

**INFLUENCE OF KEEPING BEES (*APIS MELLIFERA CARNICA* POLLM.) IN COLONIES OR CAGED ON THE FORMATION OF CONDITIONED REFLEX ON THE QUEEN BEE PHEROMONES**

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S u m m a r y

Worker honeybees (*Apis mellifera carnica* Pollm.) were investigated. They were kept in a bee colony or in small cages in a thermostat from 0.5 to 3.0 h without food until the beginning of conditioning trials. Ethanol extract of mated bee queens ( $1 \cdot 10^{-3}$  Qeq) was used as a conditioning stimulus and 50% sugar solution as reward. The ability of a bee to respond to queen extract (from  $1 \cdot 10^{-12}$  to  $1 \cdot 10^{-3}$  Qeq) calibrated according to (*E*)-9-oxo-2-decenoic acid was investigated.

The results revealed that conditions, under which worker honeybees were kept prior to conditioning, influenced the formation of the reflexes to the pheromones of the bee queen. They affected not only the ability to form this reflex, but the course of its formation as well:

- a) the conditioned reflex to queen extract can be successfully formed in 93.2% of worker honeybees kept without food for 0.5 h after they have been taken away from a colony (the stimulus and reward have to be delivered on average  $2.9 \pm 0.44$  times, bees responded on average to  $5.7 \pm 0.11$  queen extract doses).
- b) the formation of the olfactory reflex to queen extract in worker honeybees taken from the colony and kept in a thermostat depends on the length of their existence without food: if they were kept without food for 0.5 h, the conditioned reflex was trained only in 10.0% of the individuals; if they were kept without food for an hour, the percentage of the individuals in which conditioned reflex occurred reached 59.1% (the stimulus and reward have to be delivered on average  $4.1 \pm 0.07$  times, bees responded on average to  $4.3 \pm 0.01$  queen extract doses), if 2.0 h - the number of individuals in which we managed to form the conditioned reflex grew up to 82.8% (the stimulus and reward should be offered on average  $2.3 \pm 0.50$  times, bees responded on average to  $5.3 \pm 0.20$  queen extract doses). If the bees were kept without food for 3.0 h, the conditioned reflex was trained in 0.0 % of them, because they were very weak or dead.

**Keywords.** *Apis mellifera carnica* Poll., olfactory reflex, conditioning, conditioned stimulus, reward.

## INTRODUCTION

It is well known that keeping conditions, age and species are important factors in the formation of a conditioned reflex (Mac-Farland, 1988). While studying the mechanisms of pheromone perception in worker honeybees, experiments are carried out with individuals kept under various conditions (bee colonies, cages kept in a thermostat, etc.). However, usually neither bee keeping conditions nor a possible effect of these conditions on study results are mentioned.

Living conditions in a cage differ from those in a bee colony. First of all, a worker bee living in a cage is isolated from bee colony's life and cannot participate in it; besides, it's feeding also changes greatly. These changes are reflected in its honey bag. The honey bag of a worker bee living in a hive usually does not weigh much (about 0.6 mg), while in an isolated worker bee it considerably increases. For instance, the honey bag of a worker bee kept in a cage for 4 days weighs on average 13.9 mg and for 9 days about 9.3 mg (Eskov, 1995). These facts show that before applying the method of a conditioned reflex in determining worker bee sensitivity to queen's pheromone, the conditions of worker bee keeping should be accounted for.

## METHODS

Worker bees *Apis mellifera carnica* Pollm. were studied. They were kept: a) in a bee colony, and in small cages in a thermostat; b) without feeding for 0.5, 1, 2, 3 hours before starting their training for the conditioned reflex.

Because of bad weather conditions, the bee colony was fed 50% sugar solution. In the thermostat, the temperature was +30°C, the bees were fed candy and given water all the time. In one cage were kept 30 - 50 bees.

