THE INFLUENCE OF HONEY ON (E)-9-oxodec-2-enoic acid CONTENT IN THE ALIMENTARY CANAL OF WORKER BEES (Apis mellifera L.)

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Summary

This study examines honey influence on (E)-9-oxodec-2-enoic acid (9-ODA) content in the alimentary canal of worker bees and its mechanism. It was tested on more than 40 samples with 10-20 worker bees in every sample. The 9-ODA analysis was carried out using gas chromatography. The results demonstrate that feeding and type of food influence the content of 9-ODA in the alimentary canal of worker bees that lick the queen. Honey decreases the content of 9-ODA both in vivo and in vitro and the process proceeds rather rapidly: in vivo, the 9-ODA content decreases to 15% in 20 min and in vitro to 63.5% in 5 min. The temperature inactivates the substance (or substances) and decreases the content of 9-ODA in honey. The alimentary canal of worker bees does not secrete a substance (or substances) to influence the content of 9-ODA in it.

Keywords: Apis mellifera L., worker bee, queen, mandibular gland, pheromone, (E)-9-oxodec-2-enoic acid, 9-ODA, enzyme, honey, alimentary canal.

INTRODUCTION

Our present understanding of queen mandibular gland pheromones is the product of multi-year research (Butler 1959, Verheijen-Voogd 1959, Pain 1961, Skirkevičius 1965, Free 1987, Winston 1987, Slessor et al 1988, Kaminski 1990, Wossler 2002). However, despite a significant progress, the mechanisms of transmitting queen pheromones in a bee colony is not yet fully understood or appreciated. One serious problems is the lack of information on the content of the queen’s pheromone after it enters the alimentary canal of a worker bee (licker).

It is known that usually one in every ten worker bees in the court licks the queen (Allen 1957, Skirkevičius 1965, 1968). Workers lick their queen when she is laying eggs and when she is not moving over the comb (Skirkevičius 1965, 1968, 1984; Free 1987), i.e. the licking lasts as long as the queen does not change her location. It is supposed that workers obtain the pheromone by licking the queen’s body and that the pheromones become distributed among workers in regurgitated food (Butler 1954, Winston 1987, Caron 1999, Graham 2003). Thus, the queen’s pheromones mix with food. This raises the question: is it a coincidence or does it have any biological significance?

There is evidence that if the queen’s pheromones are mixed with food containing sugar in concentrations of 5-10%, it loses its inhibiting effect (Verheijen-Voogd 1959, Van Erp 1960, Pain 1961 a,b). It has long been established that (E)-9-oxodec-2-enoic acid (9-ODA) - one of the most abundant components of the
queen mandibular gland pheromone - is converted into other substances in the alimentary canal of a worker bee. However, a small content of this acid remains in the alimentary canal of a worker bee for quite a long time: from 24 to 72 hours (Johnston et al. 1965). Later, the idea emerged that food must deactivate queen pheromones in the alimentary canal of a licker (Skirkevičius and Skirkevičiene 1981) and some enzymatic deactivation mechanism may take part in this process (Pain and Barbier 1981). Free (1987) doubts whether the queen's pheromone remains biologically active after a worker bee has swallowed it. To date, there has been no research that can contradict or corroborate either assumption.

Thus, the described results of studies (Verheijen-Voogd 1959; Van Erp 1960; Pain 1961 a,b; Johnston et al. 1965) and assumptions (Skirkevičius, Skirkevičiene 1981; Pain and Barbier 1981; Free 1987) cast doubt whether the queen's pheromone mix with food in the alimentary canal just for transmission through trophallaxis among the workers in a colony.

Only comprehensive studies can contradict or corroborate this doubt. Depending on the situation, special limitations were made intended to simplify research. First of all, not all components of queen pheromone were studied, but only 9-ODA as one of the best known components of the queen mandibular gland pheromone. Second, only honey was studied and it was assumed, that if the content of 9-ODA does not decrease after the inactivity of honey enzyme, and then process must be of enzymatic origin (the second hypothesis).

The purpose of this study was to test the first (Experiment I) and the second (Experiment II) hypothesis, i.e. it tested honey influences on 9-ODA content in the alimentary canal of a worker bee and its mechanism.

