Apidologie 30 (1999) 367–374

Original article

Influence of body fluid from pin-killed honey bee pupae on hygienic behavior

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(Received 24 October 1998; revised 5 March 1999; accepted 18 May 1999)

Abstract – Hygienic behavior in honey bees can be tested by piercing the brood with a pin; however, there is concern that variability in the quantity of fluids that leaks from the pupae could influence test results. Colonies of *Apis mellifera carnica* were tested to evaluate this possibility. We made four repetitions of four treatments and one control in each of three colonies. The order of degree of hygienic behavior was: pin-killed capped worker brood with a drop of body fluid injected underneath the cell capping > pin-killed capped worker brood > undamaged capped brood with a drop of body fluid injected underneath the cell capping > control or a drop of pupal body fluid placed on the cell cappings. All of the differences were significant (Tukey test, $P < 0.05$) except the body fluid on the cell cap, which gave the same results as the control. The addition, inside worker brood cells, of pupal body fluid had a significant effect on honey bee hygienic behavior, both in normal brood and in pin-killed brood. © Inra/DIB/AGIB/Elsevier, Paris

***Apis mellifera carnica* / hygienic behavior / body fluid / pin-killing method**

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1. INTRODUCTION

Honey bee workers (*Apis mellifera* L.) are able to recognize diseased, damaged or dead brood inside capped cells and remove them through hygienic or cleaning behavior [4, 5]. Gramacho and Gonçalves [2], working with Africanized honey bees *Apis mellifera scutellata,* made a comparative study of the freeze-killed and pin-killed brood assays to test hygienic behavior of workers. They found no significant differences between the two methods. They considered the pin-killing method developed by Newton and Ostasiewski [3] more useful than the freeze-killing method because it is easier to use in the field and in the laboratory and less expensive to implement than the freezing method. Newton and Ostasiewski [3] had suggested the pin-killing assay as a means to test for honey bee colony resistance to American foulbrood (*Paenibacillus larvae*). However, Spivak and Downey [6] prefer the freeze-killing method, because they found that the pin-killed brood was quickly removed by virtually all colonies, and was therefore not a good discriminatory test. Taber and Gilliam [11], Spivak and Gilliam [7] and Spivak and Downey [6] made important contributions and improvements to the methodology and field assays of hygienic behavior of honey bees. Spivak and Reuter [8] compared, through the freeze-killing method, colonies with naturally mated queens from a hygienic line of Italian honey bees to colonies from a commercial line of Italian bees not selected for hygienic behavior. They showed that the hygienic colonies removed significantly more freeze-killed brood and had less chalkbrood (a brood disease caused by the fungus *Ascosphaera apis*) than the commercial colonies. This was the first study carried out in the USA to evaluate hygienic stock in large field colonies. Also Spivak and Reuter [9, 10] recently described a method of freezing brood with liquid nitrogen and suggested that this is the best screening procedure for hygienic behavior studies. However, we did not find this method convenient or simple for routine use. It is necessary to carry a liquid nitrogen tank and to take several safety precautions (protective clothing, heavy gloves, boots, glasses, etc.) to avoid accidents with this extremely cold liquid. The main advantage is that it does not require the cutting out and removal of pieces of brood comb, to be frozen overnight and later returned to the colony.

The pin-killing method kills or damages the brood by perforating and killing the pupa within the brood cell, which can be easily performed in the field or in the laboratory. This process produces a small hole in the capped cell and the treated brood exudes body fluid. Sometimes this fluid is visible on the cell cap. The presence or absence of this fluid, and the volume of such fluid, could be an additional non-standard stimulus for hygienic behavior. We were concerned that this would affect test results.

The objective of our study was to determine the effect of the body fluid of the pupa, which appears after piercing with a pin, on the hygienic behavior of worker bees.

