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Olfactory learning in worker honeybees from queenright and queenless colonies (*Apis mellifera carnica* Pollm.)

Algirdas Skirkevičius[1]*,

Laima Blažytė-Čereškienė[2]

[1] Vilnius Pedagogical University, Studentų 39, LT-08106 Vilnius, Lithuania

[2] Institute of Ecology of Vilnius University, Akademijos 2, LT-08412 Vilnius, Lithuania

In order to study the significance of queen loss in a honeybee colony for the ontogeny of queen pheromone perception, the conditioned proboscis extension response to queen extract (dose of 0.001 queen equivalent) in worker honeybees (*Apis mellifera carnica* Pollm.) was investigated. Worker bees (newly emerged, 1-, 2-, 3-, 4-, 5-, 6-, 7-, 8-, 9-, 10-, 12-, 14-, 16-, 18- and 20-day-old) from specially formed queenright (with a mated and egg-laying queen) and queenless colonies were studied.

Our results revealed a great improvement in olfactory learning of queenright workers during the first three days of adult life. The olfactory learning in queenless worker bees reached its maximum only on the 10th day, i. e. seven days later than in queenright bees. Differences in the levels of conditioned response to the queen extract of queenright and queenless workers were revealed within two to nine days of adult development. Similar differences were revealed in the rate of conditioning of queenright and queenless workers of the same age in the period from three to eight days (except in the rate of conditioning of 6-day-old bees). The conditioned response of workers over 10 days of age was high irrespective of the queen's presence in the colony.

Key words: *Apis mellifera*, proboscis extension response (PER), conditioning, honeybee queen, queen pheromone, age

INTRODUCTION

A mated queen not only lays eggs in a bee colony, but also produces and releases vital pheromones that exert a profound influence on the most important life activities of the colony [1–5]. Its loss in a honeybee colony influences workers' behaviour [4, 5] and the sensitivity of their antennal receptors to queen extract odour [4–6], or affects the maturation of the antennal lobes of the worker bee brain [7], etc. However, there are many questions that are still unanswered. The role of queen pheromone in the ontogeny of worker bees' learning is one of these questions.

In this respect, we believe that the method of conditioned proboscis extension may be useful. The point is that the age of worker honeybees has an influence on their ability to learn to discriminate olfactory stimuli [8–12]. Thus, if queen loss in a honeybee colony influences queen pheromone perception of workers of different age, we can conclude that the present pheromone (the most essential chemical signal) is responsible for the ontogeny of its olfactory system in a bee colony.

To study the significance of queen loss in a honeybee colony for the ontogeny of queen pheromone perception, the conditioned proboscis extension response to queen extract in worker honeybees (*Apis mellifera carnica* Pollm.) was investigated, including the level of conditioned response and the rate of conditioning.

* Corresponding author. E-mail: algskirk@ktl.mii.lt

METHODS

Honeybees

The experiments were conducted on worker honeybees (*Apis mellifera carnica* Pollm.) of a known age. Combs with pre-emerging brood were removed from the honeybee colony and placed in a thermostat where a temperature of +30 °C was maintained. Emerging workers were collected every day (Fig. 1) and introduced into special one-frame (205 × 130 mm) observation hives with an automatically regulated temperature (Fig. 2).

One day the workers were introduced into a queenright colony and the other day into a queenless one. Every second day, 50 to 300 young over-night emerged workers were placed in the colonies. The queenright colony was started with 180 workers, and the formed colony comprised about 1900 bees. The queenless colony was set up with 260 workers, and the formed colony contained about 2000 bees. The queenright colony was enlarging: the queen laid eggs and the colony reared the worker brood. The second colony was diminishing: the queen was absent, and workers reared the drone brood.

