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Original article

Ontogeny of cuticular chemosensory cues in worker honey bees *Apis mellifera*

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Summary — Cuticular-wax composition of worker honey bees changes with age (Blomquist *et al.*, 1980). Genetic variation in cuticular-wax composition and other chemicals on the surface of the cuticle *(e.g.,* fatty acids) may provide workers with recognition cues for kin-related interactions that take place within the hive (Getz, 1988). We show here, using a proboscis-extension differential-conditioning paradigm, that a forager can detect shifts in the composition of chemicals (not necessarily waxes) residing on the cuticle of other workers; at least 2 of these shifts occur within the first week of adult life. This complicates the question of the role of cuticular chemicals as kin recognition cues.

Apis mellifera **— kin recognition — ontogeny — cuticle — chemical composition**

Résumé — Ontogenèse des signaux chimiosensoriels de la cuticule chez les ouvrières d'abeilles, *Apis mellifera*. *Les ouvrières d'abeilles possèdent une odeur unique (Getz* et al., *1988) qui leur permet de distinguer les congénères de leur colonie à l'aide de signaux chimiques. Les signaux de reconnaissance utilisés semblent posséder une composante génétique (Getz and Smith, 1983; Breed* et al., *1985). La variabilité des odeurs volatiles émanant des ouvrières (Getz* et al., *1987) et les odeurs présentes à la surface de la cuticule sont détectables par les ouvrières. Nous avons mené des expériences sur l'ontogenèse des signaux chimiosensoriels de reconnaissance en utilisant le réflexe d'extension du proboscis (Bitterman* et al., *1983), qui s'est déjà montré utile dans l'approche des problèmes de discrimination (Getz* et al., *1988).*

Les expériences ont porté sur des abeilles «testées» conditionnées à des baguettes de verre frottées sur le thorax des abeilles «source». Ces dernières sont maintenues en étuve à partir de 24 h avant l'émergence dans la ruche, tandis que les abeilles testées sont des butineuses prises dans une ruche la veille du jour où elles ont été conditionnées. Nous avons testé la capacité des abeilles à discriminer les substances chimiques obtenues en frottant les baguettes sur le thorax de 5 abeilles (toutes sœurs du même âge).

Chaque test a été fait sur 30 abeilles environ avec 2 baguettes différentes; les antennes étaient mises en contact alternativement avec les 2 baguettes et on associait une baguette (CS+) à une récompense (US+), alors que la stimulation avec la 2e baguette n'était pas récompensée. Les baguettes ont été présentées en une séquence de 16 essais à 10 min d'intervalle (cf. Methods). On

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a enregistré le nombre d'erreurs faites par une abeille pendant les essais 9 à 16. Chaque individu pouvait donc faire de 0 à 8 erreurs. On a tracé pour chaque groupe de 30 abeilles un histogramme d'erreurs et calculé l'erreur moyenne e.

La Figure 1 illustre les résultats. Les colonnes représentent l'erreur moyenne faite par un groupe d'abeilles recevant un traitement particulier. La formule i-j *signifie que des abeilles source âgées de* i *et* j *jours ont été respectivement frottées avec des baguettes CS– et CS+. La ligne de tirets représente l'erreur moyenne du groupe témoin 1—1. Les colonnes avec les hachures et les croix montrent les différences entre le groupe témoin et le groupe recevant un traitement particulier, aux niveaux respectifs de signification de* $P < 0,05$ *et de* $P < 0,005$ *(table de contingence du* χ^2 *3 x 2). Les colonnes avec pointillés ne sont pas significativement différentes.*

Les traits avec des flèches au-dessus des colonnes donnent les comparaisons par paires; l'étoile indique que les différences sont statistiquement significatives* ($P < 0,05$). *Dans les tests représentés par les 2 avant-dernières colonnes, les baguettes CS+ et CS– ont été respectivement frottées sur les thorax (T) et entre les sternites abdominaux (A) des mêmes 5 abeilles source. Le traitement représenté le plus à droite est un autre témoin (C).*

