

Search for Alternative Methods for Stimulating Bumblebee Queen of *Bombus terrestris* L. During Laboratory Rearing

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Abstract. Laboratory-reared queens need to be stimulated to oviposition by external stimuli. The most common method includes social stimulation by male cocoons, contact with a worker, and contact with another queen. In the first part of this study, the possibilities to replace a live cocoon with cocoon imitations and to increase success by adding smell stimulus of live cocoons were surveyed. Seventeen variants were tested in 4 experiments. The best results were achieved with stuffed male cocoons, but wool and polystyrene cocoons were also used. Although these cocoons were able to trigger continuous incubation behavior, not even a monthly incubation of these imitations initiated oviposition. The fact that incubation does not lead to laying eggs is a somewhat surprising finding which has not been published yet. It also seems that the addition of a smell stimulus fails to reinforce stimulation if the tactile and olfactory stimuli are not concentrated into a single object. In the second part, we surveyed whether the presence of a worker with prevented physical contact has the same stimulating effect on the neighboring queen as the presence of another queen. The presence of a worker without physical contact with the queen did not stimulate laying of eggs and, moreover, it was found that the presence of another queen has no stimulatory effect if the physical contact is prevented.

Key words: *Bombus terrestris*, laboratory rearing, stimulation, cocoon imitation, incubation.

INTRODUCTION

Bumblebees belong to the major pollinators of a number of plants (Kearns and Inouye, 1997; Sajjad *et al.*, 2008). Their ability to perfectly adapt to the limited space of greenhouses and plant-breeding cages is now a days widely utilized in the production of dozens of crops, *e.g.* tomatoes, peppers, cucumbers, soft fruits, stone fruits and seeds. Year-round demand on bumblebee colonies led to the worldwide emergence of laboratory rearings that mostly focus on the species of *Bombus terrestris* (Velthuis and Van Doorn, 2006).

Nests are established by the queen that usually needs stimulation to trigger the nesting instinct and oviposition in artificial rearing conditions.

To stimulate the queens, a method of 2 queens, which, however, led to aggressive behavior of the queens and to subsequent killing of one of them, or insertion of a bumblebee brood, most commonly a male cocoon, were used (Sladen, 1912; Alford, 1975; Duchateau *et al.*, 1994). A worker of

B. terrestris is also added to the queen of the same species as well as to the queens of some other closely related species of *B. hypocrita* and *B. ignitus* (Ono *et al.*, 1994; van Doorn in Velthuis and Van Doorn, 2006). According to Gretenkord and Drescher (1997), the most successful method of stimulation is adding a combination of larvae and workers. Tasei and Aupinel (1994) found that abiotic factors such as photoperiodic regime also influence oviposition. Beginning of brooding behaviour and oviposition were significantly faster in the L8:D16 regime compared with other tested regimes.

Ptáček (1983, 1985, 1991) described completely a new method when 3-7 freshly hatched worker honey bees (*Apis mellifera*) were placed close to the queen. This method proved the best with the species of *B. terrestris*. For other species surveyed (*B. lapidarius*, *B. lucorum*, *B. hypnorum*, *B. pratorum*, *B. pascuorum*, *B. hortorum*, and *B. ruderatus*), the queens laid eggs independent of the presence or absence of bees. However, Yoon *et al.* (1999) found that stimulation by *A. mellifera* has positive effect on the oviposition (timing, quantity of eggs) and size of the colonies of *Bombus ignitus*.

Röseler and Röseler (1984) found that CO₂ treatment influences corpora allata, induces oogenesis and inhibits formation of reserves for

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hibernation. That is the reason why narcotization by CO₂ can be used to wake up the queen of hibernation earlier than it is natural for her and also acts as a stimulus for nesting.

Katayama and Ochiai (1988) and Yoneda (2008) found that queens can be stimulated even by old dead male cocoons. Kwon *et al.* (2003) nevertheless, reported that the highest stimulatory effect is provided by cocoons not older than two days and that the orientation of the cocoon has an effect on the number of individuals of the first generation as well as on the subsequent development of the nest.