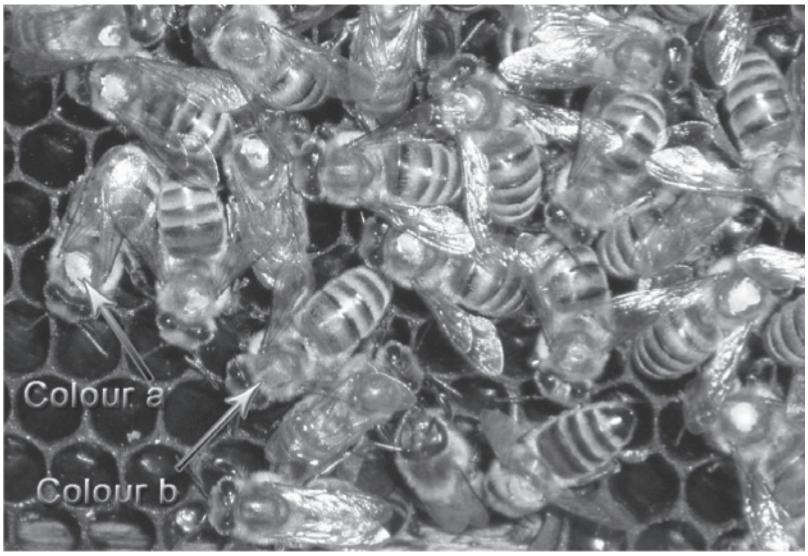

Fig. 1. Colour-marked worker honeybees

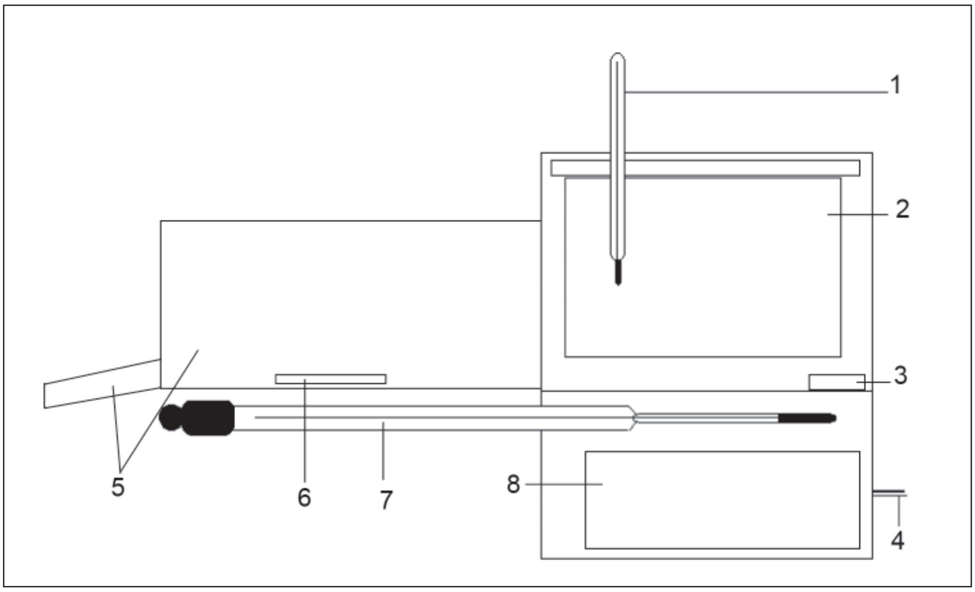

Fig. 2. One-frame observation hive with automatically regulated temperature:

1 – thermometer, *2* – frame (205 × 130 mm) with comb, *3* – candy, *4* – wire, *5* – passage, *6* – drinking place, *7* – contact-thermometer, *8* – space-heater

Fig. 3. Worker bees fixed in a test-stand (conditioning of proboscis extension response)

Worker bees could freely fly out and forage. Additionally they were fed on candy and water. We maintained a temperature of +30 °C in the observation hives.

We tested the groups of newly-emerged (over 2–3 h), 0.5 (overnight emerged), 1-, 2-, 3-, 4-, 5-, 6-, 7-, 8-, 9-, 10-, 12-, 14-, 16-, 18- and 20-day-old bees. Newly-emerged and 0.5-day-old bees were collected from the combs in the thermostat, and bees of other ages were collected from the experimental colonies. After training, bees were not returned into the colonies. The experiments were conducted in July–August.

Stimuli

The conditioned stimulus was the odour of the mated honeybee queen's extract, the dose of which corresponded to 0.001 queen equivalent (Qeq). The extract was prepared by extracting mated egg-laying queens in ethanol. The collected material was kept in a refrigerator at a temperature of 4 °C. The extract contained 0.1 μg *E*-9-oxo-2-decenoic acid (established by Dr V. Apšegaitė at the Institute of Ecology of Vilnius University). This component is dominant in the mandibular gland pheromone of a mated honeybee queen [13–15]. The queen extract was used to make the stimulus reflect the quality and quantity of the real honeybee queen pheromone [2, 5, 13].

