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RESEARCH ARTICLE

Separation of different pollen types by chemotactile sensing in *Bombus terrestris*

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ABSTRACT

When tasting food, animals rely on chemical and tactile cues, which determine the animal's decision on whether to eat food. As food nutritional composition has enormous consequences for the survival of animals, food items should generally be tasted before they are eaten or collected for later consumption. Even though recent studies have confirmed the importance of, for example, gustatory cues, compared with olfaction only little is known about the representation of chemotactile stimuli at the receptor level (let alone higher brain centers) in animals other than vertebrates. To better understand how invertebrates may process chemotactile cues, we used bumblebees as a model species and combined electroantennographical (EAG) recordings with a novel technique for chemotactile antennal stimulation in bees. The recorded EAG responses to chemotactile stimulation clearly separated volatile compounds by both compound identity and concentration, and could be successfully applied to test the receptor activity evoked by different types of pollen. We found that two different pollen types (apple and almond; which were readily distinguished by bumblebees in a classical conditioning task) evoked significantly distinct neural activity already at the antennal receptor level. Our novel stimulation technique therefore enables investigation of chemotactile sensing, which is highly important for assessing food nutritional quality while foraging. It can further be applied to test other chemosensory behaviors, such as mate or nest mate recognition, or to investigate whether toxic substances, e.g. in pollen, affect neuronal separation of different food types.

KEY WORDS: EAG recording, Chemotactile, Pollen

INTRODUCTION

All organisms depend on nutrients to sustain metabolic processes and thus for survival. The nutritional content of food typically differs between food items, as do nutritional requirements of consumers, rendering nutritional quality assessment essential for choosing food that best meets current needs. Such quality assessment can be either indirect, e.g. via saturation, nausea or hunger, or direct, e.g. via sensory input including tactile, gustatory and olfactory stimuli. Stimulation by either one or a combination of these different sensory modalities (e.g. during food tasting) forms a common percept and will henceforth be referred to as chemotactile stimulation.

Even though insects in particular rely predominantly on chemotactile sensing to detect harmful compounds (de Brito Sanchez et al., 2005; Falibene et al., 2015), recognize mates (Blomquist and Bagnères, 2010; Leonhardt et al., 2016; Wilson, 1971), differentiate between colony members and foreigners (Fletcher and Michener, 1987; Leonhardt et al., 2016; Wilson, 1971) or determine food quality (de Brito Sanchez, 2011; Ruedenauer et al., 2015), surprisingly little is known on the neuronal processing of chemotactile information or the contribution of different sensory modalities (i.e. tactile, gustatory and olfactory cues) in insects other than *Drosophila* (Masek and Keene, 2016; Singh, 1997; Zhang et al., 2016). In contrast to *Drosophila*, with 68 known gustatory receptor genes (Scott et al., 2001), and humans, with approximately 31 (putatively) functional gustatory receptor genes (Bachmanov et al., 2014), honeybees (*Apis mellifera*) have only 10 gustatory receptor genes, suggesting that gustatory perception may be rather weak and thus likely less important for social bees than for solitary flies (Robertson and Wanner, 2006). However, 23 functional gustatory receptor genes were identified in the bumblebee *Bombus terrestris* (Sadd et al., 2015). When additionally taking into consideration that (1) alternative splicing can further increase the number of expressed receptor proteins and (2) one receptor protein may be sensitive to a variety of ligands, overall combinatorial coding options for gustatory stimuli may be drastically increased, which suggests that processing chemotactile information is important for bumblebees (and maybe for social insects in general).

Bees obtain all micro- and macronutrients from floral resources, i.e. pollen and nectar. As pollen is the only protein and fat source for bees, intake of pollen of low nutritional quality or even contaminated pollen into the hive could weaken the colony significantly. In the light of the ongoing bee decline (Biesmeijer et al., 2006; Goulson et al., 2015), it is thus of great interest to understand how bees assess the nutritional composition and thus quality of pollen. However, studies addressing the neuronal mechanisms underlying pollen quality assessment are still missing, likely because of the lack of an appropriate methodological approach that allows researchers to record and analyze chemotactile sensing. Here, we present a novel technique allowing chemotactile stimulation while recording antennal receptor activity via electroantennography (EAG). Using this technique, we examined the neuronal processing of chemotactilely evoked signals at the neuronal periphery.

EAG measurements are commonly used to record the sum of action potentials from excited receptor cells by placing a recording electrode along the antennal nerve (Gothilf et al., 1978). EAG amplitudes provide a rough measure of the compound sensitivity of the antenna to different odor compounds [e.g. pheromonal (Gothilf et al., 1978; Olsson and Hansson, 2013) or derived from plants (Lecomte and Pouzat, 1985)]. However, until now, EAG measurements have only been used in olfaction to test reception of volatile compounds and close-range olfaction (Brandstaetter et al., 2010), whereas reception of non-volatile compounds that require physical contact (e.g. many gustatory cues, such as