Les résultats montrent que les changements ontogénétiques des signaux chimiosensoriels présents à la surface de la cuticule existent dans les 2 ruches testées. Il existe un changement entre les jours 1 et 2, mais peu de changement entre les jours 2 et 3. Puis il y a à nouveau un changement entre les jours 3 et 4 et peu de changement entre les jours 4 et 6. Il semble qu'il y ait un changement entre les jours 6 et 8, bien qu'il soit moins marqué que les 2 précédents. Le changement entre les jours 9 et 17 existe, bien que moins fort que celui entre les jours 2 et 6. Il pourrait être dû à la sécrétion de cire par les abeilles à partir du 12e jour. On ne sait pas dans quelle mesure la production de cire contamine le thorax des abeilles, car les substances à la surface du thorax et de l'abdomen des abeilles âgées de 15 jours n'ont pas la même composition.

Apis mellifera — *reconnaissance de souche — ontogenèse — cuticule — composition chimique — reconnaissance de parenté*

Zusammenfassung — Ontogenie der chemischen Merkmale bei den Arbeiterinnen der Honigbiene, *Apis mellifera*. *Honigbienen besitzen einen spezifischen Geruch (Getz* et al., *1988), der es ihnen ermöglicht, aufgrund chemischer Merkmale zwischen Nestgenossinnen zu unterscheiden. Diese chemischen Erkennungsmerkmale scheinen eine genetische Komponente zu haben (Getz und Smith, 1983; Breed* et al., *1985). Die Variabilität flüchtiger Gerüche (Getz* et al., *1988) und der Kutikula anhaftender Oberflächengerüche (Getz* et al., *1988) werden von Arbeiterinnen erkannt. In den hier beschriebenen Experimenten testeten wir die Ontogenie der chemischen Erkennungsmerkmale mit Hilfe des Rüsselreflexverhaltens (Bitterman* et al., *1983). Diese Methode wurde schon in früheren Experimenten erfolgreich für ähnliche Fragen eingesetzt (Getz* et al., *1988). In den Experimenten werden Testbienen auf den kutikularen Thoraxgeruch von Versuchsbienen konditioniert. Dieser Geruch haftet dünnen Glasstäben an, die auf den Thorax der Versuchsbienen gerieben wurden. Die Versuchsbienen wurden vor dem Schlüpfen vom Stock isoliert und im Brutschrank aufgezogen, während die Testbienen Flugbienen sind, die einen Tag vor den Experimenten am Flugloch abgefangen wurden. Wir testeten die Fähigkeit der Bienen, zwischen Glasstäben zu unterscheiden, die auf den Thorax von je 5 Versuchsbienen gerieben worden waren. (Alle 5 Versuchsbienen waren gleichaltrige Schwestern). Jeder Test wurde mit 30 Bienen und je 2 unterschiedlichen Glasstäben durchgeführt. Die Antennen der Testbienen wurden in alternierenden Testläufen mit den beiden unterschiedlichen Glasstäben berührt : ein Glasstab wurde mit einer Belohnung assoziiert (CS+) während der zweite ohne Belohnung blieb (CS–). Es wurden 16 Testläufe mit je 10 Minuten Interval durchgeführt (siehe Methoden).*

Die Anzahl der Fehler, die eine Biene zwischen dem 9. bis 16. Versuchsschritt machte, wurde registriert. Jedes Individuum konnte daher 0—8 Fehler machen.

Für jede Testgruppe von 30 Bienen wurde die Fehlerhäufigkeit und der mittlere Fehler e *berechnet. In der Zeichnung stehen die Säulen für den mittleren Fehler der von einer Bienengruppe gemacht wurde. Die Bezeichnungen* i *und* j *kennzeichnen das Alter der Versuchsbienen, auf deren Thorax die Stäbe gerieben wurden. Die gestrichelte Linie ist der mittlere Fehler der 1—1 Kontrollgruppen.*

Die schräg schraffierten und gekreutzt schraffierten Säulen zeigen die Unterschiede zwischen diesen Kontrollversuchen und der jeweiligen Versuchsgruppe auf den Signifikanzniveaus P < 0.05 und P < 0.005 an (3 x 2 chiquadrat contingency table). Gepunktete Säulen sind nicht signifikant verschieden.

Die Striche mit Pfeilen über den Säulen geben paarweise den Vergleich an. Die Sterne bezeichnen die signifikant underschiedlichen Paare (P < 0.005). Bei den Testbienen der drittletzten und zweitletzten Säule wurden die Stäbe auf Thorax (T) und Abdominalsterniten (A) der Versuchsbienen gerieben.

