# Some factors influencing the development of conditioned reflex to queenbee pheromone in worker honeybees (*Apis mellifera carnica* Pollm.)

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The following factors are important for the conditioning of honeybees:

a) conditions in which bees are kept: the conditioning reflex in bees from a colony with the mated and egg-laying queen developed better than in bees from a thermostat without queen, in which they were kept for 4 days;

b) dose of the conditioning stimulus (queenbee extract): while reducing the dose of the extract, the number of conditioned worker bees decreases too; when the doses of the queenbee extract are higher, the conditioning is better;

c) order of offering the conditioning and unconditioning stimuli: the strongest relationship between the conditioning and unconditioning stimuli is formed when the conditioning stimulus precedes the unconditioning stimulus and the interval between the stimuli is in the range of a few seconds; when the conditioning stimulus follows the unconditioning stimulus, the relationship between the stimuli is very poor and does not depend on the interval between them.

**Key words.** *Apis mellifera carnica* Pollm., olfactory reflex, conditioning, conditioning stimulus, unconditioning stimulus, relationship, queenbee extract

## INTRODUCTION

The incredible ability of honeybees to learn has been known already for a long time. The first studies into the training of conditioned reflexes were usually carried out with freely flying honeybees [5, 6, 8]. However, some researchers investigated the conditioned reflex of the extension of the proboscis [14, 16, 17].

Honeybees acquire strong conditioned reflexes, which are far stronger than those of fish or rats and are equal to the conditioned reflexes of apes [15]. Honeybees easily associate odours with food (sucrose) [5]. To cause the reaction of the extension of the proboscis in 30–80% of worker honeybees it is enough to provide them once with the conditioning and unconditioning stimuli [1, 16].

The ability of honeybees to learn in a laboratory with limited possibilities of movement allows us to control easily the procedure of the experiment, which creates good conditions for the use of different stimuli [1, 2]. This also enables us to exercise stricter control over the time and sequence of the applied stimuli [9] and to make other measurements, which are impossible to make when honeybees are flying.

There are data showing that honeybees can well distinguish between the queenbee pheromone and other odours [17]. However, we found no data on factors influencing the training of the conditioned reflex to this biologically active material. The aim of this study was to determine the influence of the conditions, in which worker honeybees are kept prior to conditioning, the dose of the queen bee extract as a conditioning stimulus, the order of the onset between the conditioning and unconditioning stimuli on the training of the conditioned reflex to queen bee pheromones in worker honeybees.

## MATERIALS AND METHODS

Worker honeybees *Apis mellifera carnica* Pollm. were studied. The objects of the research were:

a) conditions in which bees were kept;

b) doses of the queen bee extract from $1 \cdot 10^{-17}$ to $1 \cdot 10^{-3}$ queen's equivalent (Qeq); in 1 Qeq were 100 μg *E*-9-oxo-2-decenoic acid (9-ODR);

c) order of the onset of the conditioning and unconditioning stimuli.

### *Investigations of the conditions in which bees were kept*

Worker honeybees were kept in a bee colony with the mated and egg-laying queen and in small cages located in a thermostat without queen for 2–4 days.

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In one cage, from 30 to 50 bees were kept. Due to bad weather conditions, the bee colony was fed on 50% sugar solution. In the thermostat, the temperature was +30 °C; the bees were fed on candy and received water constantly.

The ethanol extract of mated bee queens equal to 0.001 Qeq was used as a conditioning stimulus. The study was carried out in July–August 1998. Foraging conditions were normal during the whole period of studies. The room temperature was +20 °C. On the whole, 78 worker bees were investigated.

*Investigations of the queen extract doses*

The bees were taken away from the bee colony with the mated and egg-laying queen. As a conditioning stimulus, we used different doses of the queen ethanol extract solutions calibrated by *E*-9-oxo-2-decenoic acid ($10^{-17}$, $10^{-16}$, $10^{-15}$, $10^{-14}$, $10^{-13}$, $10^{-11}$, $10^{-9}$, $10^{-7}$, $10^{-5}$, $10^{-4}$ and $10^{-3}$ Qeq). The study was carried out in July–August 1998. Foraging conditions were normal during the whole period of research. The room temperature was +20 °C. The total number of investigated worker bees was 484.

*Investigations of the order of the onset of conditioning and unconditioning stimuli*

The bees were taken away from the bee colony with the queen. As a conditioning stimulus, we used the ethanol extract of mated bee queens, equal to 0.001 Qeq, whereas 50% sugar solution was used as a reward.