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carbohydrates, amino acids, etc.) has not yet been recorded. In ants, close-range olfactory reception of comparatively large compounds, e.g. cuticular hydrocarbon pheromones (necessary for behavioral interactions), is mediated by sensilla basiconica (Sharma et al., 2015). Chemotactile stimulation most likely activates several receptor types, including gustatory receptors that respond to non- or hardly volatile compounds. In bumblebees and other bee species, gustatory receptor cells are located within hairs or peg-like structures forming a gustatory sensillum (Esslen and Kaissling, 1976; Ågren and Hallberg, 1996). In many cases, gustatory and mechanosensory/tactile receptor neurons are located within the same sensillum, which increases the possibility of interactions between both modalities already at the neuronal periphery (Zhang et al., 2016). Most importantly, the density of gustatory sensillae is highest at the terminal antennomere (Esslen and Kaissling, 1976; Whitehead and Larsen, 1976), which is also the part of the antenna that makes first and most contact when tasting food (Scheiner et al., 2005). For conventional olfactory-induced EAG activity measurements, where volatile odor compounds are blown over the antennae to activate olfactory receptors, the distal part of the antennae (with the first two antennomeres) is usually cut to insert the recording electrode (e.g. Fonta and Masson, 1984), which renders this approach inappropriate for investigating chemotactilely induced receptor activity. In contrast to the conventional approach, our novel technique keeps the entire antenna intact. Instead of cutting the antennal tip, we inserted the recording electrode at the antennal base (Fig. 1B) and used a motor-driven stimulation device allowing chemotactile stimulation of the antennal tip under most natural conditions. To evaluate our technique in relation to conventional (airborne) EAG, we first compared signals evoked by airborne stimulation with signals obtained with an intact antennal tip using three different volatile odor compounds. In a second experimental series, we used chemotactile stimulation and presented two different types of pollen (apple and almond) which can be readily distinguished by bumblebees as shown in a previous behavioral experiment (Ruedenauer et al., 2015). We tested the hypothesis that both pollen types evoke distinct neuronal receptor activity at the antenna.

MATERIALS AND METHODS

Animal treatment

Two *Bombus terrestris* (Linnaeus 1758) colonies were obtained (Behr, Kampen, Germany) and kept in two-chambered wooden boxes (240×210×110 mm per chamber) in a climate chamber (25°C, 50% humidity, 12 h:12 h light:dark cycle). They had *ad libitum* access to Apiinvert (a mixture of sucrose, fructose and glucose) and bee-collected pollen (Naturwaren Niederrhein GmBH, Goch-Asperden, Germany). For the experiments, individual workers were captured and chilled on ice for 15 min. As the size of a bumblebee determines its antennal sensitivity and differently sized workers may carry out different tasks (Spaethe et al., 2007), we randomly chose bees of different sizes (ranging from ca. 150 to 300 mg) to cover the full spectrum of antennal sensitivity.

For EAG recordings, heads were cut off and fixed with dental wax on plastic brackets. The wax was also used to fix the scapi of the antennae to prevent movement (Fig. 1A). For chemotactile

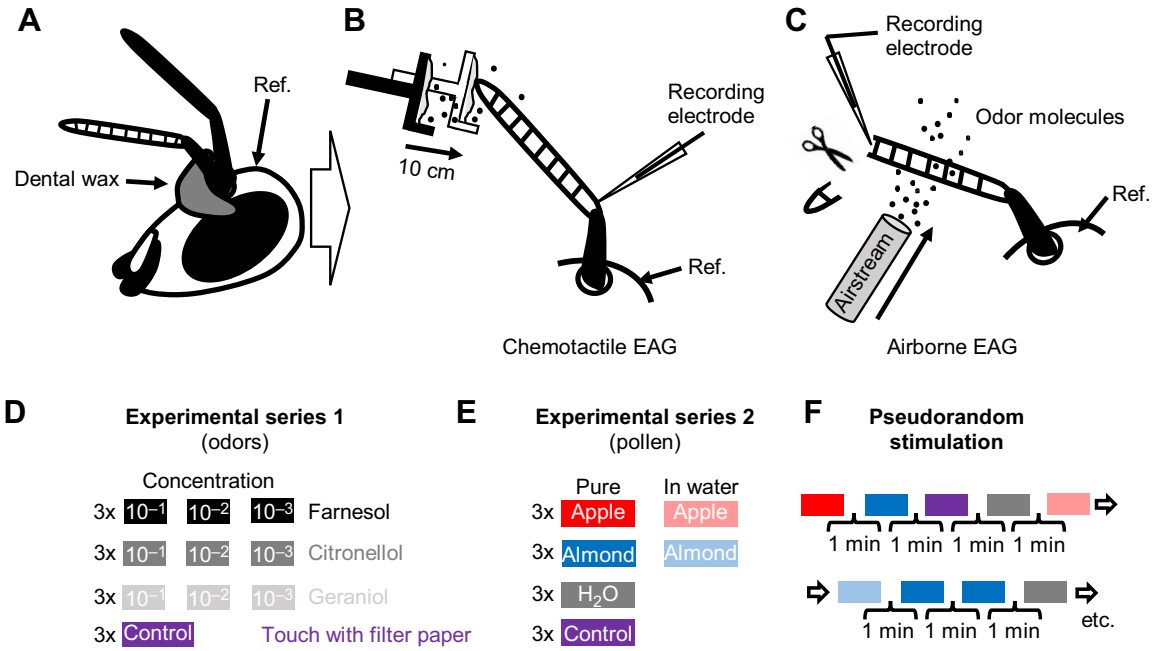

Fig. 1. Two types of electroantennograms (EAG) were measured in bumblebees. (A) The head of the bee was placed on a plastic block. The scapus of the antenna was fixed with dental wax and the reference electrode (Ref.) was inserted through the head capsule close to the optical lobes to reach brain tissue. (B) For chemotactile stimulation, the recording electrode was placed in the most proximal antennomere of the flagellum. Prior to stimulation, the stimulus was positioned ca. 15 cm from the antenna. For stimulation, the tip of the antenna was touched softly with a filter paper moisturized with volatile compound dissolved in paraffin (experimental series 1) or the pollen paste (experimental series 2). (C) For conventional airborne olfactory stimulation, the tip of the antenna was cut in the second antennomere of the flagellum and the recording electrode was placed there. The stimulus was presented via an airstream. (D) The chemotactile stimulation procedure was established by comparing the EAG response to farnesol, citronellol and geraniol at three different concentrations (10^{-1}, 10^{-2} and 10^{-3}), either presented through an airstream (C) or by chemotactile stimulation (B). (E) Two pollen types (apple and almond) were presented in two different concentrations (pure and 10^{-2} solved in water). As control stimuli, the antennae were touched with and without water. (F) We used a 1 min inter-trial interval. All stimuli were repeated three times in a pseudorandomized sequence, meaning the stimulation was randomly presented, but one stimulus did not occur more than two times successively.

