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Response of workers (*Apis mellifera carnica* Pollm.) by proboscis extension to queen extract odour before conditioning procedure

A. Skirkevièius[1*],

L. Blaþytë-Èereðkienë[2]

[1] *Vilnius Pedagogical University, Studentu 39, LT-2034, Vilnius, Lithuania*

[2] *Institute of Ecology, Vilnius University, Akademijos 2, LT-2600 Vilnius, Lithuania*

We investigated the proboscis extension response (PER) of worker bees *Apis mellifera carnica* Pollm. to the odour of the mated honeybee queen's extract (dose of 0.001 queen equivalent). The experiments were carried out in April–August 2001 and 2002. In total, 542 workers were studied.

The experimental results show that the honey bee queen extract (dose, 0.001 queen equivalent) odour elicited proboscis extension response (PER) even in 40% (on average in 20.8%) of workers before the conditioning procedure. The changes occurring in the honeybee colony over April–August had no influence on the average number of odour stimulations required for eliciting PER and to the average number of responses elicited by repeated stimulations. However, they influenced on the level (the number of individuals) of PER.

The PER that elicits the honeybee queen extract before the conditioning procedure cannot be regarded as spontaneous, because the experimentor can change its level by artificial means (removing part of young workers from honeybee colony).

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

INTRODUCTION

Classical conditioning of the proboscis extension reflex of honeybees *Apis mellifera* L has proven to be an effective paradigm to analyse the physiology and psychological rules that underlie behavioural plasticity [1, 2], learning and memory at all levels [3, 4, etc]. However, olfactory stimulus sometimes can elicit proboscis extension response (PER) even in 56% of honeybees before the conditioning procedure [2, 5].

Various authors adhere to different opinions about these workers. Some of them ignore such workers [6, 7], others [8–10] presume that such workers can distort the results of research, and they remove them. This is because the reason for the appearance of workers with PER is unknown so far.

Forasmuch as the number of such workers is quite large and PER is used as a method, it is essential to study them. First of all it is necessary to know whether according to some of the authors [4, 6, 8, 11], proboscis extension before a food reward is applied is a spontaneous or not spontaneous response. We assume that if the following PER is a spontaneous response, its level should not change to the same olfactory stimulus after the condition of worker bees (or other conditions) are changed. If the level of PER is changed the response cannot be called spontaneous, because it depends on a certain fator. Such factor may be state of the bee colony, its activity in different seasons, and so on.

The aim of the present study was to check our assumption on the nature of proboscis extension to odour before the conditioning procedure. To this end, we investigated the responses of the worker bees to the same dose of queen extract odour (pheromone) during different seasons and defined the importance of the state of the colony in this process, too.

The odour of the queen is vital for the functioning of the colony and is not of a seasonal character. Gerber et al. [2] maintain that the odours of food sources have the seasonal character and influence the eliciting of PER. Thus, the use of queen

* Corresponding author: Algirdas Skirkevièius, Vilnius Pedagogical University, Studentu 39, LT-2034, Vilnius, Lithuania, e-mail: algskirk@ktl.mii.lt

extract odour allows us to eliminate the seasonality of the odour and to escape its influence on the results of the experiment.

We turned attention to three attributes that well describe responses of bee workers: (1) the number of stimulations required for eliciting the first PER; (2) the number of stimulations, repetition of which elicited PER; (3) the number of workers that showed PER.

We hope that the present study will contribute to our understanding of the reasons of PER before the conditioning procedure.

MATERIAL AND METHODS

The study was carried out in Lithuania during 2 years (in April–August of 2001 and 2002). In the first half of each month, 40–60 individuals were investigated. In total, 542 workers were studied. We tested workers that were taken from two honeybee *Apis mellifera carnica* Pollm colonies. The first colony was unreformed (control), the second colony was reformed (it contained a decreased number of young workers).

Description of honeybee colonies. *The first (control) honeybee colony.* It was hived in a 16-frame (435 × 300 mm) standard hive. The colony contained brood at all stages, a sufficient number of workers to cover the brood nest adequately, the mated egg-laying queen, honey and pollen.