The unconditioned stimulus was a 30% sucrose solution.

Conditioning

For experiments, worker bees were prepared by the methods described above [16]. They were caged (the cage was 160 mm in length and 30 mm in diameter). The cages with bees were placed into a refrigerator and kept there for a few minutes in order to reduce the bees' activity. The wings of immobile bees were pressed with special holders, and the bees were fixed in a test-stand (Fig. 3). Seventeen age groups, each consisting of a minimum of 35 bees, were trained in total.

Prior to experiments, honeybees had been tested for the unconditioned reflex, i. e. the extension of the proboscis immediately after touching the antennae with a drop of sucrose solution. Bees that did not exhibit the reflex were discarded.

Conditioning consisted of pairing odour presentation to a restrained bee with a subsequent sugar reward [17]. Before each training, 0.01 ml of ethanol solution of queen extract had been placed on a glass stick. The solvent (ethanol) was allowed to evaporate for 5 min. Then the stick with the olfactory stimulus was delivered to the worker's antennae at a distance of 5 mm and kept for 5 s. To make the workers respond with proboscis extension, their antennae were contacted with sucrose solution. The workers were allowed to lick the sucrose solution for about 1 s. The whole procedure lasted for approximately 6 s. Each bee received a series of 10 paired odour–sucrose presentations with an inter-trial interval of 2 min. The bees that in the first trial responded to the conditioning odour by extending their proboscis prior to the sucrose reward were not included in this study. Conditioned response was recorded only when a worker extended the proboscis within 5 s and it crossed the virtual line between the open mandibles. The experiments were conducted at an exhaust system at a temperature of 18–25 °C.

Statistical data analysis

We estimated the conditioned response level (in the 10th learning trial) and the rate of conditioning. The conditioned response level was expressed by the percentage

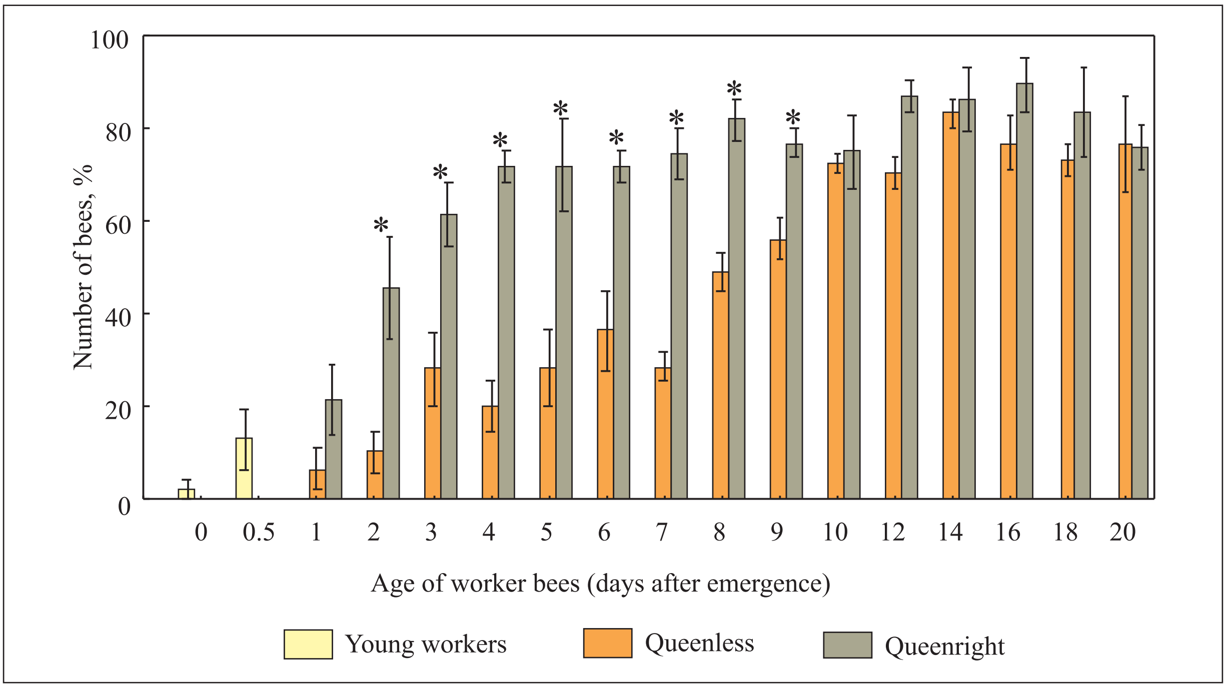

Fig. 4. Age-related changes in the level of conditioned responses (proboscis extension) of worker bees to queen extract odour (0.001 Qeq).