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stimulation, the recording electrode was positioned at the basalmost antennomere of the flagellum and the antenna was stimulated at the tip (Fig. 1B). For conventional airborne odor stimulation, the antenna was cut with a fine scissor within the second antennomere, and the recording electrode was inserted into the opening (Fig. 1C). Odorants were blown over the remaining antennomeres. In both cases, the reference electrode was inserted into the head capsule, between the left ocellus and compound eye.

Airborne (conventional) stimulation

All experiments were performed at ∼24°C. For airborne olfactory stimulation, we used citronellol, farnesol and geraniol dissolved in paraffin (10^{-2} concentration). The compounds were applied using a stimulus controller (CS-55, Syntech, Hilversum, The Netherlands) generating a continuous airflow of 1 l min^{-1} added with a stimulus flow of 0.5 l min^{-1}. Two stimulus chambers were inserted into the airstream (stimulus chamber one and two). Prior to odor stimulation, an airflow of 0.5 l min^{-1} was blown over an empty filter paper placed in stimulus chamber one. For providing the stimulus, the airflow switched from stimulus chamber one (blank pipette) to stimulus chamber two (stimulus pipette), equipped with a filter paper containing the test compound. After 0.5 s of stimulation, the airflow switched back to stimulus chamber one. All stimuli were presented three times per individual in a pseudorandomized order.

Chemotactile stimulation

To present chemotactile stimuli to individual bumblebees, we slightly modified the stimulation technique established for close-range olfactory stimulation by Brandstaetter et al. (2010). Our tactile stimulation device consisted of a metal arm moved forwards and backwards by a servo motor (Blue Bird Technology Co., Taichung, Taiwan). The arm held a copper stick equipped with a copper plate at the tip (as used in Ruedenauer et al., 2015), which was electrically grounded to prevent noise. We placed a filter paper with 3 µl of the current stimulus (or an empty filter paper as control) on the plate. To prevent antennal movement artefacts, the device was set to gently touch the tip of the antenna, but not move it. Arm movement was controlled via the TTL output of the same stimulus controller as used in olfactory stimulation (CS-55, Syntech), which was also used to synchronize the recording software. Each chemotactile EAG stimulation lasted for 1 s. To compare our chemotactile stimulation setup with the conventional airborne stimulation setup, we tested the same three compounds (citronellol, farnesol and geraniol diluted in paraffin oil; Sigma-Aldrich, Taufkirchen, Germany) at three concentrations (10^{-1}, 10^{-2} and 10^{-3}; by weight; experimental series 1; Fig. 1D) in both setups. To test chemotactile cue stimulation, we used hand-collected apple (*Malus domestica*, Rosaceae) and almond (*Prunus dulcis*, Rosaceae) pollen (Firman Pollen, Yakima, WA, USA) as natural stimuli in two application forms – as paste and as an extract in water (10^{-2}, experimental series 2; Fig. 1E). The 'pollen paste' was used to make pure pollen stick to the filter paper, reduce variation in surface texture of different pollen stimuli and enable a more even contact between antenna and stimulus. It was produced by grinding 5 g of pollen and mixing it with 1 ml of deionized water. Pollen extracts were produced by adding 10 µg of pollen to 1 ml of deionized water and extracting pollen for 1 day, with the supernatant used as stimulus. A droplet (3 µl) of each extract tested was placed on a filter paper (Hartenstein, Würzburg, Germany) on top of tactile stimulation arm (Fig. 1B). The filter paper was replaced by a fresh one after each trial. As adequate controls, the chemotactile stimulus (filter paper) and water (water+filter paper) were presented alone. All stimuli were presented three times per individual in a pseudorandomized order.

Electrophysiology

Recording electrodes were glass capillaries [World Precision Instruments (WPI), Sarasota, FL, USA] pulled with a DMZ Universal Puller (Zeitz-Instruments Vertrieb GmbH, Martinsried, Germany) and filled with potassium chloride solution (1 mol l^{-1}). Using a micromanipulator (WPI), the recording electrode was inserted either into the first antennal antennomere above the scapus of the antenna (chemotactile EAG; Fig. 1B) or into the hole resulting from cutting the antenna in the second antennomere (airborne EAG; Fig. 1C). The reference electrode (silver wire Ø=25 µm) was inserted into the head capsule between the left ocellus and compound eye (Fig. 1A–C). The measured voltage difference was 10-fold amplified by an amplifier (Neuroprobe Amplifier 1600, A-M Systems, Sequim, WA, USA), high-pass filtered (above 50 Hz, Kemo VBF 8, Kemo Inc., Greenville, SC, USA) and digitized by an acquisition board (Labtrax 4/16, WPI). All data were recorded with LabScribe v3 (WPI).

