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Original article

Selective blocking of contact chemosensilla in *Apis mellifera*

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Abstract – The aim of this study was to investigate the effect of $ZnSO_4$ on the function of contact chemosensitive and olfactory sensilla of the worker honeybee antennae. The effect of $ZnSO_4$ on contact chemosensitive sensilla was tested behaviorally using the proboscis extension response and that on olfactory sensilla using electroantennogram recordings. We showed that antennal $ZnSO_4$-treatment significantly reduced the sugar-elicited proboscis extension response but did not reduce olfactory evoked electroantennogram responses. Both results indicate that $ZnSO_4$ selectively blocks contact-chemosensory and not olfactory perception. We suggest that $ZnSO_4$ ablation will be a powerful tool to investigate the role of contact chemosensory and olfactory sensilla in short range communication within the honeybee colony.

honeybee / olfaction / contact chemoreception / PER / EAG

1. INTRODUCTION

Chemical signals play an important role in the colony communication of honeybees, within and outside the hive (von Buttel-Reepen, 1900; von Frisch, 1965; Free, 1987; Seeley, 1998). Volatile odors of the Nasanov and sting glands are used to attract and alert nestmates over long distances, whereas odors of low volatility are thought to be used in nestmate and kin recognition, as well as in the detection of comb wax cues (Breed and Stiller, 1992; Breed, 1998; Fröhlich et al., 2000). Due to the low-volatility of the substances used in nestmate and kin recognition, it has been hypothesized that they are perceived by contact chemosensory and not by olfactory sensilla; however, experimental evidence does not exist. To understand whether honeybees use different chemosensory channels in different behavioral contexts, it is desirable to have a tool to selectively ablate specific chemosensory sensilla types. In crickets, Balakrishnan and Pollack (1997) established a method using zinc sulfate

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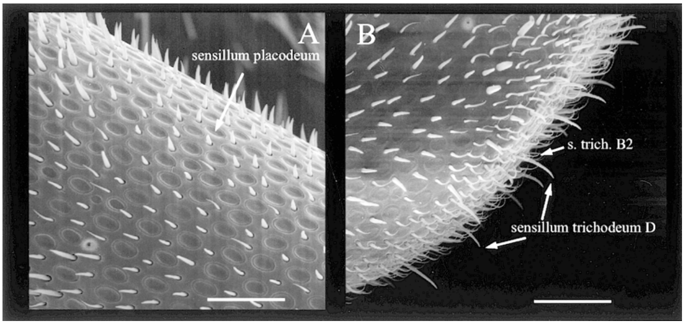

Figure 1. SEM-photograph of honeybee worker antenna showing 1A) sensillum placodeum and 1B) sensillum trichodeum D on the ventral side of the tip flagellar segment. Scale bar for both figures = 50 µm.

($ZnSO_4$) to silence antennal contact chemosensitive sensilla. In the present study, we investigated the effect of this treatment on the functioning of olfactory and contact-chemosensory sensilla of the honeybee antenna.

About 75% of circa 6500 sensilla per antenna are chemosensory (von Frisch, 1921; Lacher, 1964; Esslen and Kaissling, 1976). The main olfactory sensilla are the sensilla placodea or pore plates (Fig. 1A). Each sensillum placodeum consists of a 9 × 6 µm thin oval cuticular plate with numerous minute pores and is innervated by 15 to 30 neurons (Esslen and Kaissling, 1976). Electrophysiological recordings revealed that the receptor neurons of the sensilla placodea respond to a variety of plant and flower odors as well as to the components of the honeybee pheromones (Lacher and Schneider, 1963; Esslen and Kaissling, 1976; Vareschi, 1972). The sensillum trichodeum D (Fig. 1B) is the only morphologically identified contact chemosensitive sensillum on the honeybee antenna (Martin and Lindauer, 1966; Esslen and Kaissling, 1976). The characteristic terminal tip pore is the only passage for molecules to the inner lumen of the sensillum (Ozaki and Tominaga, 1999). The sensillum trichodeum D is the largest sensillum on the honeybee antenna, ranging between 23 µm and 43 µm. In comparison to the pore plates that are distributed over the entire antennal flagellum, the sensilla trichodea D are primarily located on the tip segment of the flagellum (Esslen and Kaissling, 1976). It is assumed that the sensilla trichodea D are used to examine the chemical composition of the wax during comb building (Martin and Lindauer, 1966). Esslen and Kaissling (1976) proposed that they are responsible for the perception of sugars. Additionally, they might perceive wax components of low volatility.