The conditioning and unconditioning stimuli were offered in different sequences and at different time of the onset of stimuli: a) unconditionning stimulus was delivered 5, 8, 10, 12 and 20 s after the onset of the conditioning stimulus; b) conditioning stimulus was delivered 3, 5, 7 and 15 seconds after the onset of the conditioning stimulus. The test trials were carried out 5 min later after conditioning. The conditioned reflex was considered to be formed, if the bee put out its proboscis at least twice after the conditioning stimulus had been presented thrice.

The study was carried out in September–October 2000. The foraging conditions were poor during the collection of these samples. The room temperature was maintained between +16 °C and +18 °C. The total number of investigated bees was 429.

*Conditioning*

In all experiments, the conditioned reflex was trained by the earlier described methods [12]. The worker honeybees were caught at the entrance to their hive. Then they were cooled briefly to immobilisation. The wings of the motionless bees were squeezed with special clips, by means of which they were hanged on a test stand. The bees warmed up in 30 min. Then the conditioned reflex was started to train. When training a bee, a glass rod was brought up to its antennae, on which 0.01 ml of queen extract (0.001 Qeq) and a drop of sugar solution were placed.

For a bee to smell the extract, the stick was kept close to the antennae for 5 s. Then the bee's antennae were touched with sugar solution in order to make it to extend reflectorily its proboscis. A reward lasted no longer than 1 second. When the bee extend its proboscis just to smell the queen extract (a conditioning stimulus), the conditioned reflex was considered to be formed. The number of conditioning stimuli necessary to form the conditioned reflex was from 1 to 10.

Up to fifteen individuals could be trained and tested simultaneously.

*Data analysis*

The obtained results were evaluated statistically [7]. All the data are presented as percentages of individuals that responded with the proboscis extension to the conditioning stimulus (N%). For each experimental group, the mean (N) of the variant and its standard error ($\pm SE_N$) were calculated. For comparison of the data between groups, the differences between the means (DM) of variants and their standard errors ($\pm SE_{DM}$) were calculated. The data were analysed using the t test or $\chi^2$ test. The results of the statistical analysis were considered significant when $P < 0.05$.

## RESULTS AND DISCUSSION

The study results have shown that the keeping conditions of honeybees, the dose of the queen bee extract and the order of the onset of the conditioning and unconditioning stimuli are important factors in the formation of a conditioned reflex.

*Effect of the conditions in which bees were kept prior the conditioning*

Worker bees from the bee colony (first group, n = = 58) and bees from the thermostat (second group, n = 20) showed different behaviour (Fig. 1).

In the first group, the conditioned reflex was successfully formed in 93.2 ± 4.38% of worker bees, and in the second group it was formed only in 10.0% (only 2 of 20 individuals had learned), *i. e.* by 83.2 ± ± 10.92% less ($P < 0.0001$).

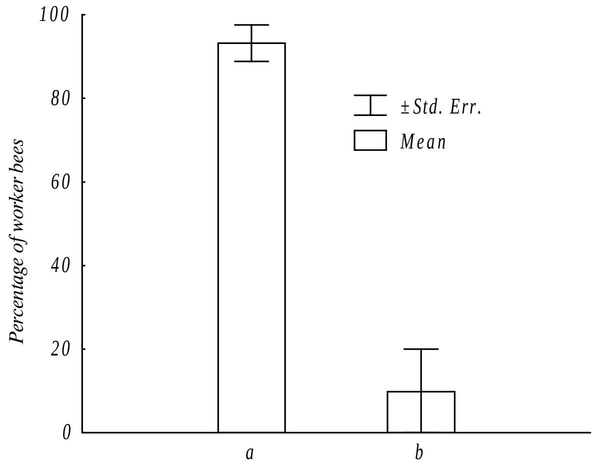


Fig. 1. The influence of the conditions in which worker honeybees (*Apis mellifera carnica* Pollm.) were kept on the training of the conditioned reflex to bee queen extract (0.001 Qeq):
*a* – bees from a colony with the mated and egg-laying queen (n = 58); *b* – bees kept in a thermostat without queen for 4 days (n = 20); N is the percentage of worker bees.