**MATERIALS AND METHODS**

**Experiment I: The influence of food on (E)-9-oxodec-2-enoic acid (9-ODA) in the alimentary canal of a worker bee.**

Initially, all workers licked the queen’s extract. After licking, some of the workers were kept unfed (group I), another group were fed a 50% sucrose solution (group II) and the rest were fed honey (group III). Two hours later, the worker bees were anaesthetized by cooling and dissected. The alimentary tract without the rectum was used for analysis (Dade 1962). Alimentary canals were triturated and extracted with hexane. Changes in the amount of the queen’s pheromone in the alimentary canal of a worker bee were estimated according to the content of 9-ODA in it.

4-12 samples were tested for each group, with 10-20 worker bees in each sample.

Natural 9-ODA was presented to worker bees, i.e. in the ethanol extract of mated egg-laying queens, because workers do not lick synthetic 9-ODA, but they lick its extract from mated egg-laying queens (Skirkevičius 1965).

As it was assumed that all tested worker bees would have an equal content of 9-ODA, the experiment was arranged as follows. The extract of mated egg-laying queens was loaded on the surface of a glass slide (8 x 35 mm) with a micropipette: the content corresponded to 1.11 µg of 9-ODA or 0.01 queen equivalent of the queen’s pheromone (Slessor et al. 1988, Apšegaite and Skirkevičius 1995). The glass slide was then placed on top of the
frame of the queenright colony until worker bees came to lick it. The lickers were covered with a small Petri dish until the worker bees licked the extract from the glass. After licking, the worker bees were unfed or fed.

**Experiment II: The effect of heating honey on (E)-9-oxodec-2-enoic acid (9-ODA) content.**

The heating of honey is known to reduce activity of most of its enzymes. Activity starts decreasing at a temperature of 60°C and disappears completely when the temperature reaches 100°C (Segel 1975). With this in mind, the following temperatures of honey were selected: +20, 50, 70, 90°C.

After heating to the required temperature, honey (1 g) was kept for about 20 min, then cooled to +20°C and 2 mg of 9-ODA was added. The obtained mixture was diluted with 10 ml of CH₂Cl₂ and kept for 5 min in the dark. Each test was repeated three times.

**Chemical analysis.**

The samples were evaporated to dryness in a rotary evaporator and methylated (Rogozinski 1964). To the methylated mixture, 0.2 ml of distilled water was added, and the aqueous layer was extracted with hexane (three times with 0.3 ml). The samples were dried and redissolved in 50 µl of hexane, of which 5 µl was then injected into the gas chromatograph. The GC analyses were performed on a Chrom-5 gas chromatograph equipped with a flame ionisation detector and a 3 m x 3 mm glass column. The stationary phase was 5% SE-30 coated on Chromaton N-AW, with a 6°C/min thermostat program ranging from 80 to 250°C. The temperature of the injector was 200°C and the temperature of the detector was 250°C. Nitrogen was used as the carrier gas at a flow rate of 30 ml/min.

**Data statistical analysis.**

Non-parametric methods were used (for small samples) for comparison of the data. To determine statistically significant differences in all experiments, the Kruskal-Wallis (H) test was used for independent samples. The Mann-Whitney (U) test followed by the Kruskal-Wallis (H) test was used to identify significant differences among the specific variables in experiments. The Spearman rank test was used to identify a correlation between the temperature of honey and the content of 9-ODA. All means are presented ± one standard error. All statistical tests were performed with Statistica software. The results of the statistical analysis were regarded as significant at p ≤ 0.05.

**RESULTS**

**Experiment I: The influence of food on (E)-9-oxodec-2-enoic acid (9-ODA) in the alimentary canal of a worker bee.**

The results showed that the content of 9-ODA in the alimentary canal of a worker bee depended not only on receiving food, but also on the sort of food it received after licking the queen’s extract: the Kruskal-Wallis test (H = 9.95, df = 2, N = 28, p = 0.007) showed statistically significant differences (Fig. 1).