The second (reformed) honeybee colony. It was hived in a 16-frame (435 × 300 mm) standard hive. The colony contained: brood at all stages, a sufficient number of workers to cover the brood nest adequately, the mated egg-laying queen, honey and pollen. In this honeybee colony we largely decreased the number of young workers. It was made as follows. First of all (in May) we removed five frames with sealed brood and young workers and after this we weekly removed two frames with sealed brood with a week's interval.

Preparing worker bees for experiments. Worker bees were taken from a honeybee colony and caged (the cage was 160 mm in length and 30 mm in diameter). Later cages were placed into a refrigerator and kept there for a few minutes to reduce the activity of bees so that we could easily fix them in the test-stand. The animals were used for experiments approximately 30 minutes after fixing [12].

Test. Before each test, a 0.01 ml drop of ethanol solution of odorous substance was placed on a glass stick. After a few minutes (4–5 min), when the solvent (ethanol) evaporated, the stick with olfactory stimulus was delivered to the worker's head 5 mm from the antennae (the stick did not touché the antennae). The stimulation lasted 5 seconds with intertrial intervals of 60 seconds. Each worker received five stimulation trials. In turn, each response was recorded in all workers. The experiments were conducted in the room at a temperature of 20–25 °C.

Preparation of the queen extract. Mated egg-laying queens were placed in a flask and soaked in ethanol. The collected material was kept in the refrigerator at a temperature of 4 °C [13]. The extract was calibrated according to the amount of *E*-9-oxo-2-decenoic acid (9-ODA). The queen extract containing 100–150 μg of 9-ODA [13] was equated to one queen equivalent (Qeq). For the stimulation of workers, we used 0.01 ml of the extract that contained 0.1 μg 9-ODA (established by Dr V. Apðegaitë at the Institute of Ecology, Vilnius University). Consequently, the dose of the stimulus was 0.001 of the queen equivalent.

Data analysis. The data are presented as the percentage of worker bees responding with proboscis extension to the olfactory stimulus (PE%). The histogram demonstrated the distribution of the number of stimulation trials that succeeded in eliciting the first PER of the worker with the olfactory stimulus. The distribution of the number of repetitive stimulation trials which elicited PER is shown in the same way. Also, the skewness and standard error skewness were estimated.

A Shapiro–Wilk W test was first applied to analyze how the data corresponded to the hypothesized uniform distribution. When the distribution was found to be nonuniform, for comparison of the data nonparametric methods were used. To determine statistically significant differences in the number of stimulations that elicited the first proboscis extension and the number of proboscis extensions to five stimulations, we used the Kruskal–Wallis (*H*) test for independent samples. The Mann–Whitney (U) test followed by the Kruskal–Wallis (*H*) test was used to identify significant monthly differences in the number of workers with PER. The Duncan test was used to identify significant differences in the number of workers with PER between the first and second honeybee colonies.

All means are presented as ± one standard error. All statistical tests were performed with Statistica and SPSS.

RESULTS

The results of the research (in April–August of 2001 and 2002) showed that 113 workers out of 542 responded with proboscis extension to the queen extract odour prior to a food reward (i.e. before the conditioning procedure), which made up 20.8% of workers. In our experiments, there were no workers that extended proboscis in the absence of this stimulus.

Number of stimulations required for eliciting the first PER. For the first PER to be elicited, 1.9 ± 0.08 stimulations were needed. The distribution of stimulations revealed that one stimulation trial was sufficient to elicit response in 50.3% of workers (cal-

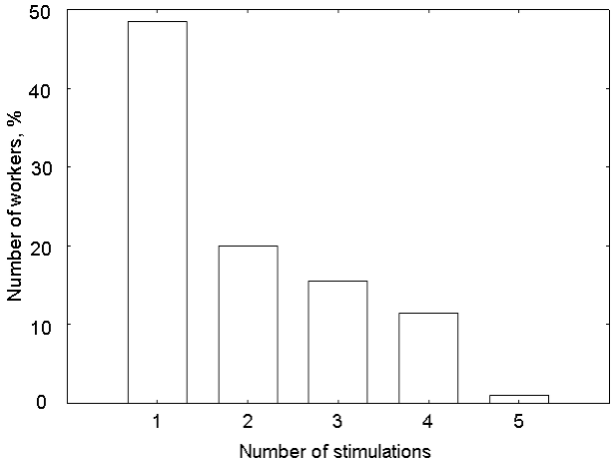

Fig. 1. Distribution of the number of stimulations that elicited the first proboscis extension response (PER) of a worker.
Stimulation with queen extract (0.001 Qeq.) was repeated five times. N = 113