Living conditions of honeybees in the cage in a thermostat differ from those in the bee colony. The caged worker bees cannot participate in the bee colony's life [13]. In addition, the feeding of a bee also changes and its honey bag considerably increases [3, 4]. Additional studies are necessary to elucidate what changed the conditioning of bees kept in the thermostat. The possible reasons for that may be as follows: nourishment, isolation from the bee colony, limited motion, etc.

*Effect of the dose of the bee queen extract on the conditioning*

In this experiment we used 11 groups of worker bees. From 4 to 5 repetitions were carried out in a group. For each group of animals, the conditioned reflex to a different dose of the bee queen extract was developed (Table 1).

The results of research have shown that the dose of the queen extract as a conditioning stimulus is important for the conditioning. With the reduction of the dose extract, the number of the trained bees also decreases (Fig. 2).

As it was possible to expect, the best conditioning was obtained with the highest doses of the queen bee extract ($10^{-3}$–$10^{-5}$ Qeq). Applying these doses, it was possible to develop the conditioned reflex in 86.8 ± 2.29% to 93.7 ± 2.71% of animals (n = 172).

With reduction of the dose of the extract to $10^{-7}$ Qeq, it was possible to form the conditioned reflex in 79.8 ± 2.23% of worker bees (n = 59), *i.e.* by 13.9 ± 3.51% less than to the dose of the extract $10^{-5}$ Qeq (P = 0.0067). While applying the

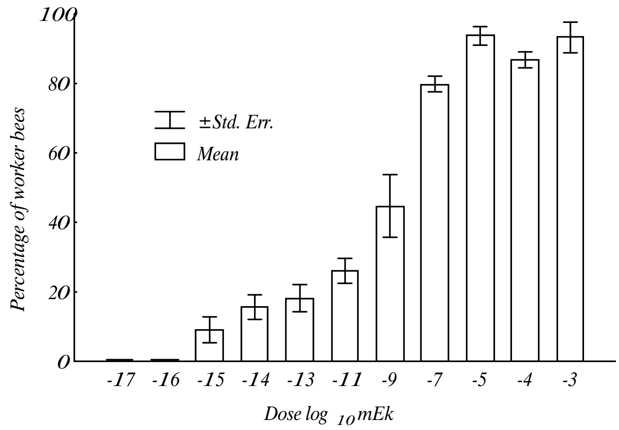


Fig. 2. The influence of the dose of a conditioning stimulus (queen extract) on the training of the conditioned reflex to the bee queen extract in worker honeybees (*Apis mellifera carnica* Pollm.) (n = 484): *N* – percentage of worker bees

Table 1. **The influence of the dose as a conditioning stimulus (CS) on training the conditioned reflex to the queen bee pheromone in worker bees (*Apis mellifera carnica* Pollm.)**

| No. | Dose of CS | Number of bees studied, | Number of bees learned | | Difference between averages, DM | | |
|---|---|---|---|---|---|---|---|
| | | | Units | n(%) ± $SE_N$ | No. | DM ± $SED_M$ | P |
| 1 | $10^{-3}$ | 58 | 54 | 93.2 ± 4.39 | – | – | – |
| 2 | $10^{-4}$ | 54 | 47 | 86.8 ± 2.29 | 1–2 | 6.4 ± 4.95 | 0.2684 |
| 3 | $10^{-5}$ | 60 | 56 | 93.7 ± 2.71 | 2–3 | 6.9 ± 3.55 | 0.1019 |
| 4 | $10^{-7}$ | 59 | 47 | 79.8 ± 2.23 | 3–4 | 13.9 ± 3.51 | 0.0067 |
| 5 | $10^{-9}$ | 66 | 31 | 44.8 ± 9.01 | 4–5 | 35.0 ± 9.28 | 0.0119 |
| 6 | $10^{-11}$ | 63 | 17 | 26.1 ± 3.56 | 5–6 | 18.7 ± 9.69 | 0.0898 |
| 7 | $10^{-13}$ | 59 | 10 | 18.2 ± 3.88 | 6–7 | 7.9 ± 5.27 | 0.1728 |
| 8 | $10^{-14}$ | 52 | 8 | 15.6 ± 3.56 | 7–8 | 2.6 ± 5.27 | 0.6509 |
| 9 | $10^{-15}$ | 62 | 6 | 9.1 ± 3.74 | 8–9 | 6.5 ± 5.16 | 0.2542 |
| 10 | $10^{-16}$ | 46 | 0 | 0.0 ± 0.00 | 9–10 | 9.1 ± 3.74 | 0.0698 |
| 11 | $10^{-17}$ | 57 | 0 | 0.0 ± 0.00 | 10–11 | 0.0 ± 0.00 | – |

dose $10^{-9}$ Qeq, the conditioned reflex was successfully formed only in 44.8 ± 9.01% of individuals (n = 66) *i.e.* it was by 35.0 ± 9.28% less than using the dose $10^{-7}$ Qeq (P = 0.0119). The least dose by which it was possible to train the conditioned reflex was $10^{-15}$ Qeq. However, such bees made only 9.1 ± 3.74% (n = 62).

