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Original article

Fight between virgin queens (*Apis mellifera*) is initiated by contact to the dorsal abdominal surface

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Abstract – To determine the nature of the stimuli involved in queen recognition, we videotaped fighting behaviour between young virgin queens and developed a bioassay. The results of the bioassay were as follows: (1) Under illumination with red light, the queens responded with stinging behaviour (stB.); thus, lack of visual stimuli did not play an essential role in releasing stB. (2) Tethered queens, narcotised queens, and dead queens were stung, demonstrating that movement was not essential for releasing stB. (3) Reduced contact between queens by placing a single screen between them reduced the stinging response, while queens separated by a double screen, blocking direct contact, had no stinging response. (4) StB. was released when queens were in contact with isolated queen abdomens or dorsal abdominal integuments. (5) Workers fitted with queen dorsal abdominal integument released stB. (6) Fifteen day old queen pupae released stB. We hypothesize that the pheromone triggering fighting behaviour is located on the queen's abdominal tergites, which is the location of the tergite glands.

honeybee queen / fighting behaviour / bioassay / tergite gland / pheromone

1. INTRODUCTION

In honeybees, the queen and her pheromones serve as the centre of the colony's social regulation. Thus the maintenance of monogyny is an important factor within the colony reproductive cycle. Generally, after swarming, several young queens are left in the nest and the elimination of all surplus queens to retain a monogynous state occurs rapidly. The dramatic fight among virgin queens of *Apis mellifera* L., resulting in the killing of one combatant, caught the attention of early researchers (Réaumur, 1741; Huber, 1792). However, the question of which stimuli or cues serve for recognition or release of fighting is still under debate (Riedel and Blum, 1972; Szabo and Smith, 1972; Lensky et al., 1991; Tiemann, 1996).

Here, we took a fresh experimental look at queen fighting. To determine the nature of the stimuli involved in queen recognition, we videotaped and analysed fighting behaviour between young virgin queens. To this end, we developed a bioassay that allowed us to test different components of the stimuli that elicit stinging behaviour under standardised conditions.

2. MATERIALS AND METHODS

2.1. Queens

Experiments were conducted in Oberursel, Germany (N 50°12'N, 8°35'E) and Toulouse, France (43°37'N, 1°26'E). Queens were reared (Ruttner, 1980) and kept in small cages (85 × 57 × 85 mm) together with 65–75 worker bees for 2 to

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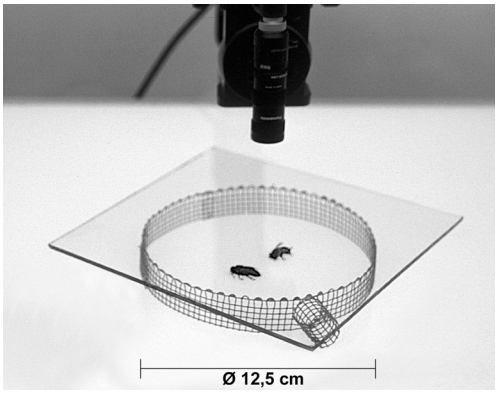

Figure 1. Arena with video camera above.

6 days under constant conditions (darkness, temperature 29 °C ± 0.5 °C, 75% RH). In the cage, bees and queen were fed sucrose (Apiinvert®) ad libitum from a 10 mL vial. All together about 1300 queens were reared and tested.

2.2. Bioassay

For the bioassay we constructed a circular arena (Ø 125 mm, height 20 mm) out of wire mesh which was placed on an translucent glass plate illuminated from below. A video camera was mounted above and recorded the behaviour of the queen in the arena which was covered by a glass plate. A small tube connected to the arena wall by a wire gate allowed us to introduce the queen at the start of the bioassay (Fig. 1).

Tests were carried out under constant conditions (temperature 29 °C ± 0.5 °C, 75% RH). Before the start of the bioassay, the test object (live, fixed, or dead queen, or body part, etc.) was placed in the arena. Then a test queen was introduced through the tube. Starting with entrance of the test queen into the arena, the behaviour of this queen was recorded for 3 minutes in each assay and stinging behaviour (see Sect. 3.1) served as test parameter. If a stinging reaction occurred during 3 min, the test result was classified as "positive", and in cases without stinging behaviour the assay was rated "negative".

Each queen was tested only once. However, afterwards the tested queens (or parts of them) were used as test objects and each test object was presented only once in a bioassay.