The bees taken away from the hive or the thermostat were kept starving for a definite period of time (0.5 - 3 h). Then they were put in a refrigerator (0°C) and were cooled to immobilise them. In motionless bees, the wings were squeezed with special clips, on which they were hung in a test stand. The bees warmed up in 30 min. Then the conditioned reflex was started to train (Skirkevičius, Blažyte, 1999).

As a conditioning stimulus, a spirit extract of mated bee queens was used, and 50% sugar solution was used as a reward. When training a bee, to its antennae a glass rod was brought, onto which 0.01 ml of queen extract (0.001 Qeq) and a drop of sugar solution were placed with the aid of a micropipette (Fig. 1).

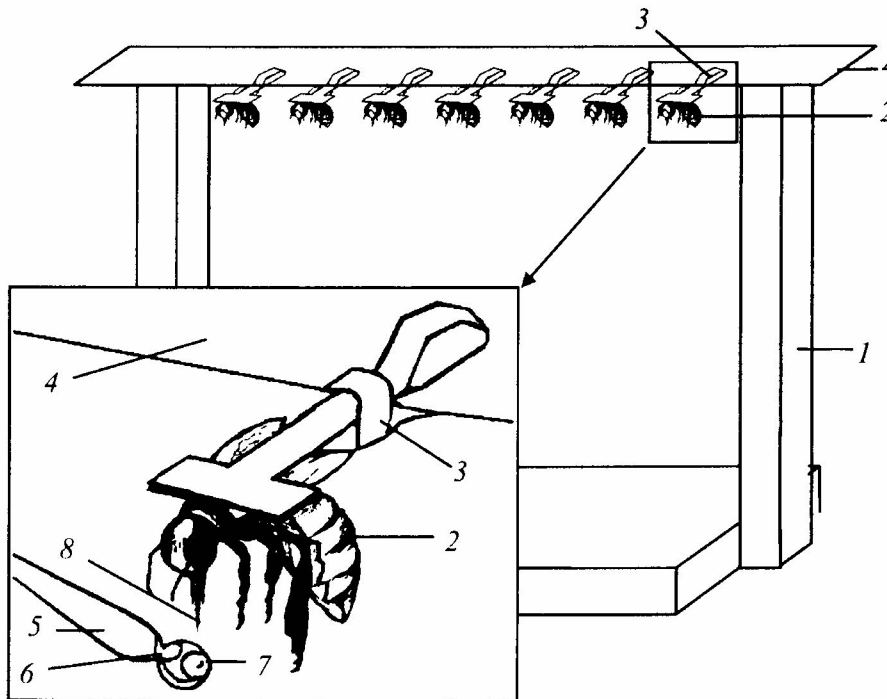


Fig. 1. Worker bees tested in a stand.

1 - test stand, 2 - a worker bee hung on her wings, 3 - bee wing clip,  
 4 - beam to hang a wing clip, 5 - glass stick to offer the stimuli, 6 - bee queen  
 extract (conditioned stimulus), 7 - sugar solution (unconditioned stimulus),  
 8 - proboscis.

Pszczoła robotnica na stanowisku badawczym

1- stanowisko badawcze, 2 - robotnica zawieszona na skrzydłach, 3 - spięte  
 skrzydło pszczoły, 4 - rama do mocowania pszczoły, 5-szklana bagietka do  
 podawania bodźców, 6 - ekstrakt z matki, 7 - syrop cukrowy, 8 - języczek

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, for the bee to put out reflectorily its proboscis. Such unconditioned reward lasted no more than 1 s. When a bee put out its proboscis just to smell the queen extract (a conditioning stimulus), the conditioned reflex was considered to be formed. The number of conditioning events was such as was necessary to form the conditioned reflex (it was 1 - 10).

Upon this reflex being formed, we checked the bees responding to queen extract solutions calibrated by (*E*)-9-oxo-2-decenoic acid from  $1 \cdot 10^{-12}$  to  $1 \cdot 10^{-3}$  Qeq. Fifteen individuals could be trained and tested simultaneously.

The study was performed in July - August 1998. All in all, 144 worker bees (58 taken directly from a bee colony and 86 kept in a thermostat) were studied. The obtained results were statistically evaluated.