While further reducing the dose of the queen extract, the bees failed to develop the conditioned reflex to the olfactory stimulus (n = 103). However, in each repetition there were some bees which put out their proboscis as soon as the stick with the odour was brought to their antennae. These insects responded also to the control stick without the odour. The relative (50% of individuals) and the absolute thresholds of the sensitivity of worker bees' pheromone receptors, which were defined applying the method of electroantennogram [11], are much higher than those obtained by the method of conditioned reflexes.

It might be that the conditioning to such low doses of queen extract is related not only to the olfaction of the bees. It is possible that vision also takes part in this process when the olfactory stimuli become weaker. Thus, the results of our study compete with the data of the research on linalool demonstrating that the conditioning increased with an increase in the concentration of odorants [10].

*The effect of the order of the outset of the conditioning and unconditioning stimuli on the conditioning*

The obtained results have shown that the strength of the relation between the conditioning and unconditioning stimuli depends on the order of the onset of the stimuli.

Two groups of worker bees were investigated. These groups differed in the order of the onest of the conditioning and unconditioning stimuli (Table 2). The group to which the conditioning stimulus was presented before the unconditioning one differed significantly from the group to which the conditioning stimulus was presented after the unconditioning one differed significantly from the group to which the conditioning stimulus was presented after the unconditioned stimulus ($\chi^2$ = 954.67; P < 0.00001) (Fig. 3 A, B).

The largest number of individuals (90.4 ± 2.88% of worker bees (n = 51)) that developed the conditioned reflex was registered when the conditioning stimulus was offered 5 s before the onset of the unconditionuse stimulus (Fig. 3 A). Therefore, we can maintain that the relationship between stimuli in this case is the strongest. When the unconditioning stimulus was presented 3 s later, *i.e.* 8 seconds after the onset of the conditioned stimulus, the number of individuals that developed the conditioned reflex decreased to 67.9 ± 5.70% (n = 46). It was by 22.45 ± 6.387% less (P = 0.0079). When the period between the onsets of the stimuli is longer, the relationship between the conditioning and unconditioning stimuli also becomes weaker. Therefore the number of bees that develop the conditioned reflex decreases, too.

When the unconditioning stimulus was offered 8 to 12 s after the conditioning stimulus, the ability of a honeybee to learn was similar (P > 0.05). When this interval became prolonged to 20 s, the number of the individuals that learned decreased to 21.7 ± ± 3.12%, *i.e.* it was by 28.3 ± 5.70% less than after the interval of 12 s (P = 0.0011).

The conditioning reflex to queen bee extract developed in 6.4 ± 4.39% to 20.7 ± 2.57% of worker bees, when the onset of the conditioning stimulus followed the onset of the unconditioning stimulus.

Table 2. **The influence of the order of the onset of the conditioning (CS) and unconditioning (US) stimuli on the conditioning of worker honeybees (*Apis mellifera carnica* Pollm.) to the bee queen extract**

| No. | Time between the onset of CS and US (s) | Number of bees studied, N | Number of bees learned | | Difference between averages, DM | | |
|---|---|---|---|---|---|---|---|
| | | | Units | N (%) ± $SE_N$ | No. | DM ± $SE_{DM}$ | P |
| | | | *CS presented before US* | | | | |
| 1 | 5 | 51 | 46 | 90.4 ± 2.88 | – | – | – |
| 2 | 8 | 46 | 31 | 67.9 ± 5.70 | 1–2 | 22.5 ± 6.39 | 0.0079 |
| 3 | 10 | 50 | 30 | 60.0 ± 5.48 | 2–3 | 7.9 ± 7.90 | 0.3461 |
| 4 | 12 | 48 | 24 | 49.9 ± 4.77 | 3–4 | 10.1 ± 7.26 | 0.2027 |
| 5 | 20 | 46 | 10 | 21.7 ± 3.12 | 4–5 | 28.3 ± 5.70 | 0.0011 |
| | | | *CS presented after US* | | | | |
| 6 | 15 | 47 | 3 | 6.4 ± 4.39 | 5–6 | 15.2 ± 5.39 | 0.0256 |
| 7 | 7 | 49 | 6 | 12.8 ± 4.39 | 6–7 | 6.4 ± 6.21 | 0.3350 |
| 8 | 5 | 48 | 6 | 12.3 ± 4.89 | 7–8 | 0.6 ± 6.57 | 0.9347 |
| 9 | 3 | 44 | 9 | 20.7 ± 2.57 | 8–9 | 8.4 ± 5.52 | 0.1664 |