Die Säule ganz rechts ist eine weitere Kontrollgruppe (C). Die Ergebnisse zeigen, dass ontogenetische Änderungen in den chemischen Merkmalen der Kutikula bei Bienen aus beiden Versuchsstöcken auftraten.

Es gibt eine Änderungen von Tag 1 auf Tag 2, aber wenig von Tag 2 auf Tag 3. Dann gibt es eine Änderung von Tag 3 auf Tag 4, aber wenig von Tag 4 auf Tag 6. Es scheint eine Änderungen von Tag 6 auf Tag 8 zu geben, sie ist jedoch nicht so stark wie die beiden vorhergehenden. Die Änderung von Tag 9 auf Tag 17 ist offensichtlich, obwohl sie nicht so stark ist, wie die von Tag 2 auf Tag 6. Die Änderungen zwischen Tag 9 und Tag 17 könnte auf der beginnenden Wachsproduktion der Bienen ab dem 12. Tag beruhen.

Es ist nicht klar, inwieweit die Wachsproduktion den Thorax eines Individuums kontaminiert, da die Ergebnisse besagen, dass die Stoffe der Thoraxoberfläche und des Abdomens von 15 Tage alten Bienen kompositionell verschieden sind.

Kutikulare Hydrogencarbonate können benutzt werden, um verschiedene Bienenrassen zu identifizieren (Carlson und Bolten, 1984; McDaniel et al., 1984, 1987) und ontogenetische Änderungen sind mit Hilfe der Gaschromatographie identifiziert worden.

Die verhaltensbiologisch nachgewiesenen ontogenetischen Änderungen der Gerüche sind von grosser Bedeutung für die Verwandtschafts-Unterscheidungsprozesse innerhalb der Bienenvölker.

Apis mellifera — *Verwandtenerkennen — Ontogenie — Kutikula — chemische Merkmale*

Introduction

Social interactions between *A. mellifera* honey bees may be far more complex than is currently thought. Each worker appears to possess a unique odor (Getz *et al.*, 1986, 1988), making it possible for workers to discriminate among nestmates using odors as cues. Furthermore, because queens mate with many males, each worker in a colony belongs to one of a dozen or so concurrently existing genetic lines [1] or subfamilies (Laidlaw and Page, 1984). Individuals within subfamilies are related, on average, by 3/4 rather than 1/2 because of haplodiploidy. Therefore, these individuals are referred to as super- rather than full-sisters (Page and Laidlaw, 1988). Each worker encounters both super- and half-sisters while performing daily chores within her hive.

Guard bees defend their hives against the entry of foreign bees who may try to rob their store of honey. Apart from discrimination between nest and non-nestmates, it now has been established that workers can discriminate between their super- and half-sisters in a variety of situations (although these results relate to highly manipulated hives so that the implications for feral colonies are uncertain; reviewed by Breed and Bennett, 1987; Getz, 1988; in press). The recognition cues appear to have a genetic component (Breed, 1983; Getz and Smith, 1983; Breed *et al.*, 1985), and workers may use their own cues as a basis for

[1] These lines are determined by the father, and thus are often referred to as patrilines. This convention is not entirely in keeping with the matri- and patriline terminology used by breeders.

evaluating their kinship to others (Getz and Smith, 1986). Variability of volatile odors emanating from individual workers (Getz *et al.*, 1988) are detectable by worker honey bees.

Within a colony, the relatively non-volatile chemosensory cues residing on the cuticle may be much more easily associated with a particular individual than highly volatile odors produced by, for example, the Nasanov or mandibular glands; although some transfer of cuticular chemicals may take place as individuals groom or rub against one another (Breed *et al.*, 1985; Getz and Smith, 1986; Breed *et al.*, 1988b). Thus observed preferential kin interactions within a colony (Frumhoff and Schneider, 1987) are unlikely to be mediated by highly volatile substances which, on release, would pervade the hive. When workers defend their hives against plundering by workers from a foreign colony, however, both volatile odors and cuticular substances may play a role. There are a number of facets to this discrimination behavior, one of which is the ontogeny of the chemosensory cues that mediate recognition.