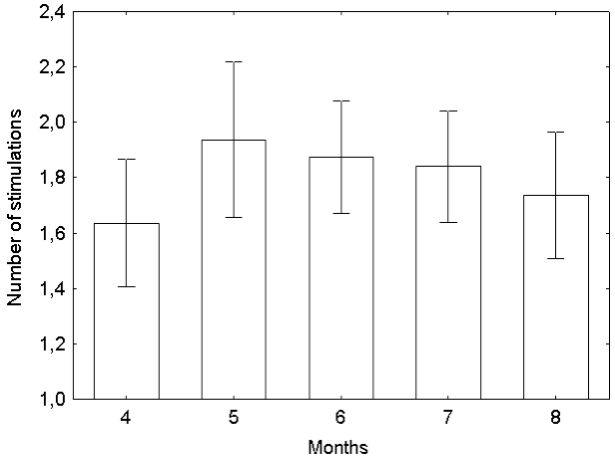

Fig. 2. Dynamics of the average number of stimulations that elicited the first PER in different months.
Columns represent the average number of stimulations. Whiskers indicate the standard error of mean. Stimulation with queen extract (0.001 Qeq) was repeated five times. N = 113

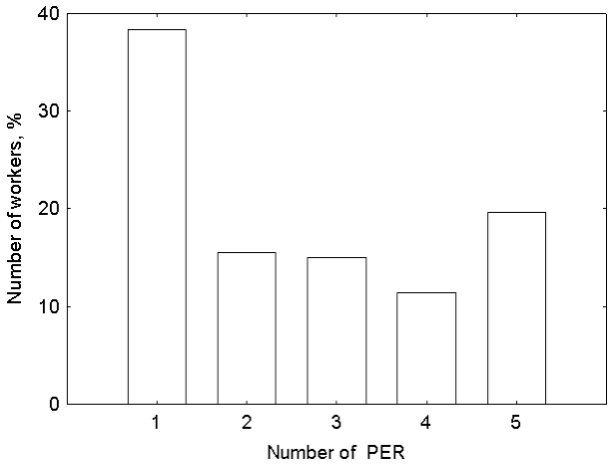

Fig. 3. Distribution of the number of stimulation repetitions which elicited PER.
N = 113. For other explanations, see Fig. 1

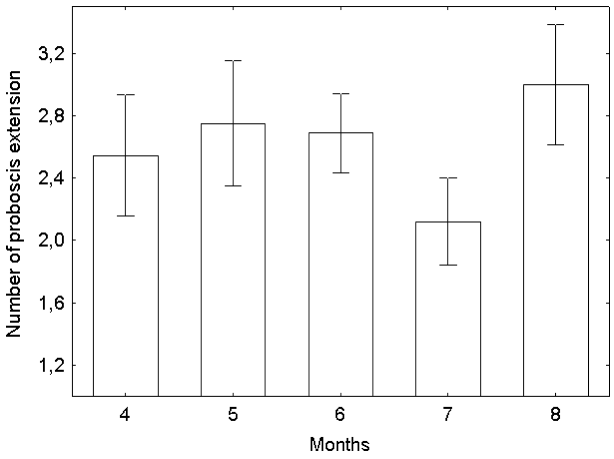

Fig. 4. Seasonal variation of the average number of proboscis extension to honeybee queen extract odour (0.001 Qeq). Columns represent the average number of proboscis extensions. Whiskers indicate the standard error of mean. For other explanations, see Fig. 2