Without stimulation, queens of the buff-tailed bumblebee reared in a laboratory, nest very rarely. Přidal and Hofbauer (1998) believe that the need of stimulation and a longer period of activation of laboratory-reared queens are caused by incomplete awakening from hibernation.

Similarly, according to our own experience we know that the addition of stimulatory material (either another individual or brood) is not necessary and the queen can lay eggs on the pad or on the feeder. However, the addition of a cocoon usually significantly increases the success rate of nest establishment and decreases the period of time a queen needs to nest. The reason why the cocoons stimulate the queen so effectively is not exactly understood. It is believed that the shape, smell, and temperature of the cocoon may play an important role. Laying eggs is mostly preceded by incubation which is usually a signal that the queen has adopted the incubated object. In this phase, the queen lies on the cocoon or around the feeder and moves her abdomen in a rhythmical manner to produce heat. The maintenance of a high brood temperature would permit the more rapid development of the poikilothermic eggs, larvae and pupae (Heinrich, 1972b). Přidal and Hofbauer (1998) found that queens that had already had an egg cell, but destroyed it, accept the given cocoon faster than the first one.

All of the above described methods of stimulation (excluding CO₂ stimulation) require a bumblebee colony in an advanced stage of development, from which the cocoons or imagines can be removed, or a bee hive, which can be problematic due to the seasonality of bees and

bumblebees. Likewise, transporting individuals or broods between nests carries the risk of infection with it. In this article, we publish the results of testing several types of materials and smell stimuli in order to stimulate the laboratory-reared queens of *Bombus terrestris*.

MATERIALS AND METHODS

In the experiments, fertilized queens *Bombus terrestris* from laboratory rearing after 3-6 month-long hibernation in a refrigerator (4±3°C) were used. The experiments were carried out from the 3rd to the 6th day of awakening from hibernation. Until this time they were left together in rearing boxes at room with natural light in 20°C, 50% RH and they were supplied with sugar syrup. The queens were kept under standard laboratory conditions (darkness, 27°C, 60 % RH), had available pollen and sugar syrup *ad libitum*.

All experimental data were categorical. Fisher's exact test was used for comparing individual groups in 2 x 2 contingency tables. Whenever more than two groups were compared significance was at first tested with Chi-square test. All of the statistical analyses were performed using Statistica 10 Software (StatSoft Inc., 2011).

Experiment No. 1 - Cocoon material

In this experiment 25 queens were offered artificial male cocoons made of sheep's wool, sheep's wool which had absorbed the nest smell, sheep's wool which was coated by wax, sheep's wool from another queen, plasticine, Fimo® (modeling clay), putty eraser, bumblebee male cocoons out of which larva or pupa was removed and which were stuffed with sheep's wool (Table I). Before the experiment, several woolen cocoons were placed in a nest for 2.5 days to absorb the smell of the nest and other woolen cocoons were coated with melted bumblebee wax. The queens had generally available 2-3 types of material in the rearing box. This combination of cocoon materials was on a random basis, and since it is not probable that the presence of a particular type of cocoon influenced incubation or establishing egg cells on another type of cocoon, we do not consider necessary to define all the combinations. The

number of individual types of cocoons is given in Table I. Three weeks later, stimulation by these cocoons was terminated and the queens were provided with live male cocoons to make sure that queens are capable of oviposition under standard conditions.

Table I.- Recorded incubation of cocoons from various materials (n = number of tested queens; m = number of tested cocoons - one queen had 3 different types of cocoons available).

Experiment No. 1 (n=25)	No. of incubated cocoons
Wool (m=10)	0
Wool which had absorbed the nest smell (m=10)	1
Wool which was coated by wax (m=10)	1
Wool from another queen (m=1)	1
Plasticine (m=10)	0
Fimo® (m=10)	0
Putty eraser (m=10)	0
Male stuffed cocoons (m=10)	4

Table II.- Recorded incubation of foam and polystyrene cocoons and number of them used for oviposition (n = number of tested queens).