2. MATERIALS AND METHODS

We used three strong colonies of *Apis mellifera carnica* Pollm., each maintained in two full-size Langstroth boxes, at the Beekeeping Laboratory of the University of Hohenheim (Landesanstalt für Bienenkunde), in Stuttgart, Germany. A comb containing sealed pupae aged 11–15 days was used for each test, with four repetitions for each treatment. Each comb was used at the same time for four treatments and a control. Five areas identical in size (3.5 cm × 6 cm) were delimited to include 50 cells of the sealed worker brood (*figure 1*). The treatments were as follows: A = pin-killing method (worker brood cells perforated with a no. 1 insect pin; B = one drop (4 μL) of pupal body fluid placed on the capping of an otherwise undisturbed brood cell; C = pin-killing method and introduction of 4 μL of body fluid inside the brood cell through the pin hole; D = control (normal undisturbed brood); E = introduction of body fluid (4 μL) inside the capped brood cell. In treatment E, part of the cell

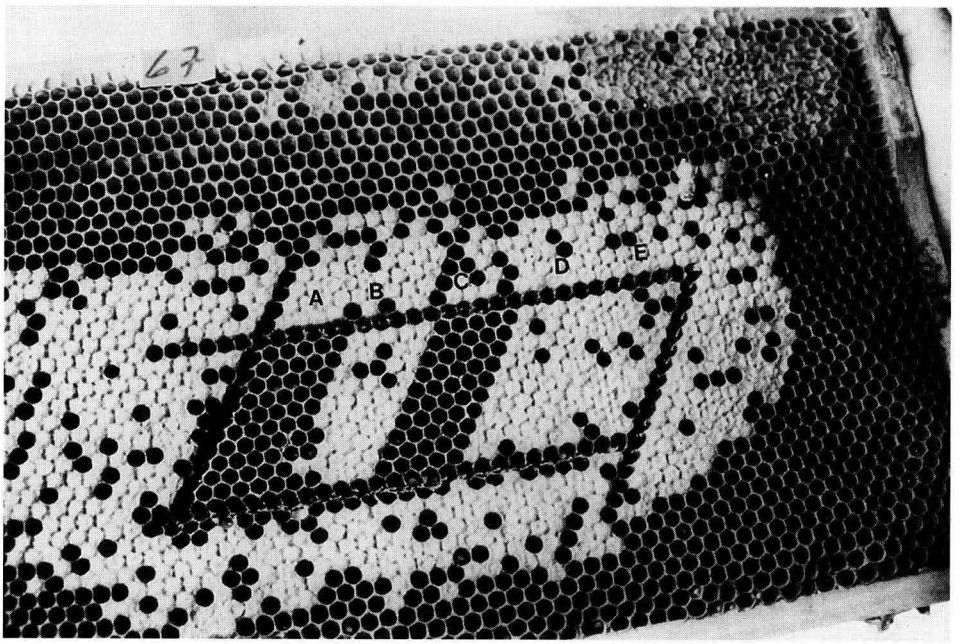

Figure 1. Sealed worker brood comb showing in the center, from left to right, the five delimited and identical areas (3.5 cm × 6.0 cm) with 50 cells each used for the treatments A (pin-killing method), B (body fluid on the capped brood cells), C (pin-killing method plus body fluid inside capped cells), the control D (normal, live brood) and E (body fluid inside the capped brood cells).

capping was lifted at the edge with fine-tipped forceps and the body fluid injected through the hole, taking care to avoid touching the pupa, and then resealing the cell capping.

The body fluid used for treatments B and E was obtained by cutting worker pupae aged 11–15 days with a glass pipette, and letting the fluid drip into small sterilized glass flasks. These were kept on ice (0 °C) for a few minutes, until the test was performed. The body fluid was then allowed to warm to room temperature just before it was used.

For the pin-killing of the brood (treatments A and C) a no. 1 insect pin was used to pierce the sealed brood cells through the center of the cell cap, penetrating the body of the pupa until the pin reached the base of the cell. The sealed worker brood comb was then returned to the respective hive. Twenty-four hours later each 'treated' comb was brought to the laboratory to check for uncapped cells and to see if the brood was completely removed by the workers, characterizing hygienic or cleaning behavior. Hygienic behavior was calculated according to the following formula:

$$HB = \frac{EC\ (24\ h) - EC\ (0\ h)}{CC\ (0\ h)} \times 100$$

where EC = empty cells, CC = capped cells and HB = hygienic behavior (in %)

The data were transformed via arcsine of the square root of the proportion removed, for comparisons by a repeated measures analysis of variance and the Tukey test.