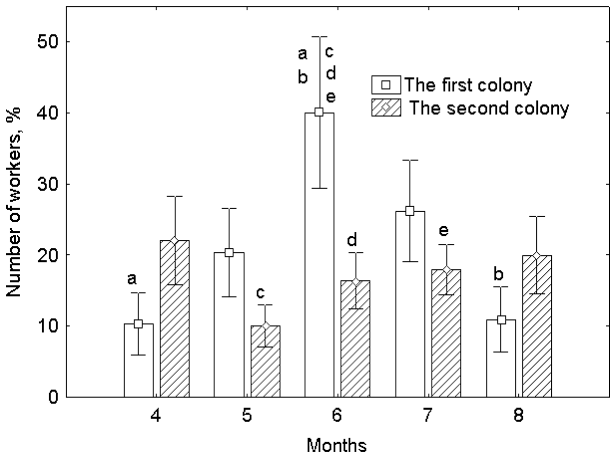

Fig. 5. Dynamics of the average number of workers that responded with proboscis extension to the odour of queen extract (0.001 Qeq.) from two colonies in April–August.
Columns represent the average percentage of bees showing PER. Whiskers indicate the standard error of mean. 4 – April, 5 – May, 6 – June, 7 – July, 8 – August. The first colony was unreformed (control). The second colony was reformed (removed part of sealed brood). Columns with different letters indicate significant differences (Mann–Whitney and Duncan tests). N = 54

culations made on the basis of all responding workers), whereas 20.7% of workers responded only to the second stimulation, 16.1% to the third, 11.9% to the fourth, and 1.0% to the fifth (Fig. 1).

The positive asymmetry is specific to this distribution (skewness = 0.85, std. err. Skewness = 0.17). It shows that some reasons might exist that predetermine the first PER at the number of stimulations lower than average. Thus, if the first stimulation did not elicit PER, then the probability of eliciting such response decreased with each repetition of stimulation. Upon the fifth stimulation, such probability nearly disappeared.

The average number of stimulations required for eliciting the PER of workers was stable from April to September: Kruskal–Wallis test (H = 11.09, df = 11, 193, p = 0.43) did not show statistically significant differences (Fig. 2). Thus, changes occurring in the honeybee colony during April–September did not affect the number of odour stimulations required for eliciting the first PER prior to a food reward.

Number of stimulations which if repeted elicited PER. The PER was elicited in 2.6 ± 0.11 of five stimulations. 38.3% of worker bees responded only to one stimulation out of five, whereas 15.5% esponded to two, 15.0% to three, 11.4% to four and 19.7% to five stimulations (calculations made on the basis of all responding workers). The positive asymmetry is specific to this distribution (skewness = 0.41, std. err. skewness = 0.17). A higher number of workers responding to a lower number of stimulations than the mean (Fig. 3) indicates that there must be some reasons suppressing PER to repeated odour stimulations.

The workers that responded more than once to repeated stimulations made up 60.7%, which is 1.6 times more than those responding to the first stimulation. The workers responding to all five stimulation trials constituted 1/3 of the workers that responded more than once.

The average number of stimulations (Fig. 4) that elicited the PER of the worker bees was stable from April to September: the Kruskal–Wallis test (H = 19.65, df = 11, 193, p = 0.05) did not show statistically significant differences (Fig. 4). Consequently, changes occurring in the honeybee colony over April–August did not affect the number of proboscis extensions elicited by odour stimulations.

Number of workers that showed PER. *The first (control) honeybee colony.* The Kruskal–Wallis test (H = 8.74; df = 4, 28; P = 0.06) showed that the number of workers responding with proboscis extension to odour tended to vary (Fig. 5) in April–August. However, a consistent pattern was obtained by the use of the Mann–Whitney test. It gives a possibility to compare results of all months in twos.

In April, honeybee queen extract elicited PER on average in 10.3% of worker bees (Fig. 5). In May, the number of these workers was 20.3%, however, the increase was not statistically significant (Mann–Whitney test: U = 5.5; N_1 = 4; N_2 = 5; P = 0.28).

Responding workers showed a particular increase in number in June, because the PER level to the queen extract odour went up to 40.1% of worker bees, *i.e.* was fourfold higher than in April and the difference was statistically significant (Mann–Whitney test: U = 2.0; N_1 = 4; N_2 = 6; P = 0.03). However, the difference of the PER level between June and May was not statistically significant (Mann–Whitney test: U = 8.0; N_1 = 5; N_2 = 6; P = 0.24).