In our study, we investigated the effect of $ZnSO_4$-treatment, established by Balakrishnan and Pollack (1997), on these two sensilla types. The functioning of the sensilla trichodea D was tested behaviorally using the sugar elicited proboscis extension response (PER), and the functioning of the sensilla placodea was tested electrophysiologically using olfactory evoked EAG responses.

2. MATERIALS AND METHODS

2.1. Bees

The experiments were conducted from July to October 1999 at the Biozentrum of the University of Würzburg, Germany. In all experiments, adult worker bees of a single colony of *Apis mellifera* L. were used. Bees leaving the hive were caught at the hive entrance to ensure that the bees had an empty honey stomach. In the laboratory, the bees were anaesthetized with CO_2 and restrained in small brass tubes commonly used in conditioning experiments (Bitterman et al., 1983).

2.2. Antennal zinc sulfate treatment

The mounted bees were anaesthetized with CO_2 to immobilize the antennae for the treatment with the heavy metal salt zinc sulfate ($ZnSO_4 \times 7\ H_2O$, Roth, Germany). Each of the two antennae were stuck entirely in a capillary filled with one $ZnSO_4$ solution solved in 0.3% Triton X (Sigma, Germany) for 15 minutes. Five groups of bees were treated with a concentration of either 0.125 M, 0.25 M, 0.5 M, 0.75 M or 1.0 M $ZnSO_4$, respectively. In a control series, we assessed the effect of the time the bees spent in the brass tubes (untreated control group) and the effect of the solvent 0.3% Triton X (Triton X group). Additionally, we tested the response probability of each antenna in bees in which only one antenna was treated with 0.25 M $ZnSO_4$ to investigate the effect of $ZnSO_4$ on the motivation to respond to sugar.

2.3. Proboscis extension response assay

Prior to the $ZnSO_4$-treatment, the proboscis extension response (PER) level of the test bees was assayed by touching the antennae with a droplet of sucrose (Minnich, 1932). A 2.0 M sucrose solution was used to ensure that the restrained foragers elicit their proboscis with a high probability (Takeda, 1961; Page, 1998). The PER response of each bee was tested three times in intervals of 5 minutes (pre-treatment test). Only bees that responded at least once in the pre-treatment test were used for the behavioral experiments. After the pre-treatment test the antennae of the bees were treated with $ZnSO_4$. One hour and 24 hours after the antennal treatment we observed the PER response as described above (post-treatment tests). After the first post-treatment test, the bees were individually marked and released in wooden cages overnight (von Frisch, 1965).

2.4. Electroantennogram recordings

We determined the effect of $ZnSO_4$ on the olfactory sensilla using olfactory evoked electroantennogram (EAG) recordings. Untreated and $ZnSO_4$ treated bees were restrained in plastic holders and the scape and pedicel of both antennae were fixed to the head capsule. Between $ZnSO_4$-treatment and EAG-recordings, the bees were allowed to recover for one hour. The distal flagellar segment of the investigated antenna was removed and a saline-filled glass electrode was placed over the tip of the antenna. From the contralateral antenna, all flagellar segments with sensory sensilla were cut off and a second saline-filled electrode (the reference electrode) was placed over the scape, pedicel and the two remaining flagellar segments. Signals were amplified using a DC-coupled amplifier (DAM 50, WPI, Germany) which facilitated a 100× amplification, then digitized and analyzed with Spike 2 (CED, United Kingdom). The peak-amplitude of each EAG response was measured (Roelofs, 1984). During the recordings, charcoal-filtered and humidified air was continuously blown over the antenna with a flow rate of 900 ml/min. To apply the stimulus, a second air stream was led through a glass tube containing a filter of 1 cm^2 loaded with 10 µl of the test odors and was pulsed into the constant air flow. In the EAG experiments we determined dose-response curves of untreated antennae and antennae treated with one $ZnSO_4$ solution of either 0.25 M, 1.0 M or 5.0 M. We used citral (0.89 g/ml), geraniol (0.88 g/ml), isoamylacetate (0.87 g/ml) and jasmine

(1 g/ml) as test odors in concentrations of 1:1000, 1:100, 1:10 and undiluted.

