THE WORKERS' (*Apis mellifera carnica* Pollm.) RESPONSE WITH PROBOSCIS EXTENSION TO THE ODOUR OF QUEEN EXTRACT

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S u m m a r y

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 monthly over a two-year period. Workers were of unknown age. In total, 1261 workers were studied. Of them, 15.3% workers showed PER before a food reward had been applied. of all responding workers, 50.3% responded to the first, 20.7% to the second, 16.1% to the third, 11.9% to the fourth and 1.0% to the fifth stimulation. of all workers, 38.3% responded only to one of five stimulation trials, whereas 15.5% to two, 15.0% to three, 11.4% to four, and 19.7% to five. Neither the average number of stimulations (1.9) required for the eliciting of PER nor the average number of responses (2.6) elicited by the repetition of odour stimulation in different months were affected by seasonal changes in the honeybee colony's activity.

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

INTRODUCTION

During the process of conditioning, an olfactory stimulus sometimes can elicit a low-level of honeybee workers' proboscis extension response (PER) before a food reward is applied. For example, the PER to linalool as an olfactory stimulus on its first delivery totalled to 17% of worker bees (Sandoz et al. 1995), to geraniol (Ray & Ferneyhough 1997), hexanal and 1-hexanol (Bhagavan et al. 1994) from 3% to 30% of worker bees. In general, olfactory stimuli elicit the PER in up to 10% of workers (Menzel & Müller 1996). The reasons why olfactory stimulus can elicit PER before a food reward are unknown. On this point, only certain assumptions exist. According to the opinion of Pelz et al. (1997), if the olfactory stimulus elicits the PER of the worker, the bee is likely to have experienced it somewhere else. Erber (1980) considers that the season is important here. Gerber et al. (1996) reported that the season predetermined the seasonal character of food sources.

The answer to the question why the olfactory stimulus can elicit the PER prior to a food reward is important not only when PER is used as a method, but also when the behaviour mechanisms of workers (in particular elicited by odours) are investigated. While looking for the answer to this difficult question, it is worthwhile to investigate the stability of PER, or first of all the dependence of PER on the activity of the bee colony in different seasons.

The aim of the present study was to investigate the responses of worker bees to

the same dose of queen extract odour during different seasons and different activity of the honeybee colony. The odour of the queen is vital for the functioning of the colony and is not of a seasonal character. This can contribute to our understanding of the reasons and origin of PER.

MATERIAL AND METHODS

Worker bees *Apis mellifera carnica* Pollm. of unknown ages were investigated. They were taken from a normal honeybee colony. the colony was hived in a 16-frame (435x300 mm) standard hive. The colonies contained brood in all stages, sufficient number of workers to cover adequately the brood nest, a mated egg-laying queen, honey and pollen. The experiments were set up in September 2000 and finished in October 2002. In the first half of each month, 30-60 individuals were investigated. In total, 1261 workers were studied.

The olfactory stimulus was the odour of the mated honeybee queen's extract, the dose of which corresponded to 0.001 of queen equivalent (Qeq). This dose is ten times lower than the threshold dose necessary for eliciting the licking response (Skirkevičius & Skirkevičienė 1996a).

The queen extract was prepared as follows. 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 (Apšegaitė & Skirkevičius 1995). 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 (Slessor et al. 1988, Apšegaitė & Skirkevičius 1995) was equated to one Qeq. Thus the extract contained 0.1 μg 9-ODA (established by Dr V. Apšegaitė, the Senior Researcher at the Laboratory of Chemical Ecology and Behaviour, Institute of Ecology, Vilnius University).

Worker bees were prepared for experiments applying the previously described methods (Skirkevičius et al., 2000). At first, workers were 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 in order to reduce the bees' activity so that we could easily fix them in the test-stand. The animals were used for experiments approximately 30 minutes after fixing.

Before every test, a 0.01 ml drop of ethanol solution of odorous substance was placed on a glass stick. After a few minutes, after the solvent (ethanol) evaporated, the stick with olfactory stimulus was delivered to the worker's antennae. Each worker received five stimulation trials. The stimulation lasted for 5 seconds with inter-trial intervals of 60 seconds. The experiments were conducted in the room at a temperature of 16 – 25°C.