3. RESULTS

There was a significant difference among the treatments (*table I*, $F = 57.7$, DF = 4, $P < 0.001$). Colony ($F = 2.10$, DF = 2, $P = 0.204$) and date ($F = 0.515$, DF = 3, $P = 0.687$) had no significant influence on the results.

Table I. Hygienic behavior (% brood completely removed) of three colonies of *Apis mellifera carnica*, each tested four times with five treatments, simultaneously.

	Treatments														
	A Pin-killed			B Pupal body fluid dropped on capped cells			C Pin-killed + body capped cells			D Control			E Body fluid injected inside capped cells		
	Colony no.														
Dates	12	67	68	12	67	68	12	67	68	12	67	68	12	67	68
August 1 (1996)	41.60	97.80	12.50	0.00	0.00	0.00	91.50	97.60	37.50	0.00	0.00	0.00	42.50	47.72	34.00
August 5	79.00	74.50	66.00	0.00	0.00	0.00	93.20	95.60	92.00	0.00	0.00	0.00	20.80	30.20	35.40
August 21	95.60	93.20	88.00	0.00	0.00	0.00	100.00	97.70	100.00	2.30	0.00	4.20	30.70	2.00	6.20
September 5	45.50	100.00	52.10	0.00	4.30	2.20	97.00	100.00	62.22	2.80	2.10	0.00	43.20	18.70	6.00
Average	65.43	91.38	54.65	0.00	1.08	0.55	95.43	97.73	72.93	1.28	0.53	1.05	34.30	24.66	20.40
s.d.	26.20	11.60	31.75	0.00	2.15	1.10	3.82	1.80	28.67	1.49	1.05	2.10	10.67	19.25	16.52
Mean for each treatment	70.5 %			0.5 %			88.7 %			1.0 %			26.4 %		

All of the five treatment groups gave significantly different hygienic behavior means (Tukey test, $P < 0.05$), except for the control compared to the body fluid placed on the cell capping. The sequence of results was: pin-killed + body fluid > pin-killed > body fluid inside capped cells > body fluid on capped cells or control (*table I*). Treatment A (pin-killing method) and treatment C (pin-killing method plus body fluid inside the capped cells) provoked significantly more brood removal than in the control (*table I*, Tukey test, $P < 0.001$). Treatment C (pin-killed plus body fluid injected inside the cells) provoked a 18 % higher brood removal rate than treatment A (pin-killed brood), which is a significant difference (Tukey test, $P < 0.01$). The body fluid placed on the capped cells had no influence on the hygienic behavior of the worker bees, when compared to the control (Tukey test, $P = 0.976$).

Placement of body fluid inside the capped brood cells, without deliberately piercing the pupa (E), provoked about 25 % removal behavior (*table I*). The increase over the control was significant (Tukey test, $P < 0.01$).

4. DISCUSSION AND CONCLUSION

When a pupa is pierced, a whitish fluid is exuded. This 'body fluid' is composed of hemolymph, along with many fat body cells and other types of material from tissue which has been damaged. In a recent paper [6] 'hemolymph' was extracted from live and dead (frozen) pupae to determine the effect on hygienic behavior; however, this was actually what we chose to call body fluid, because it is not possible by normal methods to obtain clean hemolymph from live pupae.

We found that pupal body fluid placed inside the capped cells was an important stimulus for hygienic behavior. Spivak and Downey [6] found similar effects when they placed pupal body fluid (which they called hemolymph) on pupae in an artificial comb, though when this material was taken from frozen pupae the effect was significantly stronger than when fluid was taken from live pupae. Hemolymph removed from dead pupae would be contaminated with burst fat body and hemolymph cells, which may have been the reason for the difference in reaction.

The significant effects of introducing a drop of pupal body fluid inside pin-killed brood in our experiment show that this fluid has an important effect on hygienic behavior when exposed inside the brood cell. This means that fluid that leaks from pin-killed pupae is an important stimulus. In our experiment the addition of extra pupal body fluid increased the stimulus for the bees to remove the brood, whether perforated or not.