2.3. Different test conditions/treatment

2.3.1. Visual stimuli

To test whether visual stimuli play an important role in eliciting stinging behaviour, we used red light of 715 nm to illuminate the arena. There was no other light source in the room. We introduced two queens consecutively into the test arena. Recording started when the second queen entered the arena.

2.3.2. Movement stimuli

The influence of movement on eliciting stinging behaviour was analysed by fixing a live queen in the arena so she could not move. A queen was fixed by crossing insect pins between the thorax and abdomen, which restricted her to the substrate without harming her. We also tested queens that had been narcotised by CO_2 and queens that were freeze-killed before the test.

2.3.3. Perception of stimuli through screen

A piece (2 cm) of the wall of the arena was replaced by a test screen and a queen that had been narcortised for 10 minutes under CO_2 was fixed to the outside of the arena with the dorsal side in direct contact to the test screen. During the bioassay, the test queen inside the arena was separated from the narcotised queen by the test screen. We recorded whether the test queen reacted towards the narcotised queen behind the test screen with stinging behaviour. A positive stinging response was recorded if the test queen seized the wire of the test screen with her mandibles, bent her abdomen and pressed the sting chamber against the screen, and the sting protruded. In the case of larger mesh sizes (2.5 mm, 5.7 mm), the sting touched and penetrated the probe queen (behind the test screen). We tested screens of different mesh size (5.7 mm; 2.5 mm and micro screen with 0.075 mm × 0.125 mm). Further, we tested a double screen (mesh 2.5 mm) with a spacing of 0.5 mm.

2.4. Location of releaser

To determine which body parts released stinging behaviour we tethered a live queen by the petiole to the wall (wire mesh) of the test arena, so that her abdomen protruded into the arena and the head and thorax protruded outside the arena. In the next bioassay, a queen was fixed to the arena wall the other way around (with the head and thorax inside and abdomen outside the arena). Next, we placed an isolated abdomen of a freeze killed queen as test object in the arena. Further, we isolated the dorsal integument of queen's abdomen by carefully removing the heart and attached alary muscles, leaving the epidermis, tergites, intersegmental

membranes and subepidermal glands intact and tested it in the bioassay.

The isolated dorsal abdominal integument of a queen was put over the abdomen of a worker bee which had been immobilized by CO_2. The transplant was held in position by elasticity of the tergites and gluing effects of the fresh tissue underneath the queen's integument. We tested worker bees with attached queen abdominal integuments and, as controls, worker bees with attached dorsal abdominal integuments from drone bees.

2.5. Ontogeny

It is well documented that the main glands of the honeybee queen develop and commence production of pheromones at different ages (Free, 1987). Therefore, determination of critical age at which a pupa or a young queen is able to release a stinging response might yield information on the source of recognition stimuli. In these bioassays, queen cells were carefully opened at the base and the pupa in each was removed by turning of the cell and then was placed in the arena. We tested queen pupae of different ages: 11, 12, 12,5, 13, 13,5, 14, 14,5, 15, 16, 16,5 days after the egg was laid.

3. RESULTS

3.1. Behaviour

When two live queens were introduced into the arena, the behaviour of the queen winning the fight was analysed from video recordings. Twenty-three pairs of queens were recorded and in 10 pairs (selected due to visibility) behavioural analysis for time and distances were conducted. The following pattern emerged as typical.

After introduction, a queen showed high exploratory activity. With vibrating wings she moved quickly, changing direction frequently. About 27 s later the wings stopped and the queen slowed down gradually. The antennae moved continuously in different directions. We did not notice any behavioural reaction towards the second queen in the arena at distances between 30 mm and 5 mm (20 observations, 10 pairs of queens). At a closer range (<3.2 mm) the queen pointed her antennae towards the other queen and moved forward to establish contact (10 pairs of queens) (Fig. 2, pict. a). Often the first contact was directed toward the abdominal tergites

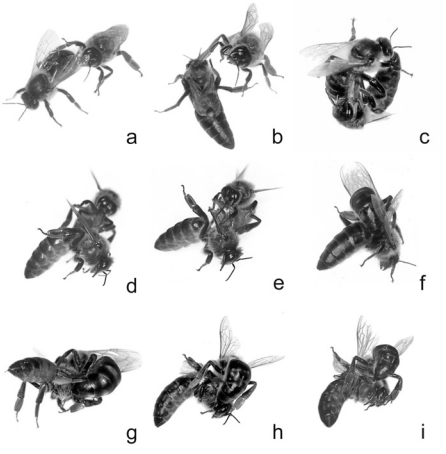

Figure 2. Behavioural sequence of queen fight (see text for details).