The data are presented as the percentage of worker bees responding with proboscis extensions to the olfactory stimulus (PE%). The histogram demonstrates 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 measure of location (mean, median, mode), measure of dispersion (variance, standard deviation) and measure of frequency distribution forms (kurtosis, std. err. kurtosis, skewness, std. err. skewness) were estimated.

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 and One-way ANOVA.

Linear regression was used to determine the relation between x (month) and y

(number of stimulations or number of proboscis extensions).

RESULTS

Out of 1261 workers, 193 responded with proboscis extension to the queen extract odour prior to a food reward (i.e. before the conditioning procedure), which makes up 15.3% of workers. The distribution of stimulation number revealed that one stimulation trial was sufficient to elicit respond of 50.3% of workers (calculations 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 (mean=1.9, SE=0.08, median=1, mode=1, variance=1.23, std. dev.=1.11, kurtosis= =-0.52, std. err. kurtosis=0.348, skewness=0.85, std. err. skewness=0.174). 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.

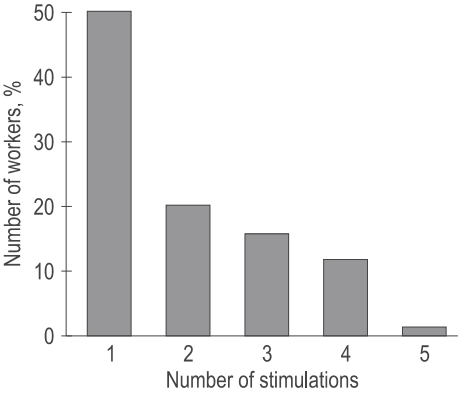

Fig. 1. Distribution of the number of stimulations that elicited the first PER of a worker. The stimulation with queen extract (0.001 Qeq.) was repeated five times. Data obtained in 2000-2002 are presented. N=193.

The average number of stimulations required for eliciting the PER of workers was stable from January to December: Kruskal-Wallis test (H=11.09, df=11, 193, p=0.4352) and One-way ANOVA test (F=0.77; df=11, 181; p=0.6684) did not show statistically significant differences (Fig. 2), whereas statistical estimation of the relation between x (month) and y (number of stimulations), expressed as a linear regression equation (y=2.1556- -0.0203*x), showed that month does not influence the number of stimulations (r=-0.0159, p=0.8267). Thus, seasonal

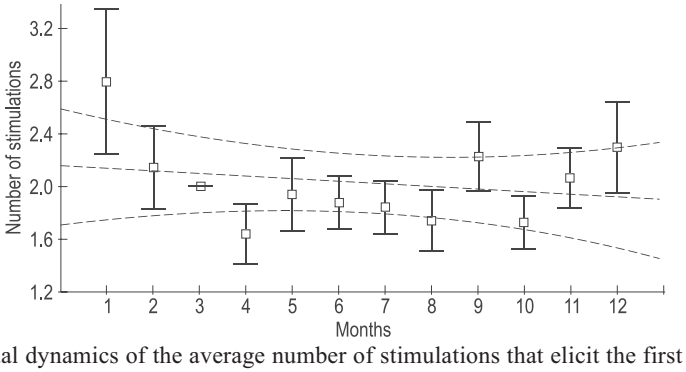

Fig. 2. Annual dynamics of the average number of stimulations that elicit the first PER. Points represent the average number of stimulations. Whiskers indicate the standard error of mean. The right line represents the estimated model (y=2,1556-0,0203*x). Crooked lines rep-resent a 95% confidence interval for the regression line. Stimulation with queen extract (0.001 Qeq) was repeated five times. The total data for the period 2000–2002 are presented. N=193.

changes occurring in the honeybee colony during the year did not affect the number of odour stimulations required for eliciting the first PER prior to a food reward.