Data analysis

As the baseline signal before stimulus onset varied between recording situations, we applied a baseline correction to compare between animals: we calculated the mean voltage signal 500 ms before stimulus onset and subtracted its value from the complete recording trace. We then calculated the mean EAG signal from the three repetitions per stimulus obtained for each animal and compound tested. Because chemotactile stimulation using volatile compounds resulted in negative on peaks and positive off peaks, we calculated compound- and concentration-dependent differences between the stimuli-induced EAG amplitudes at both time points. We extracted the stimulus-dependent minima during the 500 ms following stimulus onset and the maxima during the 500 ms following stimulus offset for all recorded animals (Fig. 2B). The distribution of stimulus-dependent minima (onset) and maxima (offset) was visualized and tested for significant differences by performing a repeated-measures ANOVA with bee identity nested within compound and compound as the repeated factor. Because airborne and pollen EAGs produced only one peak, the analysis was reduced to either maxima or minima of this peak in the first time period after stimulus onset. To test for differences between different compounds tested, the ANOVA was followed by a Tukey's test. All statistics were performed using R v3.1.2 (R Foundation for Statistical Computing, Vienna, Austria). Data were visualized using MATLAB (MathWorks, Natick, MA, USA).

To visualize the separation of the chemotactilely induced activity, we pooled all mean EAG signals and calculated stimulus-dependent population vectors by constructing the n-dimensional EAG vector v^a at each time point during the 8 s of recording for a given stimulus configuration a (stimulus identity) and a population of n animals. All population vectors together were used in a principal component analysis (PCA). To keep the temporal aspect of the EAG signals intact, PCA was performed by taking into account time as the source of sample points, and number of recordings as the dimension of the original component space. The first three principal components (PC1–PC3) were used to visualize the pollen separation in EAG recordings.

RESULTS

Different volatile compounds induce different EAG responses

To ensure that the chemotactile stimulation device can be used to separate EAG activity induced by different compounds, we first stimulated bumblebees with three volatile compounds (farnesol,

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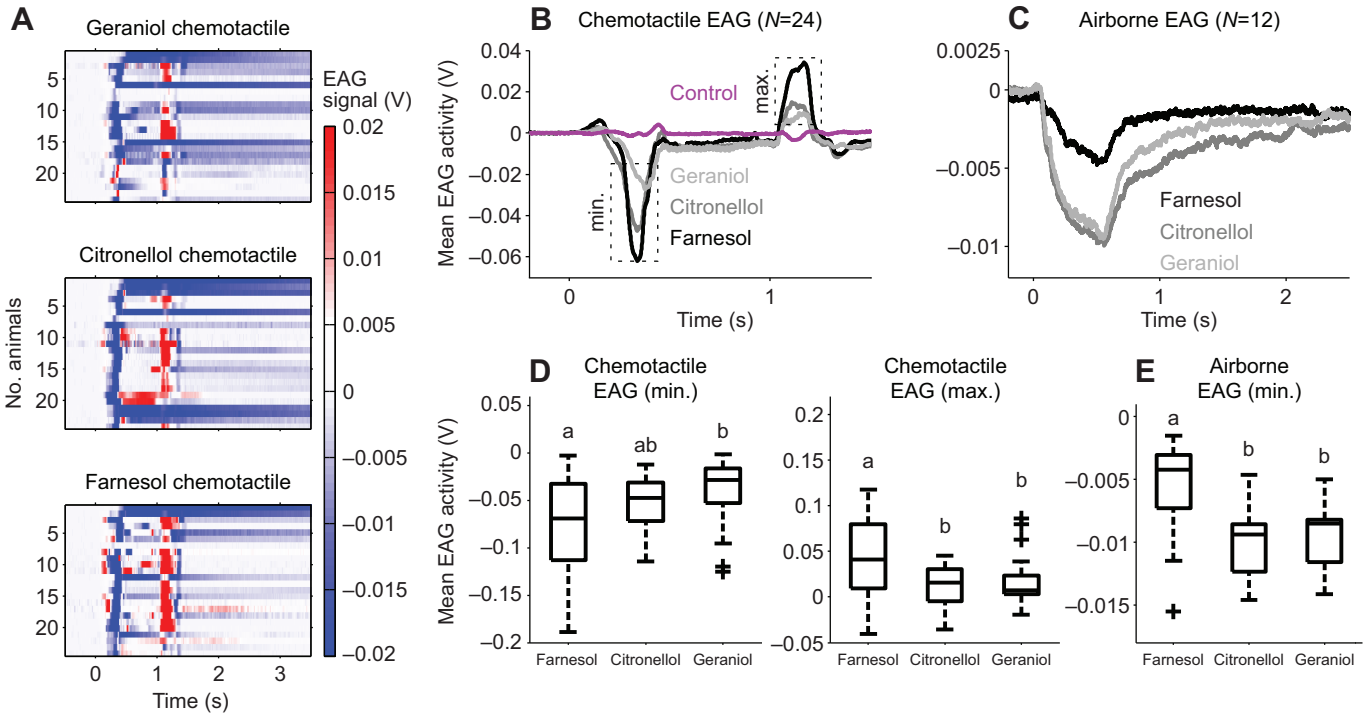

