared to resorb hydrophylic and polar pyrrolizidine alkaloid-N-oxides from their food with the help of a specific carrier system [5]. We now asked: Does *S. epilais* also use a carrier for CG uptake?

The larvae were collected from oleander bushes in Florida (USA) and later also reared on this plant in our laboratories. We analyzed the distribution of CG in larvae, their faeces, and imagines of *S. epilais* by HPLC (Table 1) and found that CG were partially resorbed from the diet. The major part of CG stored were detected in the integument, similar to the situation in *Danaus* and *Oncopeltus* [3, 4].

For the uptake experiments we dissected the guts of late larvae, sliced them open with scissors, and cut the midgut in pieces of 1 mm length, which were then incubated in a modified Ringer solution. Experiments were started with the addition of tritiated digoxin (specific activity 976 GBq/mmol, supplied by NEN, Dreieich, FRG).

Table 1. Cardenolide content of *Syntomeida epilais* and its food plant. Cardenolides were extracted with 50% EtOH, purified by solid-phase chromatography (Chem elut, Analytichem) and analyzed by HPLC. HPLC conditions: Milton Roy gradient instrument (MP 3000); column: Lichrospher 100 RP 18 (5 μm); gradient: MeOH/H_2O from 40:60% to 90:10%; UV-detector at 225 nm. Oleandrin was used as an external standard. n.d. = not determined. Quantitative determinations were made from 3 to 6 specimens each

	Cardenolide content	
	[μg/animal]	[μg/g fresh weight]
Last instar larvae (A)	325[a]	n.d.
L2 – L4 larvae (B)		
Gut	17	n.d.
Haemolymph	16	n.d.
Integument/fat body	93	n.d.
Integument	76	1300
Exuviae	36	1400
Faeces	n.d.	4500
Imagines	605	1600
Meconium	17	n.d.
Tachinid larvae	12	n.d.
Nerium oleander		
leaves USA		6000
Heidelberg		4300

[a]CG content of last instar larvae (A) was higher than that of the smaller L2 – L4 larvae (B) which were employed to study the tissue distribution of CG. This difference reflects the different weights of the respective larvae