38.3% of worker bees responded only to one stimulation out of five, whereas 15.5% 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), respectively. The positive asymmetry is specific to this distribution (mean=2.6, SE=0.11, median=2, mode=1, variance=2.43, std. dev.=1.55, kurtosis=-1.36, std. err. kurtosis=0.348, skewness=0.41, std. err. skewness=0.174). The higher number of workers responding to a lower number of stimulations than the mean (Fig. 3) indicates that there must be some reasons suppressing the PER to repeated odour stimulations.

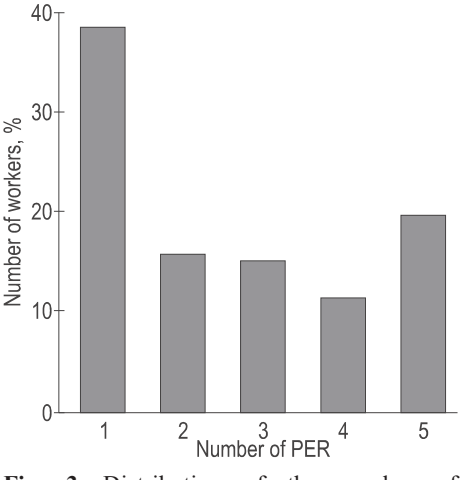

Fig. 3. Distribution of the number of stimulations, repetition of which elicited the PER. Stimulation with queen extract (0.001 Qeq) was repeated five times. Data for 2000–2002 are presented. N=193.

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.

On average, only 2.6±0.11 of five stimulations (Fig. 4) elicited the PER of the worker bees. This number was stable from January to December: The Kruskal-Wallis test (H=19.65, df=11, 193, p=0.0504) did not show statistically significant differences (Fig. 4) and the statistical estimation of the relation between x (month) and y (number of responses), expressed as linear regression equation (y=4.4110+ +0.00165*x), showed that month does not influence number of responses (r=0.0405, p=0.9813). Consequently, the seasonal changes occurring in the honeybee colony over the year did not affect the number of proboscis extensions elicited by odour stimulations.

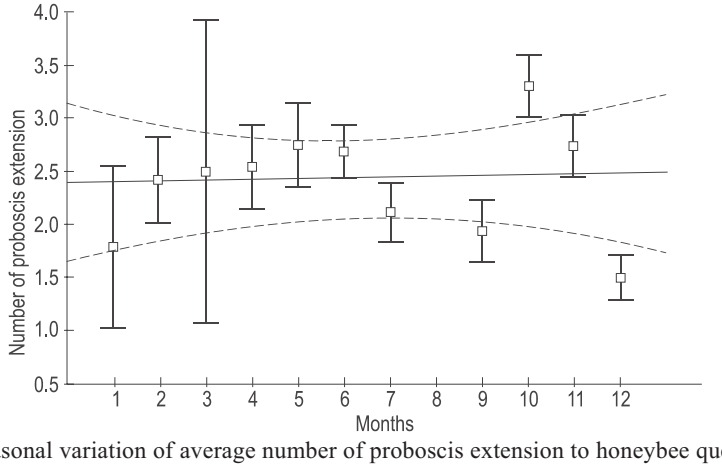

Fig. 4. Seasonal variation of average number of proboscis extension to honeybee queen extract odour (0.001 Qeq). Points represent the average number of proboscis extensions. Whiskers indicate the standard error of mean. The right line represents the estimated model (y =4.4110+0.00165*x). Crooked lines represent a 95% confidence interval for the regression line. Total data for the period 2000–2002 are presented. N=193.

DISCUSSION

The data of our research demonstrate that 15.3% of workers respond with proboscis extension when they are stimulated with a queen bee extract (dose 0.001 Qeq). About one half of such workers respond with proboscis extension when they are stimulated for the first time. If the worker did not respond with proboscis extension upon being stimulated for the first time, then each repetition of stimulation decreased the probability of the extension twice (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 the repeated stimulation (Fig. 2). The 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. Consequently, proboscis extension is the result of stimulation with the olfactory stimulus and naturally it can be called proboscis extension response (PER), excluding the case of accidental or spontaneous response. The term "spontaneous" originates from the Latin word 'spontaneus', which means self-contained or destitute of any clear relation with external or internal changes in the body (Gazenko 1987 et al.). Thus, the current presumption (Erber 1980; Sandoz et al., 1995; Menzel & Műller, 1996) that this local motor response can be regarded as spontaneous is highly questionable.