Experiment No. 2 (n=15)	No. of incubated cocoons	No. of cocoons used for oviposition
Foam	0	0
Polystyrene	9	1

Experiment No. 2 - Foam and polystyrene cocoons

In this experiment 15 queens were offered artificial male cocoons made of polystyrene and foam elements. Size and shape of the artificial male cocoons were comparable with average *B. terrestris* male cocoons. 3 weeks later, stimulation by these cocoons was terminated and the queens were provided with live male cocoons to make sure that queens are capable of oviposition under standard conditions.

With respect to time sequence, this experiment was performed as the last one of all the experiments.

Experiment No. 3 - Stimulatory effect of brood smell

Based on the results of experiment No. 1, in

this experiment I focused on stuffed cocoons. The variants are summarized in Table III. On the 4th day after activation, 3 queens (M1-M3) were offered male cocoons stuffed with sheep's wool. Three queens (N1-N3) were offered male cocoons stuffed with sheep's wool attached to a grid under which two live male cocoons were placed. Four queens (O1-O4) were offered stuffed cocoons from the experiment focusing on monitoring the material preferences, which had been previously incubated by another queen. The cocoons were attached to a grid below which there was an increased number of living male cocoons, namely 6 to 7.

After 6 days, the live cocoons for N and O queens were replaced by fresher living ones. Four queens (P1-P4) had 2 freshly stuffed cocoons and 7-8 live male cocoons under the grid. Three queens (Q1-Q3) had only 7-8 live cocoons in a cage and no cocoons elsewhere. Three queens (R1-R3) had only used stuffed cocoons with direct access. After 7 days, the live cocoons for P and Q queens were replaced by fresher ones. A control group of eight queens was offered live male cocoons.

Thirty days later, stimulation by these cocoons was terminated and the queens were provided with live male cocoons to make sure that queens are capable of oviposition under standard conditions.

Experiment No. 4 - Stimulatory effect of another female's smell in combination with Fimo®

In pairs, 30 queens (S) were placed in 15 jars, size 270x190x80 mm (l.w.h.), which were partitioned in the middle by a double plastic grid. This grid enabled smell, sound, and visual contact, but prevented physical contact.

Another 15 (T) queens were placed in a jar as described above, but instead of the other queen there was a one-day-old worker placed in the other half of the jar.

Control group: 15 queens were placed in a jar, size 170x130x750 mm, which was approximately the same area as each female in the separated pairs.

All queens had also available cocoons made of Fimo®.

Three weeks later, this method of stimulation was terminated and the queens were provided with

Table III. Summary of stimulatory variants, incubation, and oviposition (n = number of tested queens).

Experiment No. 3	Stuffed male cocoons	2 live cocoons under the grid	7-8 live cocoons under the grid	incubation	oviposition	egg cell on the pad
M – stuffed male cocoons (n = 3)	Yes			3	2	0
N – stuffed male cocoons + 2 live cocoons under the grid (n = 3)	Yes	Yes		2	1	0
O – used stuffed male cocoons + 7-8 live cocoons under the grid (n = 4)	Yes		Yes	0	0	2
P – stuffed male cocoons + 7-8 under the grid (n=4)	Yes		Yes	4	0	1
Q – 7-8 live cocoons under the grid (n = 3)			Yes	0	0	0
R – used stuffed male cocoons (n = 3)	Yes			0	0	0
Control – live male cocoons (n = 8)				8	8	

Table IV.- Overview of incubation and oviposition in stimulating by smell of another female.

Experiment No. 4	number of incubated Fimo® cocoons	number of Fimo® cocoons used for oviposition	number of egg cells (with eggs) established on the pad
S – queen-queen pair (30 queens, n = 15)	0	0	1
T – queen-worker pair (15 queens, n = 15)	0	1	2
Control – queen alone (15 queens, n = 15)	0	1	2

live male cocoons to make sure that queens are capable of oviposition under standard conditions,.

Experiment No. 5 - Frozen cocoons

Four queens were offered male cocoons that were frozen at -18°C for 24 hours before the application. Seven days later, stimulation by these cocoons was terminated and these queens were provided with live male cocoons to make sure that queens are capable of oviposition under standard conditions.

RESULTS

Cocoon material

The number of material types used for the production of cocoons and their incubation by queens is shown in Table I.