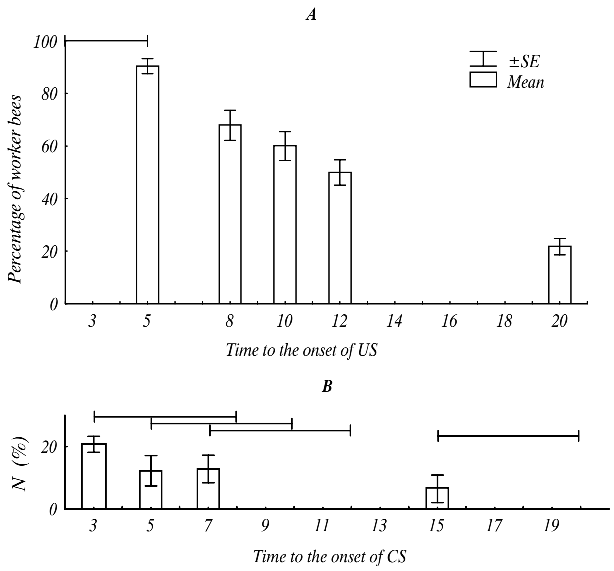


Fig. 3. The influence of the order of onsets of conditioning (CS) and unconditioning (US) stimuli on the conditioning of worker honeybees (*Apis mellifera carnica* Pollm.) to bee queen pheromone (n = 429): *A* – onset of CS preceding the onset of US by interval shown in the X axis; *B* – onset of CS following the onset of US by interval shown in the X axis; continuous lines at the top mean the time of stimulation by CS (5 s); *N* – percentage of worker bees

So, a relationship between the stimuli is very poor and it does not depend on the interval between the onsets of the stimuli ($P > 0.05$) (Fig. 3 B).

While comparing the results of research on geranial [9] with our results obtained during the research on the queenbee extract, no essential differences have been found. We can state that the strongest relationship between the conditioning and unconditioning stimuli was generated when the onset of the conditioning stimulus preceded the onset of the unconditioning stimulus and the interval between them was in a range of a few seconds. Besides, this relationship does not depend on the value of a substance in the life of honeybee colony.

## CONCLUSIONS

The following factors are important for the conditioning of honeybees:

a) conditions in which bees are kept: the conditioned reflex in bees from a colony with the mated and egg-laying queen develops better than in bees from a thermostat without the queen, in which they were kept for 4 days;

b) dose of the conditioning stimulus (queenbee extract): while reducing the dose of the extract, the number of conditioned worker bees decreases, too; when the doses of the queenbee extract are higher, the conditioning is better;

c) order of offering the conditioning and unconditioning stimuli: the strongest relationship between the stimuli is formed when the conditioning stimulus is offered before the unconditioning stimulus and the interval between the onset of stimuli is in the range of a few seconds; when the conditioning stimulus followed the unconditioning stimulus, the relationship between the stimuli was very poor and did not depend on the interval between the stimuli.