Fig. 2. Compound identity separation during chemotactile stimulation. (A) The averaged EAG signals of 24 bumblebees (*y*-axis) in response to the middle concentrations (10^{-2}) of geraniol, citronellol and farnesol are shown color-coded (see color bar). Chemotactile stimulation starts at time zero and lasted for 1 s. Typical responses started with a voltage decrease (blue) after stimulus onset (0 s) and ended with an increase (red) after stimulus offset (1 s). Mean EAG activity was calculated from the recorded animals for (B) chemotactile stimulation (*N*=24) and (C) conventional airborne stimulation (*N*=12). Odor presentation started at time 0 s and lasted for 500 ms. Purely tactile stimulations were presented to control for stimulation artefacts (purple, left). The induced responses were strongest for farnesol (black) followed by citronellol (grey) and geraniol (light grey) at the onset (min.) as well as at the offset (max.) of the chemotactile stimulation. The conventional airborne stimulation (C) led to an inverted order with respect to the odor-induced response strength, meaning that farnesol showed the lowest response (also shown in E). (D) Boxplot of compound-dependent EAG minima (stimulus onset, left) and maxima (stimulus offset, right) of the 24 animals. The central mark is the median, the edges of the box are the 25th and 75th percentiles, the whiskers extend to the most extreme data points and outliers are plotted individually (black cross). The stimulus-induced minima as well as the maxima distributions were significantly different (RM-ANOVA, $P<0.05$). Individual pairwise differences of the minima and maxima were tested using a Tukey test. Different letters indicate significant differences ($P<0.05$). (E) Odor-induced EAG minima distributions after airborne stimulation showed significant differences (RM-ANOVA, $P=0.004$). Same statistical tests as in D.

citronellol and geraniol) in three concentrations that can be separated using conventional (airborne) EAG recordings (Fig. 2C, E). All stimuli evoked reliable responses in all animals (Fig. 2A). The expected tactile stimulation artefact remained negligible. A typical chemotactile EAG response to volatile compounds consisted of two prominent phases (Fig. 2A,B). One was the negative signal after stimulus onset (blue; Fig. 2A), the other the positive signal after stimulus offset (red; Fig. 2A). We concentrated on the middle concentration (10^{-2}) and extracted the minima of the mean EAG activity in every single animal in a 500 ms time window after stimulus onset, and the maxima in a 500 ms time window after stimulus offset (Fig. 2B). EAG minima and maxima differed significantly between the three compounds tested, allowing compound differentiation in both time windows (Fig. 2D). In all cases, farnesol triggered the strongest and geraniol the weakest response (for details, see Fig. 2D). Overall, the different compounds evoked distinct EAG activities, allowing a compound identity separation at the onset and/or offset.

Different concentrations of volatile compounds induce different EAG responses

To determine whether compound concentration was also represented in the EAG signal induced by chemotactile stimulation, we again analyzed both the minima after stimulus onset and the maxima after stimulus offset. The differences in the signals induced by chemotactile stimulation were significant between different concentrations of all three volatile compounds tested (for details, see Fig. 3B–D). For geraniol (Fig. 3D), a significant concentration-dependent EAG signal could be found only at the stimulus onset, but not at the offset.

Pollen of different plant species induces different EAG responses

After making sure that our chemotactile stimulation allowed separation of different compounds as well as distinction of different concentrations of these compounds, we tested chemotactilely induced EAG signals in response to different species of pollen. Interestingly, when testing pollen paste as a chemotactile cue, the two pollen types evoked clear and very distinct EAG signals with opposite polarity in almost all tested animals (Fig. 4A). Almond pollen evoked a negative response, whereas apple pollen evoked a positive response (Fig. 4A). To make sure that the polarity of the EAG signal did not reflect stimulus-specific activation of muscles in the antenna, we cut the antennal nerve in a subset of three animals. Repeating the experiments in those animals produced very similar results (data not shown). The pollen extracts evoked similar, but weaker responses compared with pollen paste (Fig. 4B). Water also evoked a negative response similar to that of almond pollen (Fig. 4B). Interestingly, chemotactile stimulation with pollen, extracts and water (all

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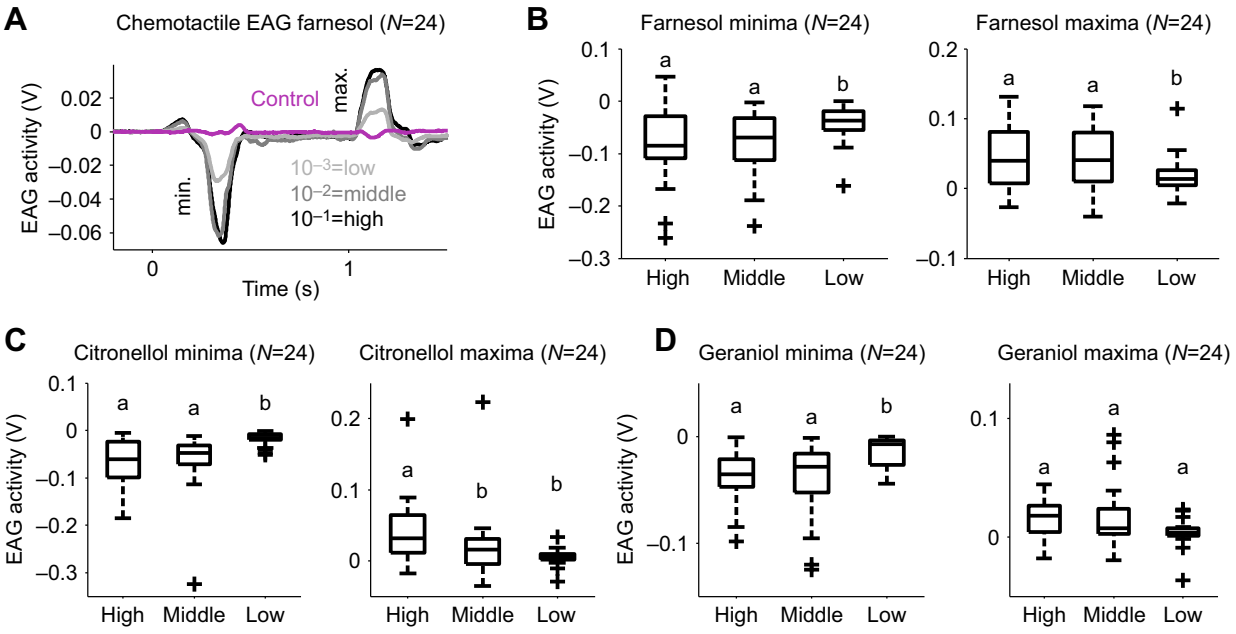