(Fig. 2, pict. c). Immediately, the queen's mandibles opened and she turned towards her rival's wing base (Fig. 2, pict. b-e). At the same moment the legs (tarsi) caught hold of target queen (Fig. 2, pict. e). In this way, the queen gained a secure grip on the competitors body and stinging behaviour was initiated (Fig. 2, pict. f). The abdomen bent towards the rival queen's thorax (Fig. 2, pict. g). The abdominal tip moved until an intersegmental membrane was found. There the sting penetrated (Fig. 2, pict. h-i). After 53.94 s (min = 1.44 s, max = 175.04 s) the queen left her defeated rival. In four cases out of 10, up to three further attacks towards the dying (stung) queen were observed within 3 min.

Among the queen's complex behavioural pattern, stinging was the most distinct element of fighting behaviour. In particular, a queen's stinging reaction consisted of bending the abdomen with its tip towards a target object, opening the sting chamber, and protruding the sting. The above definition of stinging response (abdomen bending + sting protrusion) served as test parameter in our subsequent bioassay.

3.2. Test results

3.2.1. Different test conditions/treatments

To determine whether visual stimuli play a crucial role in eliciting stinging, we

Table I. Queen stinging response under different test conditions.

Condition/treatment	Total number of queen pairs	Number of positive responses	*
light	12	12	a
red light (715 nm)	12	12	a
fixed with insect pins	12	12	a
CO_2 immobilised	12	12	a
dead (freeze-killed)	12	9	a
screen (mesh 5.7 mm)	20	3	b
screen (mesh 2.5 mm)	20	3	b
double screen	20	0	b
micro screen	20	0	b

*Different letters indicate significant difference among treatments ($P \leq 0.0001$, 2×2 contingency test).

illuminated the arena with red light. In all cases (n = 12) the queen pairs responded with stinging behaviour and there was no significant difference in the frequency of stinging compared to when the arena was lit with a day light fluorescent tube (Tab. I).

To determine the role of movement, we gradually reduced the motility of the probe queen. Queens fixed with 2 insect pins crossed between the thorax and abdomen tested positive in all cases (n = 12). Queens immobilised by CO_2 narcosis released stinging behaviour in all cases (n = 12). Also dead queens (killed by freezing; n = 12) tested positive in 9 cases. There were no significant differences among untreated, CO_2 immobilised or dead queens (Tab. I).

When probe (narcotised) queens were secured behind 5.7 mm and 2.5 mm mesh screens, the test queen contacted the probe queen with her antennae through the test screen. However, stinging responses were reduced significantly ($P < 0.0001$, 2×2 contingency test).

Antennal contacts to probe queens behind a double screen (spacing 5 mm between screens) or a micro screen were blocked. Probe queens secured behind the double or the micro screen tested negative.

These results differ significantly from freely accessible queens (Tab. I).

3.2.2. Location of releaser

In a first set of tests (n = 40), when the queen's abdomen protruded into the arena and the head and thorax remained outside the arena, 36 tests out of 40 were positive; the test queen stung the other queen's abdomen within 3 minutes. When we arranged the probe queen in the opposite direction (queen's abdomen outside, head and thorax inside) 11 tests out of 40 were positive. The difference between these two groups was significant (Tab. II). An isolated queen abdomen placed in the arena elicited a positive response in 7 out of 12 tests, whereas an isolated head and thorax tested negative (n = 12) (Tab. II). Differences between these two groups were significant at the 5% level ($P = 0.037$, 2×2 contingency test).

Isolated dorsal abdominal integuments tested positive in all cases (n = 12). Finally, when the queen's dorsal abdominal integument was placed over the dorsal abdomen of CO_2 immobilised workers, the test queen stung the "under cover" workers in 46 out of 62 tests. Workers "fitted" with drone tergites (n = 12) and untreated workers (n = 24) tested negative in all tests (Tab. II).

3.2.3. Ontogeny of the releaser

We tested queen pupae of different ages. The first positive results (Fig. 3) occurred with

Table II. Queen stinging response towards different body parts.

Sample	Total number of queen pairs	Number of positive responses	*
abdomen outside arena	40	11	a
abdomen inside arena	40	36	b
queen without abdomen	12	0	c
isolated abdomen	12	7	d
tergal abdominal integument	12	12	b
worker	24	0	c
worker with drone tergites	12	0	c
worker with queen tergites	62	46	b

*Different letters indicate significant difference among treatments ($P \leq 0.049$, 2×2 contingency test).