Young worker bees were collected from the comb in a thermostat. There were two groups: 0 (emerged over 2–3 h, n = 51) and 0.5 (emerged overnight; n = 50); Queenless – workers from the queenless colony (n = 544); Queenright – workers from the queenright colony (n = 518); * Mann–Whitney U- test shows significant differences between queenright and queenless bees, $p < 0.05$

of bees that displayed the conditioned response. The rate of conditioning was the number of learning trials after which the conditioned response level did not increase.

Nonparametric methods were used for comparison of the data. To determine statistically significant differences in all experiments, the Kruskal–Wallis (*H*) test was used when the response levels of more than two groups were analysed. The Mann–Whitney (U) test was used to evaluate the response levels of the two groups.

All values are presented as means ± one standard error. All statistical tests were performed with Statistica software. The results of statistical analysis were considered as significant at $p \leq 0.05$.

RESULTS

Conditioned responses

The results showed that the youngest worker bees that were collected from the combs in the thermostat (there was no possibility to transfer these worker bees to experimental colonies) demonstrated a low-level learning performance. Only 2.2 ± 2.22% of newly-emerged worker bees and 12.9 ± 6.34% of 0.5-day-old bees displayed a conditioned response to queen extract odour (Fig. 4). The difference was not statistically significant (Mann–Whitney test: U = 1011, Z = –0.87, N1 = 47, N2 = 46, p = 0.39) between the levels of these conditioned responses.

Bees of other ages were collected from the formed experimental colonies. The results showed that the conditioned response to queen extract odour depended on the age of workers of queenright (Kruskal–Wallis test: H = 85.8, N = 518, df = 14, $p < 0.001$) colonies (Fig. 4). However, this dependence of behaviour was observed only in a certain period of age, i. e. during the first three days of adult life, because the level of bees displaying a conditioned response to queen extract gradually increased from 21.3 ± 7.73% in 1-day-old bees to 61.3 ± 6.81% in 3-day-old bees (Mann–Whitney U-test: U = 580, Z = –3.18, N1 = 48, N2 = 40, p = 0.001) and did not vary statistically in 3- to 20-day-old bees (Kruskal–Wallis test: H = 13.8, N = 422, df = 12; p = 0.311).

The conditioned response to queen extract odour depended on the age of workers of queenless (Kruskal–Wallis test, H = 159.4, N = 544, df = 14, $p < 0.001$) colonies, too (Fig. 4). However, the olfactory learning of queenless worker bees reached its maximum only on the 10th day of adult life, because the response level of these workers gradually increased from 6.4 ± 4.39% in 1-day-old bees to 72.0 ± 1.97% in 10-day-old bees (Mann–Whitney U-test: U = 264, Z = –4.95, N1 = 48, N2 = 32, $p < 0.001$) and did not