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### References

1. Bitterman M. E., Menzel R., Fietz A., Schäfer S. Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *J. Comp. Physiol*. 1983. No. 97(2). P. 107–119.
2. Erber J. The search for neural correlates of learning in the honeybee. *Neuroethol. Behav. Physiology*. Berlin–Heidelberg–New York–Tokyo. 1983. P. 216–227.
3. Еськов Е. К. *Экология медоносной пчелы*. Рязань, 1995.
4. Еськов Е. К., Бабкина Н. Г. *Возрастная и сезонная динамика некоторых физиологических показателей, характеризующих устойчивость медоносной пчелы к гипо- и гипертермии*. Рязань: ВИНИТИ, 1995. P. 1–30.
5. Frisch K. von. *Tanzsprache und Orientierung der Bienen*. Berlin–Heidelberg–New York, 1965.
6. Koltermann R. Lern- und Vergessenprozesse bei der Honigbiene aufgezeigt anhand von Duftdressuren. *Z. vergl. Physiol*. 1969. No. 63. P. 310–334.
7. Лакин Г. Ф. *Биометрия*. Москва, 1990.
8. Menzel R. Das Gedächtnis der Honigbiene für Spektralfarben. II. Umlernen und Mehrfach lernen. *Z. vergl. Physiol*. 1969. No. 63. P. 290–309.
9. Menzel R., Bitterman M. E. Learning by honeybees in an unnatural situation. Huber F., Markl H. (ed.) *Neuroethol. Behav. Physiol*. Berlin–Heidelberg–New York–Tokyo, 1983. P. 206–215.
10. Pelz C., Gerber B., Menzel R. Odorant intensity as a determinant for olfactory conditioning in honeybees: ro-

les in discrimination, overshadowing and memory consolidation. *J. Exper. Biol*. 1997. No. 200(4). P. 837–847.
11. Skirkevičienė Z. The role of the presence of the bee queen (*Apis mellifera* L.) in the colony in the sensitivity of worker bee olfactory receptors to the queen's pheromones. *Pheromones*. 1991. No. 1(1–4). P. 65–72.
12. Skirkevičius A., Blažytė L. The effect of keeping conditions on the formation of conditioned reflex to queen bee pheromones in worker honeybees *Apis mellifera carnica* Pollm. *Pheromones*. 1996. No. 6. P. 33–38.
13. Skirkevičius A., Blažytė L., Skirkevičienė Z. Influence of keeping bees (*Apis mellifera carnica* Pollm.) in colonies or caged on the formation of conditioned reflex on the queen bee pheromones. *Pszczelnicze zeszyty naukowe*, Rok XLIV. 2000. Nr. 2. P. 43–53.
14. Takeda K. Classical conditioned response in the honeybee. *J. Insect Physiol*. 1961. No. 6(3). P. 168.
15. Тыщенко В. П. *Физиология насекомых*. Москва, 1986.
16. Vareschi E. Duftunterscheidung bei der Honigbiene – Einzelzellableistung und Verhaltensreaktionen. *Z. vergl. Physiol*. 1971. No. 75. P. 143–173.
17. Vareschi E., Kaissling K.-E. Dressur von Bienenarbeiterinnen und Drohnen auf Pheromone und andere Duftstoffe. *Z. vergl. Physiol*. 1970. No. 66. P. 22–26.

**L. Blažytė**

**KAI KURIE VEIKSNIAI, DĖL KURIŲ DIDĖJA GEBĖJIMAS IŠUGDYTI BITĖMS DARBININKĖMS (*Apis mellifera carnica* POLLM.) SĄLYGINĮ REFLEKSĄ Į MOTINOS FEROMONUS**

S a n t r a u k a

Bičių darbininkių sąlyginiam refleksui į motinos feromonus išugdyti svarbu:

a) jų laikymo sąlygos: bitėms darbininkėms iš šeimos, turinčios apvaisintą ir kiaušinėlius dedančią motiną, sąlyginis refleksas į jos feromonus susiformuoja geriau negu bitėms darbininkėms, gyvenusioms 4 dienas termostate;

b) sąlyginio dirgiklio (motinos ekstrakto) dozė: mažėjant motinos ekstrakto dozei, mažėja besimokančių bičių darbininkių skaičius. Sąlyginis refleksas lengviau išugdomas didesnėmis motinos ekstrakto dozėmis;

c) sąlyginio ir nesąlyginio dirgiklių pateikimo tvarka: stipriausias ryšys tarp dirgiklių susidaro, kai nesąlyginis dirgiklis eina po sąlyginio, bei laiko tarpas tarp dirgiklių pateikimo yra trumpas (kelios sekundės); kai sąlyginis dirgiklis pateikiamas po nesąlyginio dirgiklio, tai sąlyginį refleksą pavyksta išugdyti tik keliems procentams bičių darbininkių (ryšys tarp dirgiklių susidaro labai silpnas ir nepriklauso nuo laiko tarpo tarp dirgiklių pateikimo).

**Raktažodžiai**: *Apis mellifera carnica* Pollm., olfaktorinis refleksas, sąlyginis dirgiklis, nesąlyginis dirgiklis, bičių motinos ekstraktas