## RESULTS AND DISCUSSION

Worker bees from a bee colony and from a thermostat showed a different behaviour when kept without food for 0.5 h (Fig. 2).

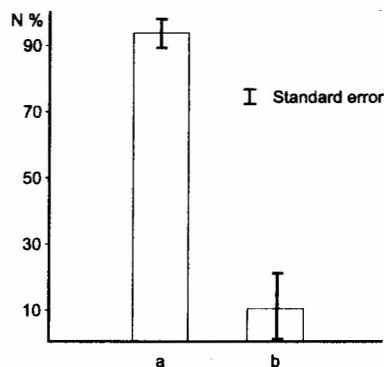


Fig. 2. Development of conditioned reflex to bee queen extract (0.001 Qeq) in worker bees (*Apis mellifera carnica* Pollm.) kept under different conditions:  
 a - bees from a colony with the mated and egg-laying queen (58 individuals studied);  
 b - bees kept in a thermostat without queen for 4 days (20 individuals studied).  
 Kształtowanie się bodźców warunkowych na ekstrakt z matek u robotnic przetrzymywanych w różnych warunkach:  
 a - pszczoły z rodziny z czerwiącą unasienioną matką (58 przebadanych);  
 b- pszczoły w cieplarnie bez matki przetrzymywane 4 dni (20 przebadanych)

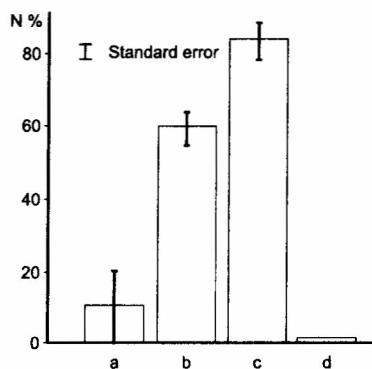


Fig. 3. Effects of the duration of starving on developing the conditioned reflex to bee queen extract in worker bees (*Apis mellifera carnica* Pollm.) (0.001 Qeq)  
 a - bees kept starving for 0.5 h (n = 20); b - bees kept starving 1 h (n = 22);  
 c - bees kept starving 2 h (n = 32); d - bees kept starving 3 h (n = 12).  
 Wpływ czasu trwania głodowania pszczół na kształtowanie się bodźców warunkowych na ekstrakt z matek: a - pszczoły trzymane bez pokarmu przez 0.5 godz. (n=20); b - pszczoły bez pokarmu przez 1 godz.(n=22); c - pszczoły bez pokarmu przez 2 godz. (n=32); d - pszczoły bez pokarmu przez 3 godz. (n=12)

The conditional reflex to queen extract was successfully formed in  $93.2 \pm 4.38\%$  of worker bees from a bee colony and only in 10% of those from a thermostat, i. e. by  $83.2 \pm 10.92\%$  less. If worker bees from a thermostat were kept starving for 1 h, the number of individuals that developed the conditioned reflex to queen extract increased to  $59.1 \pm 4.54\%$ , it was by  $49.1 \pm 10.98\%$  higher than those starved for 30 min. ( $P=0.070$ )

The number of individuals that developed the conditioned reflex to queen's pheromone in the worker bees taken from a thermostat and starving for 2 h was  $82.8 \pm 5.02\%$ , which increase of  $23.7 \pm 6.77\%$  was again significantly higher than those starved for 1h. ( $P=0.152$ ) (Fig. 3)

Thus, worker bees from a thermostat kept starving for 2 h and those from a bee colony kept starving for 0.5 h were very similar in their ability to develop the conditioned reflex to bee queen extract (Figs. 2, 3).

Worker bees from a thermostat that were kept starving for 3 h were impossible to study, for they grew weak and soon died (Fig. 3).

The time course of developing a conditioned reflex to bee queen extract in worker bees kept under different conditions was also different in respect to the number of conditioned and unconditioned stimuli offered.