2.5. Statistical analysis

The PER responsiveness between the different treatment groups was compared using the Mann-Whitney U-test and the results were corrected after Bonferroni from $\alpha < 0.05$ to $\alpha^* < 0.002$. To compare the PER responsiveness between treated and untreated antenna within single bees we utilized the Wilcoxon test. The EAG recordings were analyzed applying the Kruskal-Wallis-ANOVA. Results are presented as mean ± one standard deviation (S.D.). All statistical tests were performed with Statistica software.

3. RESULTS

3.1. Effect of $ZnSO_4$-treatment on the PER

During the entire experimental bee season (July to October, 1999) the PER responsiveness of worker honeybees (n = 303) to 2.0 M sucrose in the pre-treatment test remained constant at the high response level of 0.98 ± 0.02 (Fig. 2). Additionally, the probability of PER response of untreated bees did not decline within 24 hours. Treatment of the honeybee antennae with $ZnSO_4$ resulted in a drastic reduction of the PER responsiveness. This effect depended on the concentration of $ZnSO_4$. Additionally, the PER-reduction increased within the first 24 hours after treatment. Antennal $ZnSO_4$-concentration greater than 0.125 M led to a reduction of the response level to 0.01. The blocking effect of $ZnSO_4$ concentrations above 0.125 M were significantly stronger than the effect of the solvent 0.3% Triton X (Mann-Whitney U-test, $P < 0.001$).

Treatment of one antenna with 0.25 M $ZnSO_4$ led to a significant reduction of the PER elicited by the treated antenna (Wilcoxon, $P < 0.001$). In contrast the PER response elicited by the untreated contralateral antenna was not reduced (Fig. 3).

3.2. Effect of $ZnSO_4$-treatment on EAG amplitudes

The recorded EAG amplitudes of untreated antennae are similar to EAG recordings of worker bee antennae from other studies (Allan et al., 1987; Robinson, 1987; Vetter and Visscher, 1997). In all our experiments, the antennal $ZnSO_4$-treatment did not impair olfactory evoked EAG responses (Fig. 4A).

Moreover, the EAG peak amplitudes evoked by the test odors were not significantly different between treated and untreated

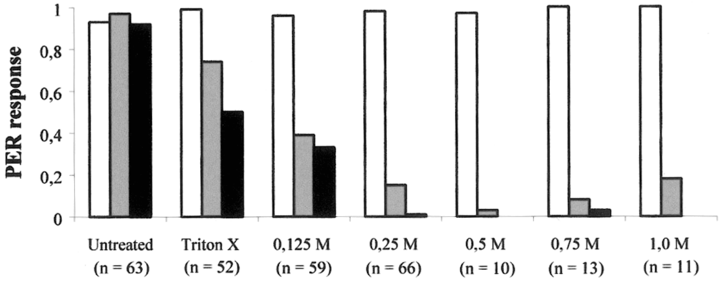

Figure 2. Concentration dependent reduction of the PER response one and 24 hours after $ZnSO_4$-treatment. White = pre-treatment, grey = 1 h after the treatment, black = 24 h after the treatment.

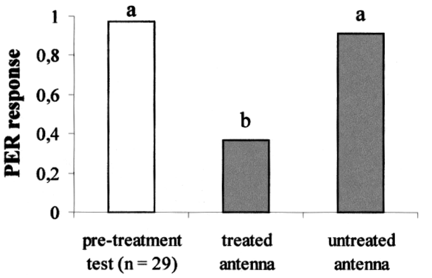

Figure 3. PER responsiveness of a 0.25 M $ZnSO_4$ treated antenna versus an untreated antenna in the same bee (n = 29). White = pre-treatment, grey = 1 h after the treatment.

antennae (Kruskal-Wallis-ANOVA, $p > 0.05$) and even treatment with the highest concentration of 5.0 M $ZnSO_4$ did not reduce the responses. Additionally, $ZnSO_4$ treated worker antennae showed normal concentration dependent dose response curves for all tested odors (Fig. 4B).