Fig. 3. Concentration separation during chemotactile stimulation. (A) All compounds were presented in three concentrations: 10^{-1} (high), 10^{-2} (middle) and 10^{-3} (low). This example shows the concentration-dependent farnesol EAG activity, averaged across all recorded bumblebees (*N*=24). Purely tactile stimulations were presented to control for stimulation artefacts (purple). The stimulus-induced responses were strongest for the highest concentration (black) followed by the middle (grey) and lowest concentrations (light grey). This concentration dependency in the EAG response was present during both the onset (min.) and the offset (max.) of the chemotactile stimulation with volatile odor compounds. (B) Boxplot of concentration-dependent EAG minima (left) and maxima (right) of the 24 animals. The central mark is the median, the edges of the box are the 25th and 75th percentiles, the whiskers extend to the most extreme data points and outliers are plotted individually (black cross). Both the concentration-induced minima and maxima distributions were significantly different (RM-ANOVA, $P<0.001$). The pairwise differences of the minima and maxima were tested using a Tukey test. Different letters indicate significant differences ($P<0.05$). (C) Same as in B, but for the different citronellol concentrations. The concentration-induced minima as well as the maxima distributions were significantly different (RM-ANOVA, $P<0.001$). (D) Same as in B, but for geraniol. A concentration-dependent EAG signal could be found at the onset (RM-ANOVA, $P<0.001$) but not at the offset (RM-ANOVA, $P=0.414$).

containing non-volatile compounds) evoked almost 10 times higher EAG amplitudes than stimulation with single volatile compounds and did not result in a bimodal on and off signal, which was seen when stimulating with single volatile compounds. Plotting the temporal evaluation of the first three principal components (PC1–PC3) revealed that all stimuli, including the pollen extracts, followed distinct trajectories (Fig. 4C). When comparing maximal amplitudes between almond and apple pollen extract, the average EAG signal showed a lower activity for apple than for almond pollen extract (Fig. 4B), but did not differ from that of pure apple pollen (Fig. 4D), whereas amplitudes were significantly different between pure almond pollen and almond pollen extract (Fig. 4D). The EAG amplitudes also differed significantly between the low apple pollen and the low almond pollen concentration as well as between pure apple and pure almond pollen (Fig. 4D).

DISCUSSION

Insects typically touch food items with their antennae or tarsae before eating or collecting it. In doing so, they receive chemotactile information, which is essential for assessing food quality, as we have recently shown for pollen-collecting bumblebees (Ruedenauer et al., 2015). Here we investigated antennal reception of chemotactile stimuli likely involved in quality assessment by analyzing whether pollen types, which can be differentiated in classical conditioning experiments (Ruedenauer et al., 2015), evoked a distinct activity already at the receptor level. We successfully established a novel technique which enables EAG recordings following robust chemotactile stimulation of the antennal tip, the predominant location of contact chemoreceptors (Haupt, 2004; Whitehead and Larsen, 1976).

Different volatile compounds and concentrations induce different EAG responses

With our chemotactile stimulation device, we were able to trigger distinct EAG signals separating different volatile compounds (Fig. 2) as well as different concentrations of the same compound (Fig. 3), which can also be separated using conventional (airborne) induced EAG recordings (Fig. 2). As fixation of the antenna does not influence sucrose taste perception (Haupt, 2004), we are very confident that the established method can be used to investigate neural representation of, for example, gustatory stimuli and other non-volatile compounds used for chemotactile sensing under semi-natural conditions.

Comparing the EAG activity induced by chemotactile stimulation with the EAG activity induced by airborne stimulation using the same odorants revealed that the signal strength was inverted. For example, farnesol induced the lowest activity when presented airborne, but evoked the highest activity when presented via touch. A similar phenomenon was observed by Brandstaetter et al. (2010), showing that close-range olfactory stimulation led to stronger signals than airborne stimulation with the same compound. Signal enhancement may therefore be a general phenomenon caused by physical properties (e.g. volatility) of individual compounds or by differences in receptor cell distribution along the antennal segments. The latter is supported by the observation that most contact chemoreceptors were mainly identified at the tip of the antennae (Haupt, 2004; Whitehead and