If a worker bee did not receive any food after licking, two hours later 0.9 ±0.07 µg of 9-ODA was found in its alimentary canal. The situation was the same when worker bees were fed a sucrose solution, because 9-ODA was at 0.9 ±0.09 µg: the Mann-Whitney test (U = 72; Z = 0.0; N₁ = 12; N₂ = 12; p = 1.00) showed statistically insignificant differences.

The situation changed radically when worker bees were fed on honey after licking. The content of 9-ODA in their alimentary canal was 0.1±0.02 µg, i.e. 0.8±0.07 µg less (Mann-Whitney test: U = 0.0; Z = 2.9; N₁ = 12; N₂ = 4; p = 0.003) than in the alimentary canal of unfed bees. Thus, honey sharply decreased 9-ODA content in the alimentary canal of a worker bee in 2 h.
Figure 1. Mean content of (E)-9-oxodec-2-enoic acid (9-ODA) in the alimentary canal of a worker bee 2 hours after feeding or fasting.
No feeding - workers were unfed (N = 12), honey - workers were fed on honey (N = 4), sucrose - workers were fed on 50% sucrose solution (N = 12).
The horizontal line shows pairs of variants compared and significance (p) of the difference between them (Mann-Whitney U test). N - number of samples (sample size = 10-20 workers).

Figure 2. The effect of heating honey from +20 to 90°C on (E)-9-oxodec-2-enoic acid (9-ODA) content decreases.
Heated to the necessary temperature, honey (1 g) was kept for about 20 min, later it was cooled to +20°C and 2 mg of 9-ODA was added and the mixture was kept for 5 min. Each test was repeated three times. Bars with different letters significantly differ in 9-ODA content decreases at different temperatures of heating honey (Mann-Whitney U test; p < 0.001).
Experiment II: The effect of heating honey on (E)-9-oxodec-2-enolic acid (9-ODA) content.

The results showed that changing the temperature of honey from +20 to 90°C decreased 9-ODA content in it: the Kruskal-Wallis test ($H = 9.66, df = 3, N = 12, p = 0.02$) showed statistically significant differences (Fig. 2).

There was a correlation between 9-ODA level and honey temperature (Spearman Rank test $0.82, p < 0.05$).

If the honey was kept at a temperature of +20°C, 9-ODA content decreased by 36.4% within 5 min (Mann-Whitney test: $U = 0.0; Z = 1.9; N_1 = 3; N_2 = 3; p = 0.04$). When the temperature of honey was increased to 50°C, the level of 9-ODA decreased by 39.4% (Mann-Whitney test: $U = 0.0; Z = 1.9; N_1 = 3; N_2 = 3; p = 0.04$) during the same period of time. Although the 9-ODA level decreased by 2.9% more at +50°C than at +20°C, this difference was not statistically significant (Mann-Whitney test: $U = 2.0; Z = 1.09; N_1 = 3; N_2 = 3; p = 0.28$). If the honey was kept at a temperature of +70°C, the content of 9-ODA decreased by 19.1% in 5 min (Mann-Whitney test: $U = 0.0; Z = 1.09; N_1 = 3; N_2 = 3; p = 0.04$). The temperature of +90°C decreased the content of 9-ODA only by 0.6%, and this decrease was not statistically significant (Mann-Whitney test: $U = 2.2; Z = 1.09; N_1 = 3; N_2 = 3; p = 0.27$). Thus, if honey had been kept at a temperature of +90°C, it did not decrease the level of 9-ODA.

The results showed that the content of 9-ODA influenced the substances in a mixture whose activity was decreased by both temperature as well as enzymes (Segel 1975). Thus, it is possible to suppose that enzymes influence the content of 9-ODA in honey.

**DISCUSSION**

The study demonstrates that honey decreases the content of 9-ODA both in vivo, and in vitro and the process is rather rapid (Figs. 1, 2). These results correspond to those of Johnston et al. (1965) who found that 9-ODA was converted into other substances in the alimentary canal of a worker bee. However, the current results demonstrate for the first time that the substance (or substances) that triggers this conversion is contained in honey.

Enzymes of honey must be such releasers, because when honey is heated (inactivation of its enzymes), the content of 9-ODA in it does not decrease (Fig. 2). This is in agreement with Pain and Barbier (1981) that some enzymatic conversion mechanism is involved in this process. However, the enzymes are unknown.