In July, honeybee queen extract elicited PER on average in 26.2% of worker bees. The difference of the PER level in June and in July was not statistically significant (Mann–Whitney test: U = 10.5; N_1 = 6; N_2 = 6; P = 0.24). In August, the level of PER decreased to 10.9% of worker bees, *i.e.* it was as high as in April (Mann–Whitney test: U = 13.0; N_1 = 4; N_2 = 7; P = 0.92). The difference between the PER level in July and in August was not statistically significant either (Mann–Whitney test: U = 11.0; N_1 = 6; N_2 = 7; P = 0.18). However, the difference of the PER level between June and August was statistically significant (Mann–Whitney test: U = 5.0; N_1 = 6; N_2 = 7; P = 0.02).

Consequently, the level of PER to the queen extract odour is not stable in April–August. In April and August it is lower than in June. Thus, it is lower in the less active period of the honeybee colony than in the active period.

The second (reformed) honeybee colony. Kruskal–Wallis test (H = 4.11; df = 4, 26; P = 0.39) showed that the number of workers responding with proboscis extension to odour was the same in April, May, June, July and August (Fig. 5).

A comparison of the results of research for both colonies revealed differences in the behaviour of their workers. The level of PER did not change in workers of the second honeybee colony and made on average 17.1% of worker bees. The level of PER changed in workers of the first honeybee colony. It was maximum in June, whereas in May and July it made on average 23.0% of worker bees, *i.e.* as high as in the second honeybee colony (Duncan test: MS = 230.01, df = 44.0, P > 0.05). In May, honeybee queen extract elicited PER on average in 16.3% of worker bees in the second honeybee colony, *i.e.* by 23.7% more in the first honeybee colony. These differences are statistically significant (Duncan test: MS = 230.01, df = 44.0, P = 0.03). Thus, the comparative analysis of workers' behavior showed that the decrease of the number of young workers in a honeybee colony suppressed the increase of the level of PER in June.

DISCUSSION

The data of our research have demonstrated that proboscis extension response (PER) even in 40% (on average in 20.8%) of honeybees which can be elicited by an olfactory stimulus (queen extract odour: dose 0.001 Qeq) before the conditioning procedure is not accidental and can be controlled.

About one half of such workers respond with proboscis extension when they are stimulated for the first time. If a worker did not respond with proboscis extension upon being stimulated for the first time, then each repetition of stimulation decreased by half the probability of the extension (Fig. 1). On the other hand, if the odour stimulation elicits the first proboscis extension, more than half (60.7%) of responding

workers respond to a repeated stimulation (Fig. 2). The positive asymmetry of histograms of the number of stimulations required for eliciting the first proboscis extension (Fig. 1) and the number of responses to five stimulations (Fig. 3) allow us to presume that the possibility of proboscis extension is controlled.

This proposition supports our results of experiment with a number of workers that responded with proboscis extension to the odour of queen extract (Fig. 5), but the effect was not equal on the all parameters tested. The changes occurring in the honeybee colony over April–August had no influence on the average number of odour stimulations required for eliciting PER (Fig. 2) and on the average number of responses elicited by repeated stimulations (Fig. 4). However, they influenced the average number of workers that responded with proboscis extension to the odour of queen extract (Fig. 5, first honeybee colony). Most importantly we managed to change this parameter by artificial means too *i.e.* by removing five frames with sealed brood and young workers (Fig. 5, second honeybee colony).

So far we have no conclusive explanation as to the biological point of this behaviour of worker bees. Nevertheless, we may suggest that proboscis extension before the conditioning procedure is the result of stimulation with an olfactory stimulus, on the one hand; on the other hand, there are some conditions necessary to elicit this response. Thus, it cannot be accidental or spontaneous. In our experiments, these were no workers to extend proboscis in the absence of any stimulus.

The term "spontaneous" originates from the Latin word 'spontaneus', which means self-contained or having no clear relation with external or internal changes in the body [14]. Thus, the current presumption [4, 6, 11] that this local motor response can be regarded as spontaneous is highly questionable.