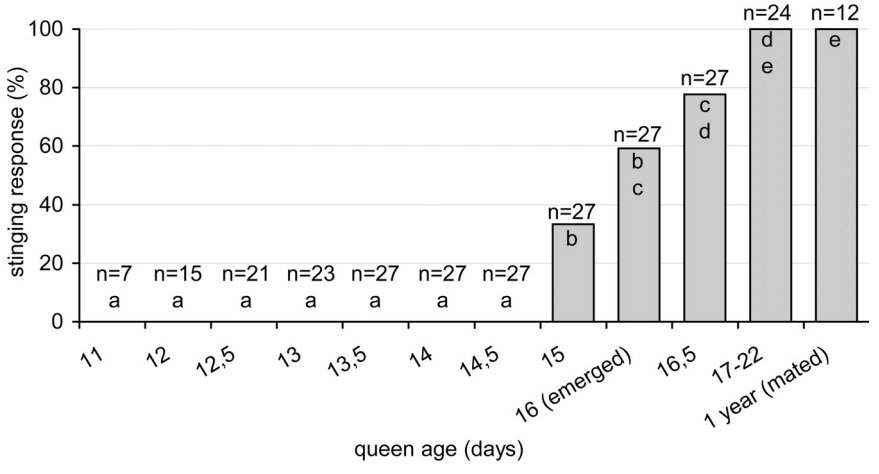

Figure 3. Test of queen pupae and queens of different age. n = number of tests, x axis = queen age (days after egg was laid), y axis = % of tests with stinging response, different letters indicate significant differences between ages at the $P \leq 0.0001$ level, 2×2 contingency test.

young imaginal queens shortly after ecdysis (15 days old). The stinging responses increased in each group up to the group of 1–6 day old queens where it reached 100%.

4. DISCUSSION

Because queen replacement has a dramatic effect on the survival and genetic structure of honey bee colonies, knowledge of the mechanisms that regulate queen competition is essential for understanding the factors that organize a honey bee society and how this social structure may have evolved. In the colony, queen duels occur under very complex and highly dynamic conditions (Gilley, 2001). The occurrence and the outcome of the fight is strongly influenced by the worker bees (Schneider et al., 2001). Some complex factors seem to be involved, such as relatedness to worker bees (Tarpy and Fletcher, 1998), and queen age and quality (Tarpy et al., 2000).

We deliberately excluded most of these factors from our test situation. The environment in our bioassay was reduced to obtain an immediate, reliable reaction to the test stimuli. Thus, the applicability of our results to colony conditions is limited and may not totally reflect queen fights under natural conditions. The behavioural fighting pattern between the queens, however, seems to be similar whether

they are in our arena or in the nest (Butz and Dietz, 1994; Gilley, 2001). The focus of our research was on the stimuli that triggered stinging once queens contacted one another. This measure represents only the final step in a complex series of interactions that regulate reproductive conflict in honey bee colonies.

Naturally, the fight between young *Apis mellifera* queens takes place in the darkness of the hive. Lensky et al. (1970) however concluded from experiments with "blinded" queens that visual stimuli are essential to release fighting behaviour. In contrast, we did not find any differences after visual perception was excluded by red light illumination. So apparently visual perception is not essential to release fighting behaviour. Further, recognition among young queens seems to be based on very close-range perception (Butz und Dietz, 1994; Riedel und Blum, 1972; Bernasconi et al., 2000). Our results demonstrate that direct contact between queens initiated fighting. Negative test results with the microscreen and the classical double screen (Butler, 1954) indicated that olfactory perception alone (without direct contact) was not sufficient to release stinging.

The literature on glands and secretions putatively involved in eliciting queen fighting suggests diverse possibilities. Riedel and Blum (1972) concluded that volatile compounds of the mandibular glands were the source of the releasers. However, Velthuis (1967), Szabo and Smith (1972) reported that queens without mandibular glands fight and recently Tiemann (1996) observed queen fights after she sealed off the mandibular glands. Lensky et al. (1970) first suspected that iso-pentyl-acetate and other volatile substances of the sting were the releasers because when the queen's sting chamber was covered with hot wax, no stinging response was observed. Later, Lensky et al. (1991) showed that iso-pentyl-acetate was not found in queens, and argued that the Koschevnikov's gland and its secretions might release queen fighting. Our results, however, point to the abdominal tergites as the major location of the releasing factor. First, a "play back" video analysis of fighting behaviour revealed that antennal contact to the dorsal surface of the opponent queen's abdomen directly preceded the fight in all cases. Further, we demonstrated that direct contact to dorsal abdominal surface of the queen was essential and sufficient to release stinging behaviour. Finally, when we transferred the abdominal integument of queens to worker bees, the "under-cover" workers were attacked and stung. Thus, contact with the queen's dorsal abdominal tergites initiated stinging behaviour.