When worker bees from a bee colony were kept starving for 0.5 h, to develop a conditioned reflex the stimulus with reward had to be offered to them on average  $2.9 \pm 0.44$  times ( $n = 58$ ). Among them, there were about 55.2% of individuals to whom 1-2 times were enough to develop the reflex (Fig. 4, a). About 37.9% of individuals needed 3-7 times for this reflex to be developed. The remaining part of individuals failed to develop the conditioned reflex even after 8-10 times of the stimulus and reward being offered to them.

To develop the conditioned reflex, worker bees from a thermostat ( $n = 22$ ) and kept starving for 1 h had to be offered the stimulus and reward on average  $4.1 \pm 0.07$  times. In this case, the distribution of worker bees with regard to the formation of conditioned reflex is different. To develop the conditioned reflex, 50% of the individuals had to be offered the stimulus and reward 1-6 times. In about 10% of individuals the conditioned reflex was developed only after even 8 times (Fig. 4., b).

Worker bees from a thermostat kept starving for 2 h had to be offered the stimulus and reward on average  $2.3 \pm 0.50$  times ( $n = 32$ ). Thus, in such a situation the development of the conditioned reflex to bee queen extract took least time. This is clearly demonstrated also by the worker bee distribution depending on the course of its formation (Fig. 4, c). Upon offering the stimulus and reward 1-3 times, the conditioned reflex was formed even in 88.9 % of individuals.

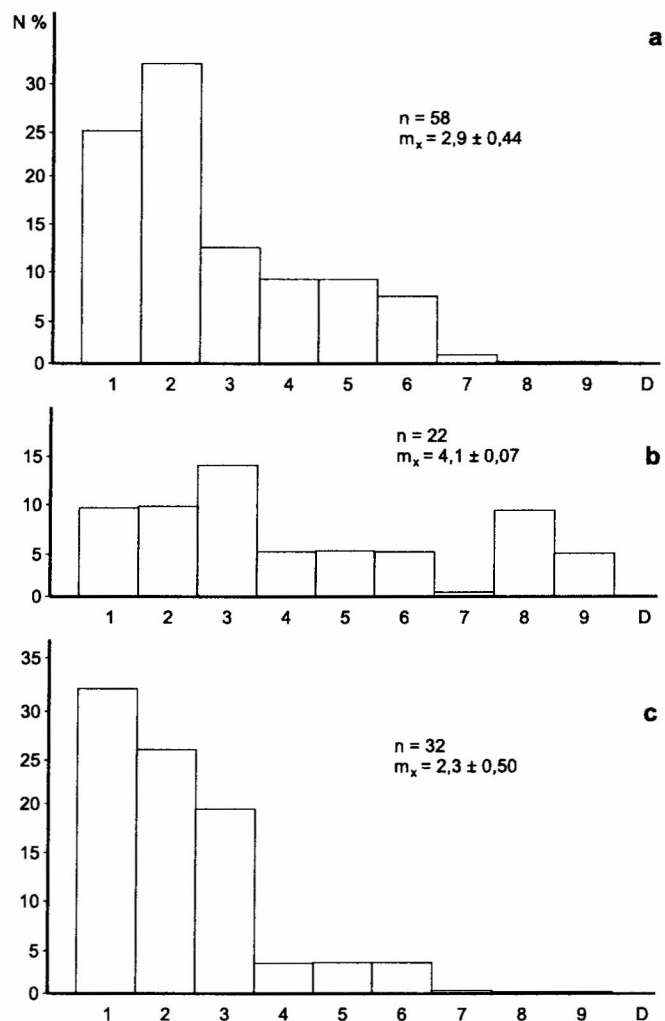


Fig. 4. The number (D) of simultaneous offering of the conditioned (bee queen extract) and unconditioned (50% sugar solution) stimuli until the formation of the conditioned reflex in worker bees.

a - bees from a colony kept starving for 0.5 h; b - bees from the thermostat kept starving for 1 h; c - bees from the thermostat kept starving for 2 h;

N - percentage of worker bees that developed the conditioned reflex to bee queen extract;  $m_x$  - average number of stimulus delivery;

$n$  - number of bees studied.