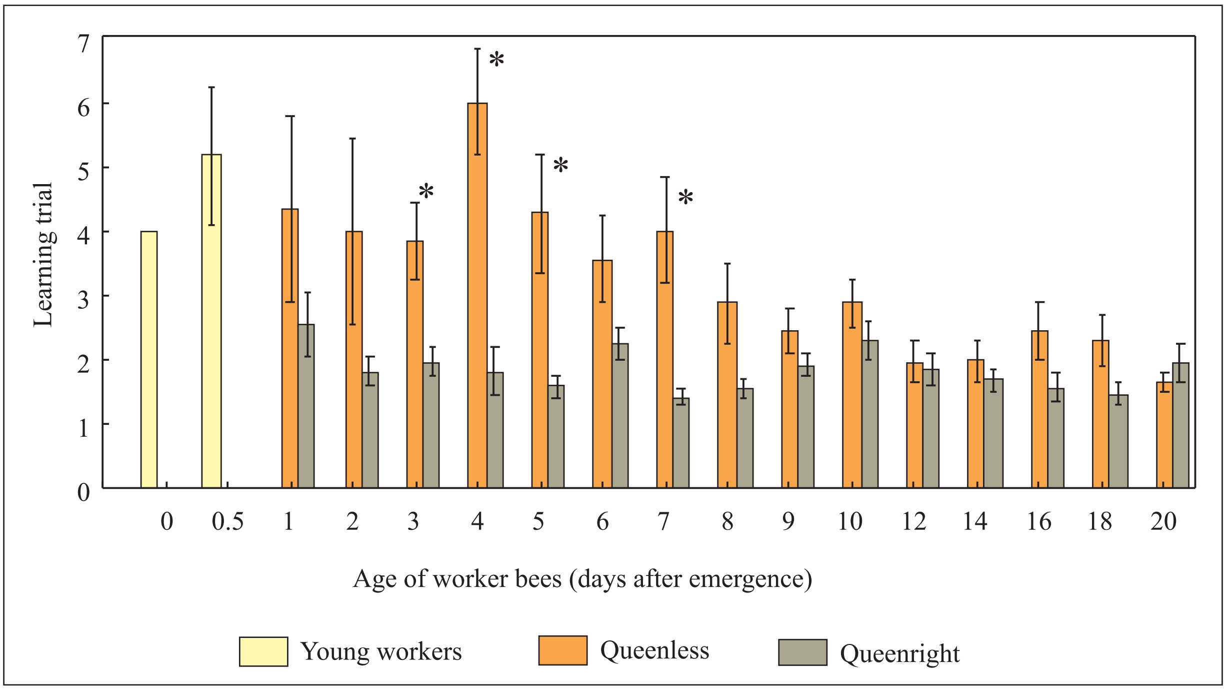

Fig. 5. Age-related changes in the rate of conditioning of worker bees which acquired the conditioned response to queen extract odour (0.001 Qeq).

Young worker bees were collected from the comb in a thermostat; there were two groups: 0 (emerged over 2–3 h, n = 51) and 0.5 (emerged overnight; n = 50) investigated; Queenless – workers from the queenless colony (n = 544); Queenright – workers from the queenright colony (n = 518); * – Mann–Whitney U- test shows significant differences between queenright and queenless bees, $p < 0.05$

vary statistically in 10- to 20-day-old bees (Kruskal–Wallis test: H = 2.2, N = 197, df = 5; $p = 0.823$).

Thus, the levels of conditioned response to queen extract odour gradually increased with the age of workers of both queenright and queenless colonies. However, the duration and time of gradual increase was absolutely diverse (Fig. 4), because the differences in the levels of conditioned response to queen extract of queenright and queenless workers were statistically significant in the period from 2 to 9 days of adult development (Mann–Whitney U-test: for all age groups from 2 to 9 days $p < 0.01$). The level of the conditioned response of workers over 10 days of age was similar, i. e. high (~80%) irrespective of the queen's presence in the colony (Mann–Whitney U-test: queenless vs. queenright in all age groups from 10 to 20 days, $p > 0.05$).

Rate of conditioning

Our results suggest that the youngest (newly emerged and 0.5-day-old) worker bees, which were collected from the combs in the thermostat (there was no possibility to transfer them to experimental colonies) and which acquired the conditioned response to queen extract odour, needed 5.0 ± 0.93 learning trials on average (Fig. 5).

Statistical analysis revealed a significant effect of age on the rate of conditioning in the workers of other age, which were collected from the formed experimental queenright colonies (Kruskal–Wallis test: H = 26.8, N = 342, df = 14, $p = 0.02$). Initially, i. e. at the age of one day, workers were conditioned during 2.5 ± 0.51 trials on average. Thus, the rate of conditioning decreased by half times in one day, but the decrease was not statistically significant (Mann–Whitney U-test: U = 15, Z = 1.8, N1 = 6, N2 = 11, $p = 0.07$). The rate of conditioning had also a tendency to decrease in 2-day-old queenright workers, because differences in the rate of conditioning between 0.5- and 2-day-old bees decreased until 1.8 trials (Mann–Whitney U-test: U = 18, Z = 2.6, N1 = 6, N2 = 21, $p = 0.009$). The rate of conditioning remained unchanged in older (2- to 20-day-old) queenright workers (Kruskal–Wallis test: H = 20.4, N = 345, df = 13, p = 0,086).