4. DISCUSSION

Our behavioral experiments revealed that $ZnSO_4$-treatment of antennae reduced the sugar elicited PER in worker honeybees. Additionally we showed that this effect was not a result of changing the motivational status of the bee. Due to the fact that the sensillum trichodeum D is the only contact chemosensitive sensillum on the bee antennae, we assume that the $ZnSO_4$-treatment led to a blocking of this sensillum type. In contrast, the EAG recording experiments did not show a reduction of the olfactory evoked responses in $ZnSO_4$-treated antennae. As all test odors are detected by the sensilla placodea, we assume that this olfactory sensillum type was not affected by the $ZnSO_4$-treatment. Both experiments indicate that $ZnSO_4$-treatment of honeybee antennae selectively blocked the contact chemosensitive sensilla while olfactory sensilla remained intact.

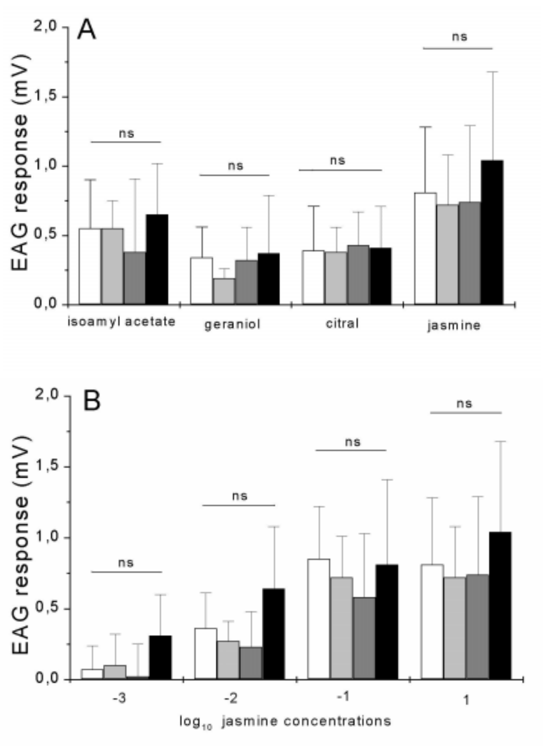

Figure 4 A. EAG response of untreated and $ZnSO_4$ treated antennae with respect to different odors. White = untreated antennae, bright grey = 0.25 M $ZnSO_4$ treated, dark grey = 1.0 M $ZnSO_4$ treated, black = 5.0 M $ZnSO_4$ treated. **B**. EAG dose-response curve of untreated and $ZnSO_4$ treated antennae with respect to different concentrations of jasmine. White = untreated antennae, bright grey = 0.25 M $ZnSO_4$ treated, dark grey = 1.0 M $ZnSO_4$ treated, black = 5.0 M $ZnSO_4$ treated.

In vertebrates, intranasal application of $ZnSO_4$ induces necrosis of olfactory receptor neurons (Hunnicut, 1939; Burd, 1993; Herzog and Otto, 1999). Bakakrishnan and Pollack (1997), who first used $ZnSO_4$ to block chemosensory neurons in insects, showed that application of $ZnSO_4$ affects contact chemosensory sensilla, but not mechanosensory sensilla which lack any pores. Selective blocking of contact chemosensory sensilla in honeybees may be a result of either differential permeability of the sensilla pores or the differential uptake and effect of $ZnSO_4$ on the receptor neurons.

Extirpation techniques are powerful tools in determining the role of identified sensilla as triggers of behavioral reactions. Specific ablation is difficult to perform where abundant sensilla of different sensory modalities appear spatially mixed, as on honeybee antennae. A great step forward was made by Balakrishnan and Pollack (1997) who applied $ZnSO_4$ to the antennae of crickets. In male crickets, antennal treatment with $ZnSO_4$ suppressed courtship initiation, a well characterized contact chemosensory evoked behavior. Our study suggests that this method could be used to investigate which kind of chemical channel is used in the various chemically elicited behaviors of the honeybee.