Breed *et al.* (1988a), using an agonistic behavioral interaction assay, have demonstrated that adults maintained in the laboratory develop kin discrimination cues within 12 h of emerging from the brood comb. Here, we investigate the ontogeny of chemosensory cues present on the dorsal surface of worker honey bee thoraces. We use a proboscis-extension reflex differential-conditioning technique that has proven useful in the past in addressing discrimination-related questions (Getz *et al.*, 1988).

Materials and Methods

Our experiments involved differentially conditioning worker honey bees (test bees) to odors using their proboscis-extension reflex (Bitterman *et al.*, 1983; Getz *et al.*, 1986).

Test bees, from a colony headed by a naturally mated queen, were collected at the hive entrance late in the afternoon. These bees were harnessed in small brass tubes with their mouthparts, antennae, and legs free to move. They were then fed until satiated with a 1.5-M sucrose solution and left overnight in the dark, at room temperature (≈ 18 °C). The following morning each bee was fed briefly 0.5—1 h before training began.

Source bees were obtained from 2 unrelated hives, labeled 1 and 2. All source bees for one run of the experiment were super-sisters. They were removed from brood comb within 24 h prior to emergence from the pupal stage (Getz and Smith, 1986), and immediately placed in 0.47-l cardboard containers in groups of 8. These groups were maintained in a dark incubator for 1—17 days (depending on the particular experiment) at 31 °C (relative humidity not controlled) and with sucrose and water provided *ad libitum*.

On the morning of an experiment, source bees were placed in a freezer for 20 min. Excess hairs were gently removed from chilled bees with a piece of tape to expose the surface of the thorax and then hollow glass rods (50-mm long, 1-mm outer diameter) were rubbed on the thorax of 5 bees from the same container (note that all bees in a container have ages that differ by less than 24 h). Two rods were paired for a run. Each rod was rubbed on bees coming from the same container, where containers were selected according to the age of the bees. In some experiments the different containers contained bees of the same age, but in most experiments the bees in the different containers were different ages. In all but 2 cases, the rods were rubbed on the dorsal surface of the source bees thoraces. In 2 cases, however, one rod was rubbed on the dorsal surface and the second rod was rubbed on the ventral surface of the abdomens of the same 5 source bees. The second rod was also twirled between the sternites of abdominal segments 4—7 where the wax glands of the bees are located.

Individual bees were differentially conditioned to respond to one of the rods but not the other. Stimulation was achieved by touching the contamined tip of a rod to both antennae over a 3-sec interval. The positively conditioned stimulus, (CS+), was immediately rewarded with a drop of 1.5-M sucrose solution

as a positive unconditioned stimulus (US+). If the bee did not extend her proboscis, extension was elicited by touching the sucrose solution of the antennae. The negative conditioned stimulus (CS–) was unrewarded and a drop of salt water (1-M NaCl) was applied to the proboscis as a deterrent (US–) if it was extended in response to the CS–. Note that if the proboscis was only partially extended or fully extended and immediately withdrawn, a negative response was scored. Thus a positive response was an unambiguous extension of the proboscis with the bee waiting to be rewarded.

In each run of the experiment, approximately 30 bees were differentially conditioned to 2 different rods by presenting one or the other of these rods in a sequence of 16 trials. This conditioning was carried out by placing each test bee in turn on a platform approximately every 10 min, and stimulating her with the CS+ or CS– rod in the following sequence (the indicated division into two 8-trial groups is only for the purposes of data analysis) :

16-trial sequence	Trial (training) :	1	2	3	4	5	6	7	8
	CS :	+	-	-	+	-	+	+	-
	Trial (evaluation) :	9	10	11	12	13	14	15	16
	CS :	+	-	-	+	-	+	+	-

The number of errors that an individual bee made during trials 9—16 (evaluation sequence) was recorded, where errors are responses (extension of the proboscis) to any of trials 10, 11, 13, or 16, or non-responses to any of trials 9, 12, 14, or 15. Each individual could thus make from 0—8 errors, and an error histogram and average error, *e*, were calculated for each group. The distribution of errors in these histograms was compared using an n x 2 chi-squared contingency-table analysis, where the value of n depends on whether the tail categories of the histograms need to be combined to meet minimum-expected-frequency criteria (Getz *et al.*, 1986).