A queen having a woolen cocoon died on the 5th day of activation, but there was no incubation recorded. This cocoon was given to a queen that already had a cocoon made of plasticine, but showed no interest in it. Several hours after the wool cocoon was inserted, incubation of this cocoon by

the queen was recorded.

On the 9th day, incubations of woolen cocoon that was previously placed in a nest and woolen cocoon coated by wax coatings of larvae were recorded.

In 4 cases, incubation of stuffed male cocoon was recorded.

In general statistical evaluation, a significant difference ($\chi^2 = 16.1252$, d.f. = 7, $p = 0.024$) between the groups (undoubtedly caused by the group with stuffed cocoons) was recorded. Nevertheless, when comparing individual groups, no significance was detected.

Foam and polystyrene cocoons

Of the 15 queens, 9 incubated a polystyrene cocoon. Only one, however, established an egg cell on it after 20 days (Table II). The remaining 14 queens established egg cells on live cocoons of *B. terrestris* some 8 days after testing the artificial cocoons. The Fisher's exact test results showed that the polystyrene cocoons were incubated significantly more frequently ($p = 0.0007$). However, in the case of egg cell establishment, the

difference completely disappeared ($p = 1$).

Stimulatory effect of brood smell

Overview of variants, incubation and oviposition is shown in Table III.

From the first day, all queens (M1 and M3) incubated the stuffed male cocoons and two of them laid eggs on the 5th day.

Queens N1 and N2 incubated the stuffed male cocoons from the 1st day, but only one of them established a nest on the 5th day. Queen N3 did neither incubate the stuffed male cocoons, nor establish a nest during the experiment duration.

Queens O1-O4 did not re-incubate the used stuffed male cocoons. On the 4th and 7th day after the exchange of live cocoons for fresh ones, however, two queens established nests on the pad.

One queen of the Q group, which only had live cocoons in the cage, managed to get into the cage and established a living egg cell on the cocoons. This queen was excluded from the experiment. One queen of the P group, which had available both, stuffed as well as live cocoons, established a nest on the pad on the 11th day.

Except for one (group R), all other queens that had available stuffed cocoons, either alone or in combination with live cocoons, incubated the cocoons, but did not establish nests.

In the control group, all queens incubated their cocoons and established nests on average on the 12th ($\sigma = 4.1$) of the day of activation.

Although the numbers in individual variants are too small for statistical comparison, I believe they have some explanatory value. For statistical purposes, incubation and egg cell establishment of the queens with stuffed cocoons ($n = 10$), queens with used stuffed cocoons ($n = 7$), and queens with live cocoons ($n = 8$) were compared, regardless of the type of variant (such as the presence of live cocoons). There was a significant difference found between incubation of stuffed cocoons and used stuffed cocoons ($p = 0.0004$), however, there was no significant difference found in incubation of stuffed cocoons and live cocoons. In contrast, with regard to establishing egg cells, only live cocoons differed significantly from stuffed cocoons ($p = 0.0128$) and used stuffed cocoons ($p = 0.007$).

Stimulatory effect of another female's smell in combination with Fimo®

Count of establishing egg cells in different variants is presented in Table IV.

Of the 30 queens placed in pairs, one queen established an egg cell on the container with sugar syrup after 20 days. After the termination of the experiment, the remaining 29 queens established egg cells on live cocoons after an average of 7 days.

Of the 15 queens in pairs with workers, one queen established an egg cell after 10 days on the cocoon made of Fimo® and 2 queens established egg cells on the pad after 4 and 6 days. Four workers established egg cells on the pad as well. Of the above, one worker established this egg cell 15 days after the queen's egg cell in the same pair. After the experiment, queens established egg cells on live cocoons after an average of 4 days.

In the control group, one queen established an egg cell on the Fimo® cocoon after 11 days and 2 queens on the pad after 4 and 5 days. The remaining queens established egg cells on average after four days of the end of stimulation on live cocoons of *B. terrestris*. No significant difference was detected between the groups with regard to establishing egg cells ($p \geq 0.05$).

Frozen cocoons

During seven days, neither incubation nor oviposition was recorded and the experiment was terminated.