Liczba (D) bodźców stosowanych pszczołom pobudzonym (ekstrakt z matki) i nie pobudzonym (50% syrop cukrowy) do czasu pojawienia się u nich odruchu warunkowego:

a - pszczoły z rodziny głodzonej przez 0.5 godz., b - pszczoły z cieplarki głodzone przez 1 godz., c - pszczoły z cieplarki głodzone przez 2 godz., N - procent robotnic z pojawiającym się odruchem warunkowym na ekstrakt z matek,  $m_x$  - średnia liczba stosowanych bodźców,  $n$  - liczba badanych pszczoł

The study results show also that bees kept under different conditions differed in their ability to respond to bee queen extract doses. Bees from a colony kept starving for 0.5 h respond on average to  $5.7 \pm 0.10$  bee queen extract doses ( $n = 58$ ). About half of individuals responded to 7-8 doses (Table, Fig. 5, a).

When worker bees from a thermostat were kept starving for 0.5 h, only two of them developed the conditioned reflex ( $n = 20$ ): one of them responded to two and the other to six queen extract doses (Table, Fig. 5, b). The behaviour of bees changed when they were kept starving for 1 h: they responded on average to  $4.3 \pm 0.01$  queen extract doses ( $n = 22$ ). In this case more than 50% of individuals responded only to 1-3 doses (Table, Fig. 5, c).

Table

Extract doses (queen equivalents) responded by worker bees (*Apis mellifera carnica* Pollm.) kept under different conditions and kept starving for different times

Wielkość dawki ekstraktu (odpowiednik matki) na którą reagowały robotnice przetrzymywane w różnych warunkach i głodzone przez różny okres

Bees kept starving until the development of conditioning reflex Czas głodzenia pszczoł (h)	Extract doses studied (Quq) Wielkość dawki ekstraktu	Number of bees studied Liczba badanych pszczoł	Extract doses responded by worker bees (Qeq) Wielkość dawki ekstraktu		Number of differentiated doses - Liczba odróżnianych dawek	
			minimum	maximum	mean $\pm$ SE Średnio	most frequent najczęściej
Bees kept in colony - Pszczoły przetrzymywane w rodzinie						
0,5	$1 \cdot 10^{-12}$ - $1 \cdot 10^{-3}$	58	$1 \cdot 10^{-11}$	$1 \cdot 10^{-3}$	$5,7 \pm 0,11$	7 - 8
Bees kept in cages in a thermostat - Pszczoły przetrzymywane w cieplarni						
0,5 <sup>1)</sup>	$1 \cdot 10^{-12}$ - $1 \cdot 10^{-3}$	20	$1 \cdot 10^{-8}$	$1 \cdot 10^{-3}$	-	2 and 6
1,0	$1 \cdot 10^{-12}$ - $1 \cdot 10^{-3}$	22	$1 \cdot 10^{-11}$	$1 \cdot 10^{-3}$	$4,3 \pm 0,01$	1 - 3
2,0	$1 \cdot 10^{-12}$ - $1 \cdot 10^{-3}$	32	$1 \cdot 10^{-11}$	$1 \cdot 10^{-3}$	$5,3 \pm 0,20$	7 - 9
3,0 <sup>2)</sup>	$1 \cdot 10^{-12}$ - $1 \cdot 10^{-3}$	12	-	-	-	-

Notes:

1) conditioned reflex developed in 2 bees only, 2) did not recover after chilling.