Results and Discussion

In the context of the experiment

Results obtained over various runs (treatments) of the experiment are illustrated in Figure 1. The details of each treatment and the significance of the results are explained in the caption. Controls for the treatments are CS+ and CS– rods prepared from different sets of 5 source bees of the same age but maintained apart in the incubator. The average error $\bar{e} = 4.2$ for the 1—1 control (represented by the broken line in the figure; see caption for an explanation) is actually the combined results of 2 runs (the separate results are : $e_1 = 4.1$, $e_2 = 4.2$, $n_1 = 27$, $\chi^2_2 = 3.8$, and $P > 0.1$). This is significantly less than the average error $\bar{e} = 4.7$ of the 3—3 control. In a previous study (Getz *et al.*, 1988), values of $e = 4.4$ and $e = 4.6$ were obtained for 2 no discrimination control runs (both rods rubbed on the thorax of the same individual worker bee). Note that no discrimination corresponds to an average error level $\bar{e}$ greater than the random error level $e = 4$ because of a number of factors, including the willingness of individual bees to extend their proboscides in anticipation of a possible reward. The result for the 3—3 control is consistent with no discrimination, while the control result is consistent with a very low level of discrimination. Thus, in comparing the treatments with the 1—1 rather than 3—3 control, we are actually being conservative in our assessment of whether discrimination is taking place in the various age comparison experiments.

The reason for a low level of discrimination in the 1—1 control is not directly apparent, although there is an explanation that depends on the level of individual odor variation in relation to the

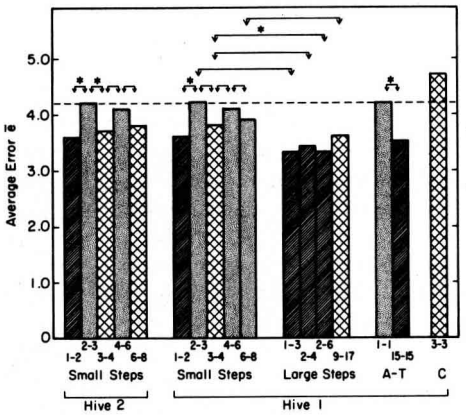

Fig. 1. The bars represent the average error, $\bar{e}$, made by a group of bees receiving a particular treatment (sample sizes range from 26—31). All treatments involve source bees of age *i* and *j*, where the label *i—j (e.g.,* 1—2) implies that *i*- and *j*-day-old source bees were respectively rubbed with CS– and CS+ rods. The dotted line is the average error obtained for the 1—1 control treatment (sample size is 59). Cross-hatched and hatched bars indicate differences between this control and the particular treatment group respectively at $P < 0.05$ and $P < 0.005$ significance levels (3 x 2 chi-squared contingency table). The significance of selected pairwise comparisons are indicated by line-and-arrows, where (*) indicates that differences are statistically significant ($P < 0.05$, 3 x 2 chi-squared contingency table) while stippled bars indicate a lack of significance ($P > 0.05$). For the third and second treatments from the right, the CS+ and CS– rods are respectively rubbed on the thoraces **(T)** and abdomens **(A)** of the same 5 source bees. The right-most treatment is another control **(C).**

number of individuals in a set of source bees. By chance, for example, 2 sets of 5 individuals may contain a spectrum of similar odor phenotypes while a third set may contain an individual that is sufficiently different to set the group apart (also see Getz, 1982). Thus the variations in cuticular chemosensory cues on the thoraces of the sets of 3-day-old individuals may by chance be "averaged out" using groups of size 5, while a residual difference remains apparent in the case of the sets of 1-day-old individuals. Hence this difference, although significant, could be due to the chance selection of particular individuals as source bees, and definite conclusions are unwarranted without further investigation.

Significantly greater levels of discrimination (smaller $\bar{e}$) were obtained in the remaining treatments except for 2—3 and 4—6 in both hives and 6—8 and 1—1-(A-T) in hive 1 (Figure 1). Testing over larger steps in the ontogeny sequence, using source bees from hive 1, yielded marginally higher levels of discrimination than comparable smaller steps, although the differences were generally not significant. Thus discriminable odor differences may get a little stronger as age differences increase, but there appears to be no linear (additive) trend in the strength of this discrimination.