A significant effect of age was found also in the workers of other age, which were collected from the formed experimental queenless colonies (Kruskal–Wallis test: H = 45.4, N = 243, df = 14, $p < 0.001$). However, the conditioning rate (on average 3.5 ± 0.67 to 6 ± 0.82 trials of the individual age group) of queenless bees did not differ from that of the youngest workers until the 8th day of their adult life (Mann–Whitney U-test, the youngest vs. queenless age groups 2- to 8-day-old, $p > 0.05$).

Nine-day-old queenless workers differed statistically from the youngest bees, because the number of learning trials required for response decreased to 2.4 ± 0.35 trials on average (Mann–Whitney U-test: $U = 23$, $Z = 2.5$, $N1 = 7$, $N2 = 19$, $p = 0.012$). This number of trials remained unchanged in 9- to 20-day-old queenless workers (Kruskal–Wallis test: $H = 11.0$, $N = 169$, $df = 6$, $p = 0.087$).

Thus, statistical analysis revealed a significant effect of age on the rate of conditioning in workers of both queenright and queenless colonies. However, the rate of conditioning developed differently, because differences in the rate of conditioning in workers of queenright and queenless colonies were statistically significant in the period from 3 to 7 days of adult development (Mann–Whitney U-test, queenright vs. queenless in all age groups from 3- to 7-day-old workers, $p < 0.05$, except for the rate of conditioning of 6-day-old bees, $p = 0.053$). The rate of conditioning in the workers over 7 days of age was similar (~2 trials) irrespective of the queen's presence in the colony (Mann–Whitney U-test: queenless vs. queenright in all age groups 8 to 20 days, $p > 0.05$). More than 80% of responding bees were conditioned with 1- to 3-trial presentation of odour with a reward.

DISCUSSION

In this study, we present new data which demonstrate that the age of workers and the presence of the queen in a honeybee colony (from which bees were removed) are factors important to olfactory learning (in our case, the odour of queen extract).

The age of workers

The youngest (newly-emerged and 0.5-day-old) workers showed a poor acquisition performance, but with age the conditioned response increased until the third day of adult life and persisted until 20 days of age (Fig. 4). Herewith, the youngest workers required 4–6 trials, i. e. more learning trials after which the conditioned response level did not increase, while the older ones needed 1–2 trials, i. e. fewer trials were sufficient (Fig. 5). Thus, workers of different age exhibited different conditioned proboscis extension responses to queen extract odour.

The results of our research agree with the results reported by other authors [9, 11, 12, 18] on odours of other origin, to which bees showed a poor learning performance only during the first 2 days of age; later workers achieved a higher level of acquisition, i. e. on the 3rd–4th day it became equal to that of older bees. However, the results of our research do not correspond to those of Bhagavan et al. [19] suggesting that the age of bees is not essential to conditioning with olfactory stimuli of floral origin (hexanal, 1-hexanol). The effect might have been different if these authors in their research programme had included bees under 5 days of age.

Now, several parallelisms are worth noting: 1) improvements in olfactory learning performance during the first days of adult life (Fig. 4) correlate in time with the increased sensitivity of workers' antennal receptors to queen pheromone [20, 21] and other olfactory stimuli [22, 23], 2) during the first days of adult life; more and more bees join the retinue of the queen [20, 24], i. e. their behaviour changes, 3) during the first week, the sensilla placodea [26] and the neuropil of the olfactory centre undergo changes [25]. Thus, it all goes to show that the growth of the possibility of conditioning with queen extract odour on the first three days past emergence should be related to the maturation of the olfactory system.

The queen's presence

The results of our investigation revealed that the ontogeny of conditioned response was closely related to the queen's presence in the colony (Figs. 4, 5). The appearance of such dependence in different ages of workers' adult life varied.

Workers older than 10 days of age were more ready to the possibility of eliciting the conditioned proboscis extension response to queen extract odour irrespective of the queen's presence in the colony. The growth of such possibility in younger bees greatly depended on their age and the queen's presence in the colony. With regard to queenright workers, the conditioned response reached its maximum on the third day. The possibility of eliciting the conditioned response in queenless workers came to its near maximum only on the 10th day of their adult life, 7 days later than in queenright workers.