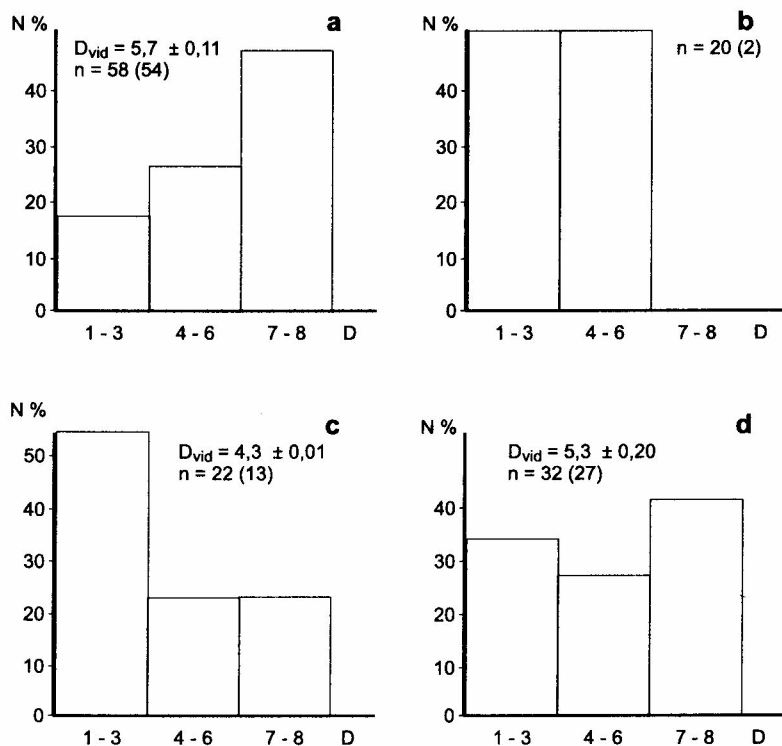
1) odruch warunkowy występował tylko u 2 pszczoł, 2) nie wykazywały oznak życia

Bees from a thermostat that were kept starving for 2 h responded on average to  $5.3 \pm 0.20$  queen extract doses. About half of individuals responded to 5-9 doses (Table, Fig. 5, d).

The analysis of the obtained results shows- that only the worker bees from a thermostat kept starving for 2 h and those from a bee colony starved for 0.5 h display a similar behaviour regarding the possibility and course of the

formation of the conditioned reflex and the number of queen extract that elicited response from the bees.

Fig. 5. The number (D) of queen extract doses that elicited response from worker bees (*Apis mellifera carnica* Pollm.), kept under different conditions (the conditioned reflex formed by 0.001 Qeq of a bee queen extract dose)



a - bees taken from colony and starved for 0.5 h; b - bees taken from thermostat and starved for 0.5 h; c - bees taken from thermostat and kept starving for 1 h; d - bees taken from thermostat and kept starving for 2 h, N - percentage of worker bees;  $D_{av}$  - average number of doses that elicited response from bees;  $n$  - number of bees studied; in brackets - number of bees that developed the conditioned reflex to queen pheromones.

Liczba (D) dawek ekstraktu z matek, na które reagowały pszczoły robotnice przetrzymywane w różnych warunkach (odruch warunkowy wywoływany przez dawkę ekstraktu 0.001 Qeq) a – pszczoły pobierane z rodzin i głodzone przez 0.5 godz., b – pszczoły pobierane z ciepłarki i głodzone przez 0.5 godz., c – pszczoły pobierane z ciepłarki i głodzone przez 1 godz., d – pszczoły pobierane z ciepłarki i głodzone przez 2 godz., N – procent pszczół,  $D_{av}$  - średnia liczba dawek, na które reagowały pszczoły,  $n$  – liczba badanych pszczół, w nawiasie liczba pszczół z odruchem warunkowym na feromony matki

Thus, the results of our study disagree with the results of the other authors (Menzel, Bitterman, 1983) that artificial conditions do not influence the



formation of conditioned reflex in bees. The reasons for such contradictory results are difficult to explain as yet. Possibly that discrepancy was caused by the different conditions in which the experimental animals were kept before the conditioning. The mentioned authors caught bees from a bee-entrance late in the afternoon, then chilled them at the laboratory, fixed into special tubes, fed and kept in the dark for a night.