We were concerned with preliminary observations that a drop of pupal body fluid sometimes appeared on the capping of pin-killed brood cells. The percentage of such cells with a visible drop of fluid varied considerably (data not shown), and could be an additional, non-uniform cue. The fact that the worker brood cells in treatment B (body fluid placed on the capped brood cells) were not damaged by the bees indicates that the deposition of body fluid on top of the capped cells does not provoke hygienic behavior. Possibly this fluid is removed by the bees before it deteriorates and can emanate odors which stimulate the bees to take further action.

There is evidence that different cohorts of bees are specialized in opening suspicious brood cells and others seal the brood cell again or remove the damaged pupae [1, 12]. This is also confirmed by our observations, as sometimes damaged brood cells were uncapped at the first examination and capped again at the second check. We found that when the capped brood cells are perforated during the pin-killing method, without damaging the brood, the workers normally do not remove these brood from the cells (data not shown). We also observed that when the capping is artificially removed without disturbing the brood inside it, the

workers reconstruct the capping without removing the brood.

Pupal body fluid placed inside brood cells increases adult honey bee hygienic behavior directed towards normal, otherwise undamaged pupae. It also significantly increases the hygienic behavior provoked by pin-killed brood. This leads us to conclude that the fluid that leaks from pin-killed pupae inside the cell is an important stimulus for brood-removal (hygienic) behavior.

ACKNOWLEDGMENTS

The authors are grateful for the support given by CAPES/MEC/DAAD, Project Probral 030/95 and to the University of Hohenheim (Landesanstalt für Bienenkunde) of Stuttgart, Germany. Our special thanks to Dr Arcadio de los Reyes Borjas of the Genetics Department of the University of São Paulo in Ribeirão Preto for help with the statistical analysis.

Résumé – Influence du liquide corporel des nymphes d'abeilles tuées par épingle sur le comportement hygiénique. Diverses techniques ont été mises au point pour mesurer le comportement hygiénique des abeilles. Les plus courantes consistent soit à congeler le couvain, soit à le tuer avec une épingle, mais toutes deux détruisent un certain nombre de cellules de couvain d'ouvrières operculé. La première méthode nécessite de découper un morceau du rayon de couvain et de l'ôter de la colonie durant 24 h, alors que la seconde méthode peut être appliquée en quelques minutes sur le terrain comme au laboratoire sans abîmer le rayon. Néanmoins on craint que la méthode de l'épingle ne soit moins précise et moins uniforme. Au cours de l'enfoncement et du retrait de l'épingle on voit souvent, sur l'épingle et sur l'opercule, du liquide corporel provenant des nymphes. Cette substance pourrait stimuler une réaction de la part des abeilles nettoyeuses, ce qui influerait sur le taux de désoperculation et d'élimination du couvain. Nous avons testé des colonies d'*Apis mellifera carnica* pour évaluer cette possibilité. Un rayon de couvain d'ouvrières operculé (c.o.o.) avec des nymphes âgées de 11 à 15 j a été utilisé pour chaque test (rayon expérimental). Cinq portions de rayon, de même taille (3,5 × 6 cm) et comprenant 50 cellules de c.o.o., ont été délimitées. Après avoir tué par épingle les nymphes, chaque rayon expérimental a été replacé dans sa colonie. 24 h plus tard chaque rayon expérimental a été apporté au labo et les cellules désoperculées, ainsi que les nymphes totalement éliminées par les abeilles, ont été dénombrées. Dans chacune des trois colonies, nous avons répété quatre fois les quatre traitements suivants (plus le témoin) : a) le c.o.o. est percé au centre avec une épingle n° 1 jusqu'à atteindre le fond de la cellule ; b) une goutte de liquide corporel de nymphe est déposée sur le sommet des cellules de couvain, sans qu'elles ne soient perforées, c) le c.o.o. est percé comme précédemment et une goutte de liquide corporel de nymphe est déposée à l'intérieur des cellules, d) cellules de couvain operculé (témoin), e) le c.o.o. n'est pas abîmé et une goutte de liquide corporel est introduite à l'intérieur des cellules. Les données ont été traitées par analyse de variance avec mesures répétées et test de Tukey, après transformation Arcsin$\sqrt{}$. Le comportement hygiénique chez les groupes expérimentaux n'était pas uniforme ($p < 0{,}0001$). Il se classait comme suit par ordre décroissant : traitement c > traitement a > traitement e > traitement d ou b. Ces différences sont toutes significatives ($p > 0{,}05$). L'addition de liquide corporel s'écoulant des blessures faites aux nymphes à l'intérieur des cellules a une action significative sur le comportement hygiénique des abeilles, bien qu'elle n'en ait aucune lorsque les gouttes de liquide sont déposées sur les opercules.