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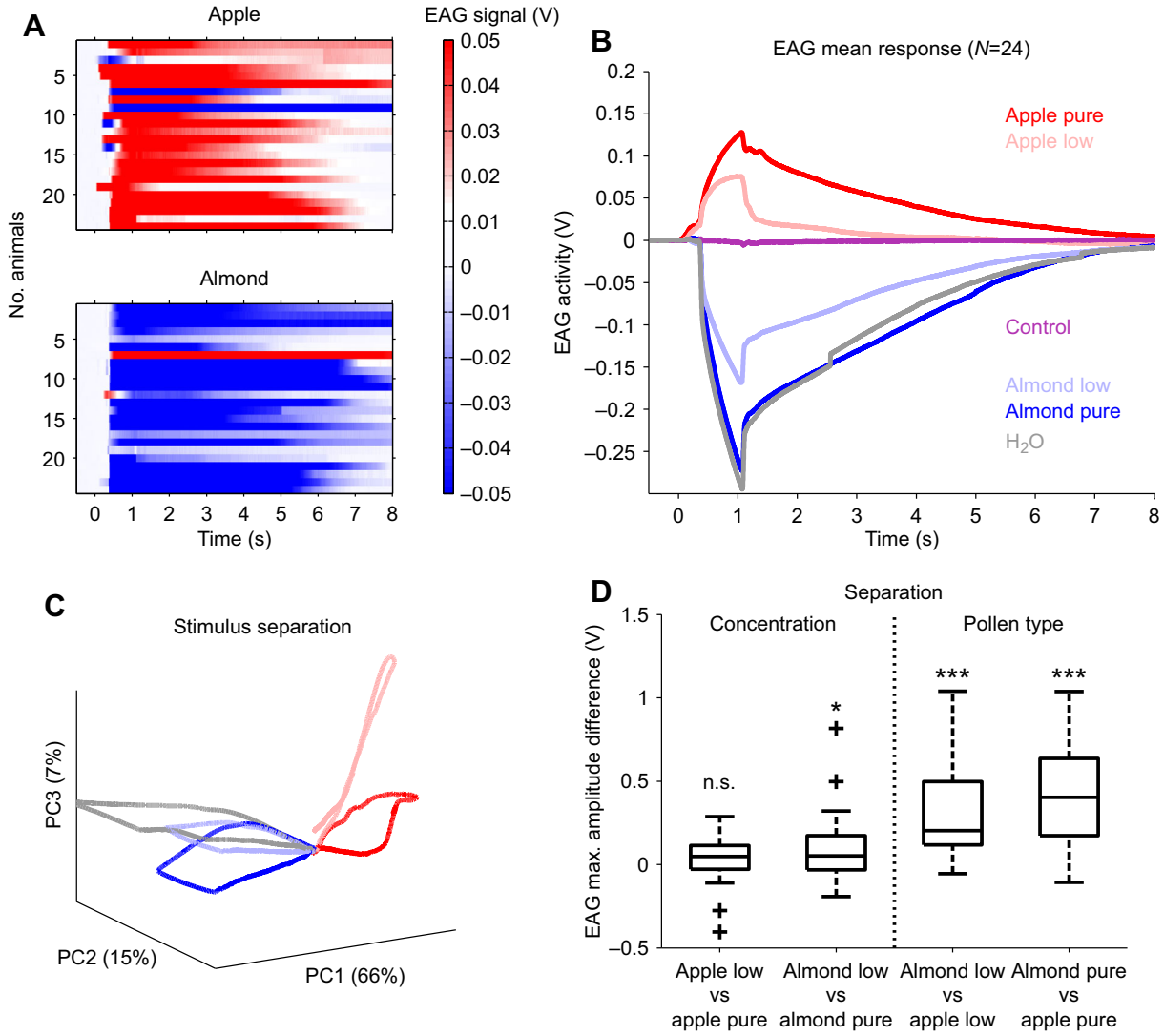

Fig. 4. Separation of apple and almond pollen in EAG activity after chemotactile stimulation. (A) Apple and almond pollen evoked opposing EAG signals. The mean EAG response (V; compare color bar) out of three repetitions per stimulus were calculated for each bumblebee (*y*-axis, *N*=24). Chemotactile stimulation starts at time zero and lasted for 1 s. (B) Pure apple pollen (red) and apple pollen extract (light red) produced positive EAG signals, while almond pollen (blue) and almond pollen extract (light blue) produced negative responses. Water (grey) produced signals similar to those of the pure almond pollen. However, all signals can be clearly separated from the touch control (purple). (C) To visualize the stimulus separation at the antenna, we applied a principal component analysis including the population vectors (two examples are shown in A) of all stimuli (see Materials and methods). The first three principal components (PC1–PC3) were plotted against each other. PC1 explained 66%, PC2 15% and PC3 7% of the variation in the data. All trajectories start at the center (color code as in B). (D) Differences between EAG maximal amplitudes were calculated in each animal. The concentration-dependent EAG amplitude difference between pure apple pollen (apple pure) and apple pollen extract (apple low) was not significantly different (sign rank test; P=0.1). For almond pollen, the concentration difference was significant (almond low versus almond pure; sign rank test; P=0.03). The difference between both pollen types was significant at the low concentration as well as for the pure pollen (sign rank test; P<0.001).

Larsen, 1976), which was cut off in the conventional (airborne) EAG situation. The generally stronger response to antennal tip chemotactile stimulation may therefore be explained by the higher number of receptors at the sensory plate on the tip of the antenna (Esslen and Kaissling, 1976), which may also have been activated by the volatile compounds tested chemotactilely. Moreover, when tested as chemotactile stimuli, volatile compounds may activate different receptors, e.g. long-range (pore-plates and sensilla trichoidea) and close-range (sensilla basiconica) olfactory sensilla and, potentially, gustatory sensilla (Fonta and Masson, 1982, 1987; Sharma et al., 2015). In addition, different mechanosensitive receptors may specify or enhance a response, each of which may enhance each other. In contrast, airstream stimulation with single volatiles most likely activates purely (long-range) olfactory receptors, but no (or much less) close-range olfactory receptors or other modalities, resulting in a generally weaker activity. However, the high molecular weight and the low volatility of farnesol compared with citronellol and geraniol may best explain the lower farnesol response with airborne olfactory compared with chemotactile stimulation, as also discussed in Strube-Bloss et al. (2015). This finding suggests that volatile, close-range and contact perception of olfactory cues may not necessarily require a specific type of receptor, but rather results from differential activation depending on the physico-chemical characteristics of the involved cues and range of the antennal contact.