The results of our investigation are in support of the opinion of Erber [11], Menzel [3] and other authors that it is essential to detect all the workers responding with proboscis extension to such conditioned stimuli prior to applying a food reward, and to remove them from investigations. Otherwise the results can be inaccurate.

CONCLUSIONS

1. Honey bee queen extract (dose, 0.001 queen equivalent) odour elicited proboscis extension response (PER) even in 40% (on average in 20.8%) of workers before the conditioning procedure.

2. The changes occurring in the honeybee colony over April–August had not influence the average number of odour stimulations required for eliciting PER and the average number of responses elicited by repeated stimulations. However, they influenced the level (the number of individuals) of PER.

3. The PER that elicits the honeybee queen extract before the conditioning procedure cannot be regarded as spontaneous, because the experimentor can change its level by artificial means (removing part of young workers from the honeybee colony).

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A. Skirkevièius, L. Blaþytë-Èereðkienë

BIÈIØ DARBININKIØ (*Apis mellifera carnica* POLLM.) ATSAKAI LIEÞUVËLIO IÐKIÐIMU, KURIUOS SUKELIA BIÈIØ MOTINOS EKSTRAKTO KVAPAS PRIEÐ SÀLYGINIO REFLEKSO FORMAVIMÀ

Santrauka

2001–2002 m. balandþio–rugpjûèio mën. buvo tiriama bièiø darbininkiø *Apis mellifera carnica* Pollm. atsakai lieþuvëlio iðkiðimu (ALI), kuriuos sukelia apvaisintø bièiø motinø eks-

trakto kvapas (dozë 0,001 motinos ekvivalento) prieð pastiprinimà maistu. Iðtirtos 542 darbininkës. 20,8% ið jø pavyko sukelti ALI. Ið visø darbininkiø, kurioms buvo sukeltas ALI, 50,3% darbininkiø sukëlë pirmas stimuliavimas, 20,7% – antras, 16,1% – treèias, 11,9% – ketvirtas, 1,0% – penktas. 38,3% darbininkiø sureagavo tik á vienà ið penkiø stimuliavimø, 15,5% – á du, 15,0% – á tris, 11,4% – á keturis, 19,7% – á penkis. Vidutinis stimuliavimø skaièius (1,9 ± 0,08), kuris sukelia ALI, nekito visà tyrimø laikotarpá (balandþio – rugpjûèio mën.). Nekito ir vidutinis skaièius (2,6 ± 0,11 ið penkiø stimuliavimø) atsakø, kuriuos sukelia pakartotiniai stimuliavimai. Taèiau pokyèiai bièiø ðeimoje padarë poveiká reaguojanèiø darbininkiø skaièiui, t. y. ALI lygmeniui. Kontrolinës bièiø ðeimos darbininkiø ALI lygmuo tyrimø laikotarpiu kito maþdaug nuo 10% (balandþio ir rugpjûèio mën.) iki 40% (birþelio mën.). Tuo tarpu bièiø ðeimos su maþesniu jaunø darbininkiø skaièiumi jis buvo apie 17% ir iðliko pastovus visà tyrimø laikotarpá. Taigi ðioje ðeimoje padaryti pakeitimai slopino ALI lygmens birþelio mën. padidëjimà, kuris buvo kontrolinëje bièiø ðeimoje. Tai rodo, kad darbininkiø ALI, kuriuos sukëlë bièiø motinø ekstrakto kvapas prieð pastiprinimà maistu, nëra atsitiktiniai. Ðá teiginá taip pat paremia stimuliavimø skaièiaus, sukëlusio pirmà ALI (asimetrijos koeficientas lygus 0,85), ir reaguojanèiø á penkis stimuliavimus darbininkiø skaièiaus (asimetrijos koeficientas lygus 0,41) histogramø teigiama asimetrija. Pateikti faktai leidþia teigti, kad ALI, kuriuos sukelia apvaisintø bièiø motinø ekstrakto kvapas prieð pastiprinimà maistu, nederëtø vadinti spontaniðkais. Darbininkes, kurioms pavyksta sukelti ALI prieð pastiprinimà maistu, ið sàlyginio reflekso tyrimø su olfaktoriniais stimulais reikia paðalinti, nes gali bûti iðkreipti tyrimø rezultatai.