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Résumé – Blocage sélectif des sensilles chimioréceptrices de contact chez *Apis mellifera*. Les stimuli chimiques revêtent une grande importance pour la communication au sein de la colonie d'abeilles domestiques (*Apis mellifera* L.). Le riche spectre en stimuli chimiques sont reçus en premier lieu par les sensilles olfactives chimioréceptrices de contact. Les cellules sensorielles olfactives des sensilla placodea sont olfactives et excitées par les molécules transportées par les molécules transportées par l'air, celles des sensilla trichodea D réagissent aux stimuli de chimioréception de contact et aux stimuli mécaniques.

Dans ce travail nous avons utilisé le traitement des antennes des ouvrières d'abeilles au sulfate de zinc $ZnSO_4$ comme outil pour étudier l'importance de l'olfaction par rapport à la chimioréception de contact dans des questions d'ordre biologique.

À l'aide d'un test comportemental (le réflexe d'extension du proboscis, PER) et d'électroantennogrammes (EAG) il a été montré que les sensilla trichodea D, mais non les sensilla placodea, sont bloquées par le $ZnSO_4$. La réduction très significative du PER à un stimulus d'eau sucrée après traitement de l'antenne au $ZnSO_4$ indique un blocage des sensilla trichodea D (test U de Mann-Whitney, $P < 0{,}001$). Par contre les réponses EAG des antennes induites par les odeurs lors d'une stimulation par le citral, le géraniol, l'acétate d'isoamyle ou le jasmin ne sont pas significativement différentes entre les antennes traitées et les non traitées (ANOVA Kruskal-Wallis, $P > 0{,}05$). Le fait que toutes les substances testées soient perçues par les sensilla placodea signifie que le $ZnSO_4$ ne les perturbe pas.

La méthode qui permet de mettre hors circuit les récepteurs chimiosensoriels de contact constitue un outil avec lequel il est possible d'étudier de manière approfondie la nature, l'importance et l'action des signaux chimiques chez l'abeille.

olfaction / chimioréception de contact / électroantennogramme / réflexe d'extension du proboscis

Zusammenfassung – Selektive Ausschaltung der Kontaktchemosensillen bei *Apis mellifera*. Chemische Reize sind für die Honigbienen von enormer Bedeutung. Das reiche Spektrum an relevanten chemischen Reizen wird in erster Linie über antennale olfaktorische und kontaktchemosensorische Sensillen aufgenommen. Dabei werden die Sinneszellen der olfaktorischen Sensilla placodea durch luftgetragene Moleküle erregt, die adäquaten Reize für die Sensilla trichodea D sind kontaktvermittelte Geschmacks- und mechanische Reize.
In dieser Arbeit stellen wir die Behandlung der Arbeiterinnenantennen von *Apis mellifera* mit Zinksulfat ($ZnSO_4$) als Werkzeug vor, um für biologisch relevante Fragestellungen die Gewichtung von Olfaktion gegenüber Kontaktchemoperzeption zu untersuchen. Eine $ZnSO_4$-Behandlung beeinflusst signifikant die antennale Kontaktchemoperzeption. Anhand von Verhaltenstests (Proboscis Extension Response, PER) und Elektroantennogrammen (EAG) wurde gezeigt, dass selektiv die Sensilla trichodea D, jedoch nicht die Sensilla placodea durch $ZnSO_4$ blockiert werden. Die hochsignifikante Reduzierung des PER auf einen Zuckerwasserstimulus nach antennaler $ZnSO_4$-Behandlung deutet auf eine Blockierung der Sensilla trichodea D hin (Mann-Whitney U-Test, $P < 0,001$). Im Gegensatz dazu sind die duftinduzierten EAG-Antworten der Antenne bei Stimulierung mit Citral, Geraniol, Isoamylacetat und Jasmin zwischen behandelten und unbehandelten Antennen nicht signifikant unterschiedlich (Kruskal-Wallis-ANOVA, $P > 0,05$). Da alle Testdüfte über die Sensilla placodea perzipiert werden bedeutet dies, dass diese durch $ZnSO_4$ nicht beeinträchtigt werden.
Die Methode des selektiven Ausschaltens der Kontaktchemorezeptoren stellt ein Werkzeug dar mit dem die Natur, die Bedeutung und die Wirkung chemischer Signale bei Honigbienen vertieft untersucht werden kann.

Honigbiene / Geruchswahrnehmung / Kontaktchemoperzeption / PER / EAG

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