DISCUSSION

In general, the presence of bumblebee larvae or cocoon stimulates bumblebees (queens, workers and young males) to incubate them (Cameron, 1985). It is instinctive behavior conditioned by the fact, that the brood needs higher temperature for its development (Heinrich, 1972a,b). Not always, however, are cocoons incubated. As already mentioned, older cocoons stimulate less than fresh cocoons, as well as cocoons with dead larvae or pupae (Kwon *et al.*, 2003). The ability to detect a dead larva or pupa may, like hygienic behavior in honeybees, produce an evolutionary advantage (Wilson-Rich *et al.*, 2009). As with honey bees, this

ability may be genetically conditioned in bumblebees and a wide range of phenotypes may occur in the population. Incubation of inanimate objects of feeders with pollen or sugar syrup could then be explained by a lower ability to recognize the dead brood in some specimens and a lower threshold for the start of incubation behavior (round shape is enough).

In the first and second experiment which tested the effect of shape and material, only cocoons made of wool which had been previously in contact with bumblebee smell, a polystyrene cocoon, and a male cocoon stuffed with wool were incubated. Whereas, the latter two significantly more often. Except the shape, the ability to maintain warmth and softness of the material, the cocoons which were incubated had, in particular, smell characteristics in common.

Therefore, the stimulatory effect of live cocoons' smell was surveyed in the third experiment. Table III demonstrates that even the age of the stuffed cocoons had a significant effect on their incubation. Older stuffed cocoons were not incubated even though the queen was simultaneously exposed to the smell of live cocoons. It seems likely that it was caused by either losing their smell or, on the contrary, by their gradual decomposition associated with release of specific substances.

Incubation behavior in females usually precedes and accelerates the commencement of laying eggs (Přidal and Hofbauer, 1998). However, is it the trigger? The performed experiments indicate that it is not.

Although the queens incubated artificial or stuffed cocoons readily, the lasting incubation led to oviposition only in exceptional cases. Even though they were also exposed to the smell of live cocoons (and demonstrably perceived them, which was proved by their efforts to access them through the grid), it was not resembled in the increase in laying eggs (Table III). However, after the experimental stimulation was terminated and the queens were provided with a live cocoon, they began to lay eggs.

In addition to the stimulatory effect of a brood, a stimulatory effect of another female – queen or worker – is also known (Sladen, 1912). In both cases, however, fighting usually occurs, which

results in the fact that only the stronger one can lay eggs. This problem was solved by Ptáček *et al.* (2000) who placed the queens together in one container, but separated them by a double grid from each other so that they cannot kill each other. The essence of stimulation by another queen or worker is not known. It is possible that the company of another female triggers the same behavior in the queen as in the queen that, for example, has lost her nest in nature. In this case, the queen usually tries to find another nest in which she fights with the domestic queen. Since even the workers are able to lay eggs and inhibit accompanying females if they are dominant, I wondered whether the method of two queens will also be effective if the second queen is replaced by a worker.

From the results it is obvious that the worker has no stimulatory effect on the queen if separated by a double barrier. Surprisingly, however, neither a separated pair of queens stimulated each other. So it seems that the results of this study are inconsistent with the method published by Ptáček *et al.* (2000) and in agreement with the experiments performed by Přidal and Hofbauer (1998). The explanation is probably the fact that Ptáček included in his experiment also queens collected outside able to nest more easily than queens reared in a laboratory. Different behavior can then be caused by either different physiological readiness for the establishment of a nest or a different reaction to the smell of another female. Nevertheless, as already mentioned, if the pair of queens or a queen and a worker is allowed physical contact, it leads to stimulation to oviposition. As previously discovered, when some workers are denied physical contact with the queen in the early stages of nest, the queen loses her dominance over them and workers' ovaries mature (Alaux *et al.*, 2007).

The performed experiments show that stimulation of queen bumblebees is more complicated than it might be seen. Did some factor prevent them from starting laying eggs, or was a key stimulus missing, or is a summation of stimuli needed? If summation is necessary, what is the contribution of individual factors (shape, material, physical properties, smell, tactile contact) to start laying eggs? And how important it is to have concentrated all these factors in a single object? To

answer these interesting questions, however, further studies focused on extensive stimuli testing are necessary.

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