***Apis mellifera carnica* / comportement hygiénique / liquide corporel / destruction par épingle**

Zusammenfassung – Einfluß der Körperflüssigkeit von im Nadeltest getöteten Bienenpuppen auf das Ausräumverhalten (hygienisches Verhalten). Verschiedene Methoden wurden entwickelt, um das Ausräumverhalten von Honigbienen zu messen. Am häufigsten wird das Einfrieren von Brut und der Nadeltest angewendet. Eine Standardanzahl von verdeckelten Brutzellen von Arbeiterinnen werden durch Einfrieren oder durch Einstiche mit der Nadel verletzt. Im ersten Fall muß ein Stück Brutwabe ausgeschnitten und 24 Stunden lang aus dem Volk entfernt werden, während im zweiten Fall der Test in wenigen Minuten unter Feld- und Laborbedingungen durchgeführt werden kann, ohne die Wabe zu zerstören. Trotzdem gibt es Bedenken, daß der Nadeltest weniger präzise und gleichmäßig ist. Beim Einstechen und herausziehen der Nadel kann man oft Körperflüssigkeit an der Nadel und auf dem Zelldeckel sehen. Diese Verunreinigung könnte eine Reaktion der putzenden Stockbienen stimulieren, die die Rate des Entdeckelns der Zellen und des Entfernens der Brut beeinflussen könnte. Die Stärke eines solchen Effekts testeten wir in Völkern von *Apis mellifera carnica*. Für jeden Test wurde eine verdeckelte Brutwabe mit Puppen im Alter zwischen 11 und 15 Tagen benutzt. Fünf gleich große Stücke (3,5 cm × 6 cm) wurden abgegrenzt, so daß sie 50 Zellen verdeckelte Arbeiterinnenbrut enthielten. Nach Tötung mit der Nadel wurde die verdeckelte Brutwabe (Versuchswabe) in das entsprechende Volk zurückgestellt. Nach 24 Stunden wurde jede Versuchswabe ins Labor geholt, um die Anzahl sowohl der geöffneten Zellen als auch der vollständig entfernten Puppen zu bestimmen. Jede der 4 folgenden Versuchsmethoden und die Kontrolle wurde in 3 Völkern 4 mal wiederholt: a) verdeckelte Zellen wurden mit nur 1 Nadel bis zum Boden durchstochen, b) ein Tropfen Körperflüssigkeit wurde auf den Zelldeckel gelegt, ohne die Zelle anzustechen; c) die verdeckelte Zelle wurde wie oben angestochen und zusätzlich ein Tropfen Körperflüssigkeit in die Zelle eingeführt; d) unbehandelte verdeckelte Zelle (Kontrolle) und e) unverletzte Puppen mit einem Tropfen Körperflüssigkeit in der Zelle.
Für eine Varianzanalyse und den Tukey-Test wurden die Quadratwurzeln der Daten über eine Arcussinus –Transformation aufbereitet. Das Ausräumverhalten war in den Versuchsgruppen unterschiedlich ($P < 0{,}0001$). Die Reihenfolge im Ausräumverhalten war: Nadeltest plus Körperflüssigkeit in der Brutzelle > Nadeltest > Körperflüssigkeit in der Brutzelle > Kontrolle oder Körperflüssigkeit auf dem Zelldeckel. Diese Unterschiede waren alle signifikant ($P < 0{,}05$). Zusätzlich hatte die Körperflüssigkeit, die aus Verletzungen der Puppe in der Brutzelle stammten, einen signifikanten Einfluß auf das Ausräumverhalten, obwohl es keinen Effekt auf dem Zelldeckel zeigte. © Inra/DIB/AGIB/Elsevier, Paris

hygienisches Verhalten / Körperflüssigkeit / Tötung durch Nadeltest / *Apis mellifera carnica*

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