The bees were tested only next morning. Perhaps the behaviour of these bees did not differ from that of worker bees just taken away from the bee colony in our study, because of a rather short period following their separation from the bee colony, those bees were kept starving overnight and they were hungry. As to our tests, bees were kept in a thermostat with ad. lib. feeding for 2-4 days. Their feeding possibilities were limited only for 0.5 to 3 h. Before training the conditioned reflex, bees were kept starving for different periods of time. According to our results, worker bees kept in a thermostat and starved for 2 h behaved in a similar way as those studied by Menzel and Bitterman (1983). Additional studies are needed to elucidate the reasons for discrepancies of the conclusions drawn by us and by the mentioned authors.

## CONCLUSIONS

Conditions of keeping worker bees influence the development of their conditioned reflex to queen extract. They are of significance not only for the possibility of developing this reflex, but also for the course of its formation and bees' reaction to different doses of queen extract:

- a) the conditioned reflex to queen extract is possible to develop, on average, in 93.2% of worker bees that upon being taken away from bee colony are kept starving for 0.5 h (the stimulus and reward have to be delivered on average  $2.9 \pm 0.44$  times, bees responded on average to  $5.7 \pm 0.11$  queen extract doses);
- b) the development of the conditioned reflex to bee queen extract in worker bees separated from a bee colony and kept in a thermostat depends on the duration of their starving: if it lasts 0.5 h, the conditioned reflex is possible to develop in only 10.0% of individuals, starving for 1 h raises this number to 59.1% (the stimulus and reward have to be delivered on average  $4.1 \pm 0.07$  times, bees responded, on average, to  $4.3 \pm 0.01$  queen extract doses); among the worker bees kept starving for 2 h, 82.8% develop this reflex (the stimulus and reward should be offered on average  $2.3 \pm 0.50$  times, bees responded on average to  $5.3 \pm 0.20$  queen extract doses). Bees kept starving for 3 h grew weak and died.

## ACKNOWLEDGEMENT

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## WPLYW POZOSTAWANIA GŁODZONYCH PSZCZÓŁ (*APIS MELLIFERA CARNICA*) W RODZINACH LUB KLATECZKACH NA POWSTAWANIE U NICH ODRUCHU WARUNKOWEGO NA FEROMONY MATKI

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### S t r e s z c z e n i e

Obserwacje prowadzono na pszczołach robotnicach rasy kraińskiej (*Apis mellifera carnica*). Znajdowały się one w rodzinach pszczelich lub małych klaticzkach przetrzymywanych w cieplarni bez pokarmu przez 0.5 do 3 godz. Po tym czasie działano na nie ekstraktem etanolowym z unasienionych matek ( $1 \times 10^{-3}$  Qeq), który stosowano jako bodziec uzależniający i dodatkowo karmiono 50% roztworem sacharozy. Badano zdolność pszczoł do reakcji na kwas 9-oxo-2-decenoowy znajdujący się w ekstrakcie z matek.

Wyniki ujawniły, że warunki w jakich pszczoły były przetrzymywane w decydującym stopniu kształtowały odruchy na feromony matki:

- a) odruch warunkowy na ekstrakt z matek może z powodzeniem wystąpić u 93.2% robotnic przetrzymywanych bez pokarmu przez 0.5 godz., później są one wyrzucane z rodziny.
- b) tworzenie się odruchu zapachowego na ekstrakt z matek u pszczoł robotnic zabranych z rodziny i przetrzymywanych w cieplarni zależy od czasu pozostawania bez pokarmu. Jeśli były one przetrzymywane bez pokarmu przez 0.5 godz. odruch warunkowy następował tylko u 10% osobników, jeśli przetrzymywano je bez pokarmu przez godzinę procent osobników, u których występował odruch warunkowy wzrastał do

59.1%, a po 2 godz. u 82,8% osobników. Przechowywanie pszczół bez pokarmu przez 3 godz nie wywoływało już żadnej reakcji gdyż pszczoły były zbyt słabe albo martwe.

**Słowa kluczowe:** *Apis mellifera carnica*, feromony matki, bodziec węchowy, odruch warunkowy.