The prominent gland system located on the dorsal side of the queen's abdomen are the tergite glands ("Tergittaschendrüsen") which were described by Renner and Baumann (1964). The products of these glands seem to possess several pheromonal functions and play an important role in retinue behaviour (Vierling and Renner, 1977a; Velthuis, 1990). The pheromones of the tergite glands are known to supplement pheromones of the mandibular glands and increase the queen's attractivity to worker bees (Wossler and Crewe, 1999a), and to drones during mating flight (Vierling and Renner, 1977b). Compared to the pheromone of the mandibular glands, the attraction of tergite glands and extracts is limited to very close range or direct contact (Vierling and Renner, 1977a). Also in our tests, stinging behaviour depended on direct contact of the queen's abdominal tergites.

The significance of the tergite glands for recognition of queens was already noticed by Velthuis (1967). Later, Butz und Dietz (1994) discussed the possibility that the tergite glands were the source of the queen recognition. Some of the compounds of the tergite glands were identified (Wossler and Crewe, 1999b). Espelie et al. (1990) isolated and proposed decyl decanoate as the active compound of tergite gland secretion. However, this compound was not tested on young workers, drones or queens. Later, Smith et al. (1993) demonstrated that the secretion of tergite gland's alkenes is triggered by natural mating.

The fight among virgin queens naturally occurs directly after emergence. Furthermore, our results demonstrate that the releaser of queen stinging behaviour becomes active with metamorphosis (just before the queen ecloses from the cell). This stage in development is significantly earlier than the queen's sexual maturation, when mating and retinue behaviour takes place (Hammann, 1957). Decyl decanoate was absent in queens younger than 2 days and other identified

products of the tergite glands occur much later (Espelie et al., 1990). We suggest that the releaser pheromone is located on the queen's tergites, and seems to be an unidentified component of the tergal gland secretion. Further work to isolate and identify the active substance is in progress.

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Résumé – Le combat entre les reines vierges d'abeilles (*Apis mellifera*) commence par le contact avec la face dorsale de l'abdomen. Le combat dramatique entre jeunes reines d'abeilles a depuis longtemps éveillé l'intérêt des chercheurs. Néanmoins la question de savoir quels stimuli servent à la reconnaissance de la reine ou à déclencher le comportement de combat, reste à ce jour non résolu. Dans cette étude nous renouvelons l'approche expérimentale de ce problème. Afin de déterminer les stimuli impliqués dans la reconnaissance de la reine, nous avons enregistré avec un magnétoscope le comportement des reines lors des combats et mis au point un test biologique qui nous permet de tester différentes composantes des stimuli qui déclenchent le comportement de piqûre.

Les expériences ont eu lieu à Oberursel, près de Francfort/Main (Allemagne), et à Toulouse (France). De jeunes reines vierges (n = 1 300) âgées de 2 à 6 jours ont été testées. Le test biologique avait lieu dans une arène (Fig. 1). L'objet testé (reine entière vivante, fixée ou morte ou une partie du corps) était placé dans l'arène, puis une reine était introduite et son comportement enregistré durant 3 min. Le résultat du test était classé comme positif si une réaction de piqûre survenait durant les 3 min, autrement il était considéré comme négatif. Chaque reine n'était testée qu'une seule fois et servait ensuite d'objet testé qui, lui aussi, n'était utilisé qu'une seule fois.