Great changes occur in the honeybee colony's life during the year. They result in an increased sensitivity of workers' olfactory receptors to the queen extract in summer (Skirkevičius & Skirkevičienė, 1996b; 1999). Consequently, workers in our experiment must have been more sensitive to queen extract odour in summer months than in winter. However, neither different sensitivity of workers to the queen extract odour, nor other changes occurring in the honeybee colony over a year influenced the average number of odour stimulations required for eliciting PER in different months (Fig. 2). The above-mentioned changes also did not influence the average number of responses elicited by repeated stimulations (Fig. 4). We have no conclusive explanation as to the biological point of this behaviour of worker bees. The analysis of the results of our investigation are in support of the opinion of Erber (1980), Menzel (1990) 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. Honeybee queen extract (dose 0.001 queen equivalent) odour elicited PER in 15.3% of workers before a food reward had been applied.
2. Of all responding workers, 50.3% responded to the first, 20.7% to the second, 16.1% to the third, 11.9% to the fourth and 1.0% to the fifth stimulation. of all workers, 38.3% responded only to one of five stimulation trials, whereas 15.5% to two, 15.0% to three, 11.4% to four, and 19.7% to five stimulation.
3. Seasonal changes in the honeybee colony's activity affect neither the average number of stimulations (1.9) required for eliciting the PER, nor the average number of responses (2.6) elicited by the repetition of odour stimulation in different months.

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REAKCJA ROBOTNIC (*Apis mellifera carnica* Pollm.) POLEGAJĄCA NA WYCIĄGNIĘCIU JĘZYCZKA NA ZAPACH EKSTRAKTU Z MATEK

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

W procesie kondycjonowania bodziec zapachowy może czasami wywołać reakcję niskiego poziomu wyciągnięcia języczka (PER) przed podaniem pokarmu. Np. reakcja PER na linalol jako na bodziec zapachowy podczas pierwszego podania wystąpiła ogółem u 17% pszczół-robotnic (Sandoz i in., 1995), a na geraniol (Ray, Ferneyhough, 1997), heksanal i 1-heksanol (Bhagavan i in., 1994) u 3 do 30% robotnic. Ogólnie, bodźce zapachowe wywołują PER u do 10% robotnic (Menzel, Müller, 1996). Nieznane są przyczyny, dla których bodziec zapachowy może wywołać reakcję PER na podanie pokarmu.

Badaliśmy reakcję wyciągnięcia języczka (PER) u robotnic *Apis mellifera carnica* Pollm. na zapach ekstraktu z zapłodnionych matek pszczelich (dawka równoważna 0,001 matki). Doświadczenia były prowadzone co miesiąc w okresie dwóch lat. Wiek robotnic był nieznany. Ogólnie przebadano 1261 robotnic.

Ustalono, że 15,3% robotnic wykazywało reakcję PER przed podaniem pokarmu. W obrębie wszystkich reagujących robotnic 50,3% reagowało na pierwszą stymulację, 20,7% na drugą, 16,1% na trzecią, 11,9% na czwartą i 1% na piątą. W obrębie wszystkich robotnic 38.3% reagowało jedynie na jedną sesję bodźcową z pięciu, 15,5% na dwie, 15,0% na trzy, 11,4% na cztery i 19,7% na pięć sesji. Sezonowe zmiany w aktywności rodzin pszczelich nie wpłynęły zarówno na średnią liczbę stymulacji (1,9) potrzebnych do wywołania reakcji PER, ani na średnią liczbę reakcji (2,6) wywołanych przez powtórzenie bodźca zapachowego w różnych miesiącach.

Słowa kluczowe: *Apis mellifera carnica*, reakcja wyciągnięcia języczka (PER), utrwalanie odruchu, feromon matki pszczelej.