The number of learning trials required for conditioning was related also to the queen's presence in the colony. Queenless 3–8-day-old bees required more learning trials than queenright bees of the same age. The rate of conditioning of workers over 10 days of age was high, irrespective of the queen's presence in the colony.

Accordingly, the queen's presence in the colony significantly accelerates the ability of young workers to learn queen extract odour.

Similar results were obtained with odours of orange and lavender [7]. After a single trial, the level of the conditioned response of queenless workers was considerably lower than that of queenright workers. Our experimental results and their analysis show that along with already known factors [25, 26] that are related to the maturation of the olfactory system, the presence of the queen in the colony is one more factor that becomes evident.

It is a long-known truth that the queen's mandibular glands secrete pheromone [1, 27, 28]. The presence of the queen in some way inhibits the sensitivity of workers' antennal olfactory receptors to pheromone extract odour. The sensitivity of queenright workers to queen extract odour is lower than that of queenless workers [6, 29]. The removal of the queen from the colony affects the maturation of the antennal lobes of the brain [7], the functioning of workers' endocrine system [30, 31] and the ontogeny of olfactory learning behaviour of young bees to floral stimuli [7, 32]. Application of synthetic queen mandibular gland pheromone [33] instead of the queen in queenless colonies accelerates the ontogeny of olfactory learning [34].

Thus, we can assume that queen pheromone affects the maturation of structures responsible for the formation of temporary relationship between a conditioned stimulus and food reinforcement. This is the reason why younger queenright bees exhibited a higher level of learning performance than queenless bees. If the structures responsible for the time relationship between the conditioned stimulus and food reinforcement are mature enough, then the presence of the queen should have less influence on the establishment of conditioned response. This presupposition is based on the results of Morgan et al. [7]: the removal of the queen from the colony does not affect the conditioning of older workers (foragers).

The data of our research on the influence of queen pheromone on olfactory conditioning are of both biological and practical significance. They help us to understand better why some authors obtain inconsistent results. In our opinion, discrepancies are due to the time-span of workers being separated from the queen (it is important what colony workers are from, how much time has passed from the moment of their removal from the colony until the beginning of the experiment). All such information would facilitate the analysis of results reported by different authors. Also, all these circumstances are very important for further research on conditioning and its application as a test.

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Algirdas Skirkevičius, Laima Blažytė-Čereškienė

SĄLYGINIO REFLEKSO IŠUGDYMAS OLFAKTORINIU STIMULU SKIRTINGO AMŽIAUS DARBININKĖMS IŠ MOTINĄ TURINČIŲ IR JOS NETURINČIŲ BIČIŲ ŠEIMŲ (*APIS MELLIFERA CARNICA* POLLM.)

Santrauka

Siekiant nustatyti jutimą bičių darbininkių ontogenėzėje, tirti sąlyginiu refleksu suformuoti darbininkių atsakai (pagal liežuvėlio iškišimą) į motinos ekstrakto (dozė – 0,001 motinos ekvivalento) kvapą. Tyrimų rezultatai rodo, kad galimybė išugdyti sąlyginį refleksą priklauso tiek nuo pačios darbininkės amžiaus, tiek nuo motinos buvimo bičių šeimoje, tačiau ši priklausomybė skirtingais darbininkių amžiaus tarpsniais yra nevienoda – išsiskiria darbininkės iki 10 parų ir vyresnės. Vyresnėms nei 10 parų darbininkėms kur kas didesnė galimybė olfaktoriniu stimulu išugdyti sąlyginį refleksą, ir tai nepriklauso nuo motinos buvimo bičių šeimoje. Tuo tarpu jaunesnėms bitėms šios galimybės didėjimas priklauso ne tik nuo jų amžiaus, bet ir nuo motinos buvimo bičių šeimoje. Darbininkėms iš šeimos su motina galimybė išugdyti sąlyginį refleksą pasiekia beveik maksimumą jau trečią parą, o darbininkėms iš šeimos be motinos – tik 10 parą, t. y. maždaug 6 paromis vėliau.

Raktažodžiai: *Apis mellifera*, atsakas pagal liežuvėlio iškišimą, sąlyginis refleksas, bičių motina, bičių motinos feromonai, amžius