Lorsque deux reines vivantes étaient introduites dans l'arène, nous n'avons pas noté de changement de comportement lorsque la distance entre les deux insectes était supérieure à 5 mm. A distance plus courte (<3,2 mm) la reine dirigeait ses antennes vers l'autre reine, le plus souvent vers la face ventrale de l'abdomen (les tergites). La reine se tournait alors vers sa rivale et le combat commençait (Fig. 2). Après élimination des stimuli visuels par un éclairage en lumière rouge, le comportement de piqûre s'est maintenu. Même des reines anesthésiées ou fraîchement mortes ont été piquées ; le mouvement n'est donc pas l'élément déclencheur. Ce n'est qu'en réduisant ou empêchant le contact, à l'aide de grilles de mailles différentes, que le comportement de piqûre s'est réduit ou a totalement disparu (Tab. I). Les tests avec des portions de corps de reines ont montré que le contact avec la face ventrale de l'abdomen suffit à déclencher le comportement de piqûre – Tab. II). Des nymphes de reines de 15 jours le déclenchent aussi (Fig. 3).

Nos résultats montrent que la phéromone qui déclenche le comportement de piqûre est localisé sur les tergites de l'abdomen de la reine et perçu par contact. L'isolement et l'identification de la substance active est en cours.

reine / comportement de combat / glande tergale / phéromone / test biologique

Zusammenfassung – Der Kampf zwischen jungen, unbegatteten Königinnen (*Apis mellifera*) wird durch Kontakte am dorsalen Abdomen eingeleitet. Der dramatische Kampf zwischen jungen Bienenköniginnen hat seit langer Zeit das Interesse von Forschern geweckt. Die Frage jedoch, welche Reize der Erkennung der Königin oder dem Auslösen des Kampfverhaltens dienen, ist bis heute nicht geklärt. So wird hier erneut ein experimenteller Einstieg unternommen zu klären, welche Reizmodalitäten bei der Königinnenerkennung involviert sind. Wir untersuchten das Verhalten von Königinnen bei ihren Kämpfen anhand von Videoaufnahmen und entwickelten einen Biotest, der uns erlaubte die entsprechenden Reize zu analysieren.

Die Versuche wurden in Oberursel und Toulouse durchgeführt. Die jungen unbegatteten Königinnen wurden im Alter von 2 bis 6 Tagen getestet. Insgesamt wurden ca. 1 300 Königinnen aufgezogen und getestet. Der Biotest wurde in einer Arena (Abb. 1) durchgeführt. Vor Beginn wurde das Testobjekt (lebende, fixierte, tote Königin oder ein Körperteil) in die Arena eingebracht. Dann wurde eine Testkönigin zugesetzt und deren Verhalten für 3 min aufgezeichnet. Wenn eine Stechreaktion auftrat wurde das Testergebnis als positiv gewertet und wenn während der 3 min kein Stechverhalten aufgezeichnet wurde, galt der Test als negativ. Königinnen wurden nur einmal als Testkönigin eingesetzt. Danach dienten sie als Testobjekte. Auch jedes Testobjekt wurde nur einmal verwendet.

Zwei junge Königinnen wurden in die Arena eingesetzt und deren Verhalten aufgenommen. Wir bemerkten keine Verhaltensänderungen, wenn die Distanz zwischen beiden Tieren mehr als 5 mm betrug. Erst auf ganz kurze Distanzen (<3,2 mm) wurden die Antennen auf die andere Königin, meist auf die Rückenseite des Abdomens, ausgerichtet. Die Königin stieß dann nach vorne und der Kampf wurde eingeleitet (Abb. 2). Nach Ausschaltung der visuellen Reize durch eine Beleuchtung mit

Rotlicht trat keine Beeinträchtigung des Stechverhaltens auf. Auch narkotisierte bzw. frisch tote Königinnen wurden gestochen. Demnach ist Bewegung kein notwendiger Auslöser für Stechverhalten. Erst durch eine Einschränkung bzw. Blockierung des Kontaktes (mit Hilfe von Drahtgittern unterschiedlicher Maschenweite) wurde das Stechverhalten vermindert bzw. ganz ausgeschaltet (Tab. I). Tests mit isolierten Körperteilen von Königinnen zeigten, dass der Kontakt zur dorsalen Rückendecke des Abdomens ausreicht um Stechverhalten auszulösen (Tab. II). Weiter wurde Stechverhalten schon von Königinnen (15 Tage alt) vor dem Schlupf aus den Zellen, direkt nach der abgeschlossenen Imaginalhäutung ausgelöst (Abb. 3).
Unsere Ergebnisse weisen darauf hin, dass das auslösende Pheromon für das Stechverhalten auf den Tergiten des Abdomens der Königin lokalisiert ist und durch Kontakt wahrgenommen wird. Die Isolation und Identifizierung der aktiven Substanz ist in Arbeit.

Königin / Kampfverhalten / Biotest / Tergitdrüse / Pheromon

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