The current study showed another important aspect of the issue: the alimentary canal of a worker bee does not secrete a substance (or substances) that may influence the content of 9-ODA, because when worker bees were fed a sucrose solution, the content of 9-ODA did not decrease (Fig. 1), while if they were fed honey, the content of 9-ODA decreased (Fig. 1). It shows that the trigger(s) of 9-ODA conversion must enter the alimentary canal of a worker bee together with honey.

The results of this study are important in discussion of the behavior of worker bees - particularly the queen licker. Usually after licking, the first behavioral act of worker bees is to get food from other worker bees (Velthuis 1972, Skirkevičius 1973). It means that the licking of certain substances from the queen causes a need for food in worker bees and this food influences the content of 9-ODA in their alimentary canal.

The results of this study (Figs. 1, 2) and others (Verheijen-Voogd 1959; Van Erp 1960, Pain 1961 a,b; Johnston et al. 1965; Skirkevičius and Skirkevičiene 1981; Pain and Barbier 1981; Free 1987) do not imply that the queen pheromone can be distributed among workers together with regurgitated food (Butler 1954, Winston 1987, Caron 1999, Graham 2003), be-
cause a picture of the mechanism blocking this possibility has begun to emerge.

CONCLUSIONS

— Feeding and the type of food influence
the content of (E)-9-oxodec-2-enoic
acid (9-ODA) in the alimentary canal
of worker bees that lick the queen.
Honey decreases the content of 9-ODA
both in vivo and in vitro, and the pro-
cess is rather rapid: in vivo 9-ODA
content decreases to 15% in 20 min
and in vitro to 63.5% in 5 min.
— The temperature inactivates the sub-
stance (or substances) and decreases
the content of 9-ODA in honey. There
is a correlation between honey temper-
ature and the content of 9-ODA in it: if
honey is kept at a higher temperature,
the level of 9-ODA in it is also higher.
— The alimentary canal of worker bees
does not secrete a substance (or sub-
stances) influencing the content of
9-ODA in it.

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WPŁYW MIODU NA ZAWARTOŚĆ KWASU (E)-9-oxodec-2-doc-ego W PRZEWODZIE POKARMOWYM PSZCZÓŁ ROBOTNIC (Apis mellifera L.)

S k i r k e v i č i u s A., R u k š ė n a s A.

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


W pracy badano wpływ miodu na zawartość kwasu (E)-9-oxodec-2-doc-enowego (9-ODA) w przewodzie pokarmowym pszczół robotnic oraz mechanizm tego zjawiska. Badania objęły ponad 40 prób zawierających po 10-20 pszczół robotnic. Analizę zawartości kwasu 9-ODA przeprowadzono przy pomocy chromatografii gazowej. Otrzymane w pracy wyniki świadczą o tym, że zarówno karmienie jak i rodzaj pokarmu wpływają na zawartość kwasu 9-ODA w przewodzie pokarmowym robotnic, które wyizolują królową. Miod obniża zawartość kwasu 9-ODA zarówno w warunkach in vivo jak i in vitro, a sam proces postępuje raczej szybko, tj. w warunkach in vivo zawartość kwasu 9-ODA zmniejsza się do 15% w ciągu 20 minut, podczas gdy w warunkach in vitro do 63.5% w ciągu 5 minut. Temperatura inaktywuje substancję (substancje), które powodują obniżenie zawartości kwasu 9-ODA. Wykazano zależność pomiędzy temperaturą miodu, a zawartością kwasu 9-ODA w miodzie, tj. zawartość kwasu 9-ODA utrzymuje się na wysokim poziomie wraz z wysoką temperaturą miodu. Przewód pokarmowy pszczół robotnic nie wydziela substancji mogącej (mogącej) wpływać na zawartość w nim kwasu 9-ODA.

Słowa kluczowe: Apis mellifera L., pszczoła robotnic, matka pszczeła, gruczoł żuchwowy, feromon, kwas (E)-9-oxodec-2-doc-enowy, 9-ODA, enzym, miód.