Results relating to ontogenetic changes in chemosensory cues present on thoraces of workers during ages 1—6 are evident and consistent across both hives. There is a change from day 1—2 while little change is apparent from day 2—3. Again, there is a change from day 3—4 while little change is apparent from day 4—6. There appears to be a change from day 6—8, although this change is not quite as strong as the previous two. A change is apparent from day 9—17 although it is not as strong as the change from day 2—6.

Workers in normal hive situations typically synthesize wax for comb production between ages 12—18 days (Gary, 1975). Comb wax is synthesized in ventral abdominal wax glands, and its composition is strikingly different from waxes produced by the cuticle (Blomquist *et al.,* 1980).

Three of the 5 source bees used in the 15—15-(A-T) treatment were observed to have produced wax scales at age 15 days despite the fact that they had been maintained outside their hive. The results of treatment 15—15-(A-T) clearly show that test bees are able to determine a difference between a rod rubbed on the thoraces (dorsal surface) and a rod rubbed over the abdomen (ventral surface and wax glands) of the same set of workers. This implies that chemicals are not transferred from one to another part of the same individual to the point where the chemicals on these 2 surfaces are homogeneous. Hence, without further investigation, one cannot conclude that the difference between 9- and 17-day-old bees observed in treatment 9—17 is due entirely to abdominal-wax-gland production. One-day-old bees, however, do have a relatively homogeneous distribution of surface waxes (1—1-(A-T) result in Figure 1) that disappears at least when abdominal-wax-gland production is in progress.

Implications for kin recognition

Several studies suggest that workers are able to discriminate between their super- and half-sister workers and that this takes place during the course of their daily activities within their hive (Getz and Smith, 1986; Frumhoff and Schneider, 1987, Getz, in press). Recognition appears to involve chemosensory cues, although the exact nature of these cues has not been identified. One could argue that it is probably easier for a worker to identify the individual producing a particular chemosensory cue if it is a relatively non-volatile chemical on the surface of the cuticle rather than a highly volatile compound. But chemicals on the cuticle of an individual are themselves not static, changing at least twice up until age 8 days and, as shown for waxes by Blomquist *et al.* (1980), changing at older stages as well.

A difficult question to resolve is where does an individual obtain the information that enables it to discriminate between super- and half-sisters in a hive containing a distributed mixture of both super- and half-sisters (Frumhoff and Schneider, 1987). It seems that the source of this information must be endogenous; that is, it must be innately known or obtained by individuals learning their own cue phenotypes (Getz and Smith, 1986; also see Getz and Chapman, 1987). If some of these recognition cues are cuticular chemicals then the day-to-day changes, evident from the results in Figure 1, confound the situation. Either intra-individual inter-temporal changes are small compared with inter-individual differences, or a relatively static subset of chemicals on the surface of the cuticle exists and is used for recognition. Certain cuticular hydrocarbons appear to play a role in species and caste recognition in the termite, *Reticulitermes virginicus* (Howard *et al.*, 1982). Perhaps the same is true for kin recognition in honey bees. It will take detailed GC analyses of cuticle washes from samples of bees varying both with respect to age and relatedness to resolve this question.

Conclusion

Within the first 8 days of adult life, we have identified at least 3 periods during which the composition of chemicals present on the surface of a worker bee undergoes changes that are discriminable by other worker bees. These changes occur between days 1 and 2, days 3 and

4, and days 6 and 8. In contrast, between days 2 and 3, and days 4 and 6, no discriminable changes occur. At least one more change takes place between days 9 and 17, which is possibly related to individuals' producing comb wax at around 12 days. It is not clear how much of this comb wax contaminates an individual's thorax since the chemicals on the surface of the thorax and abdomen of a 15-day-old individual are compositionally distinct.

Cuticular hydrocarbons have been used to identify different honey bee races (Carlson and Bolten, 1984; McDaniel *et al.*, 1984, 1987), and ontogenetic changes have been identified using gas chromatography (GC). Such changes confound the problem of classifying individual bees using GC and have important ramifications for understanding observed intracolony-kin-discrimination processes.

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