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Pollen of different plant species induces different EAG responses

Preferences, attractiveness or the ecological meaning of compounds are not necessarily represented by the responses in EAG (Eltz and Lunau, 2005). Processes in higher nervous centers can increase or decrease the intensity of signals and therefore modulate perception (Eltz and Lunau, 2005). For example, in bumblebees, farnesol, a component of the foragers' recruitment pheromone, shows a drastically increased odor separation from general odors during late phases of the response, which reflects a prolonged network computation at the antennal lobe level, even though the averaged maximal activities in antennal lobe neurons were similar for farnesol and the tested compounds (Strube-Bloss et al., 2015). Likewise, the behavioral or ecological relevance of a stimulus cannot be directly inferred from its EAG signal, but EAG signals allow for conclusions about which compounds can be received and how the signals may become modified on their way to higher nervous centers. It was therefore even more surprising that we found a separation of apple and almond pollen already at the antennal receptor level. Whereas pure almond pollen and almond pollen extract evoked a negative EAG signal, pure apple pollen and apple pollen extract evoked a positive EAG response.

Positive EAG signals have been reported many times in olfactory EAGs (Contreras et al., 1989; Haddad et al., 2010; Leskey et al., 2010; Light et al., 1988; Ramachandran et al., 1990; Schneider, 1957) and were hypothesized to be evoked by repellent compounds (Contreras et al., 1989). However, Leskey et al. (2010) reported that the odor of preferred fruits evoked a positive signal in plum curculios (*Conotrachelus nenuphar*, Coleoptera), and Knaden et al. (2012) postulated for *Drosophila melanogaster* that no reliable information about attractiveness or repellence was encoded in the neural signal before it reached the output neurons of the antennal lobe. We also found no preferences for either apple or almond pollen in a feeding experiment with *B. terrestris* (Ruedenauer et al., 2016). Hence, in our chemotactile setup, the positive signal for apple pollen does most likely not indicate repellence. The opposite signals rather support our previous findings that *B. terrestris* can differentiate both pollen types by chemosensation (Ruedenauer et al., 2015). As the recording electrode was always inserted at the same position, the signal polarity may be related to morphological differentiations along the antennal nerve, which may originate from different subsets of activated receptor neurons.

Pollen of different plant species likely activates different sets of contact chemoreceptors

Our results indicate that the same EAG amplitudes (e.g. as found for almond pollen and water) do not necessarily indicate lack of differentiation at the level of the central nervous system. Water and pure almond pollen both seem to activate the same number of receptors, resulting in a similar EAG signal strength. However, the receptor types may differ, which is supported by the decreased overall response amplitude to the almond pollen extract, which was distinct from pure water and apple pollen extract. If activation of water-sensitive receptors dominated the response, both pollen extracts would have induced the same activity. The distinct EAG activity observed for pollen and pollen extracts therefore suggests that the different pollen types activate different ensembles of contact or even olfactory chemoreceptors. Such activation of different receptor subsets probably explains the almost five times higher EAG signal and the lack of a bimodal signal for chemotactile stimulation when compared with stimulation with single volatile compounds, where each compound likely activated predominantly or exclusively only one olfactory receptor type (as discussed earlier). However, it should be kept in mind that the pollen pastes and especially the extracts used in our experiment differ from the (dry) pollen on floral anthers as encountered by bees in nature. So far, we cannot make any inferences on the precise compounds in pollen, which dominated the signal. However, as the signal produced by the extracts resembles the signal of the pollen paste, only weaker, it is very likely that water-soluble substances are responsible. Potential candidates are, amongst others, amino acids and their metabolites, which we will investigate in future studies.

Implications and conclusions

As pollen is the only protein and lipid source for bees, intake of substances such as toxic pollen into the hive could severely harm the colony, unless it is detected and handled accordingly by mixing it with other pollen types, for example (Eckhardt et al., 2014). This could have economic consequences for beekeepers and farmers relying on bees for honey production and pollination, respectively (Biesmeijer et al., 2006; Kremen et al., 2002). We therefore need to better understand how bees discriminate pollen of different quality in order to assess whether and how such discrimination may be affected by natural (e.g. secondary plant compounds; Eckhardt et al., 2014) or human-distributed (e.g. pesticides; Marletto et al., 2003; Whitehorn et al., 2012) substances in the environment. With our novel technique (which enables chemotactile stimulation while recording antennal receptor activity), we could demonstrate that different pollen types (apple and almond, which were readily distinguished by bumblebees in a classical conditioning task; Ruedenauer et al., 2015) evoked significantly distinct neural activity already at the antennal receptor level. We can now use this characterization of the neural response to test whether, for example, toxic pollen affects neuronal separation of different pollen types.

Moreover, the established method for chemotactile stimulation opens entirely new possibilities for investigating neuronal principles underlying contact chemoreception at both the peripheral and central nervous system levels. It can thus be used for investigating reception and perception of a variety of non-volatile compounds, such as macronutrients used for nutritional quality assessment, or cuticular long-chained hydrocarbons, which play a crucial role for social interactions, particularly in social insects (Leonhardt et al., 2016).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

F.A.R. and M.F.S.-B. conceived the experimental concept and established the recordings. F.A.R. performed the chemotactile experiments. F.S. performed the conventional airborne experiments. F.A.R. and M.F.S.-B. analyzed the data in consultation with S.D.L. and W.R. F.A.R. drafted the manuscript. F.A.R., S.D.L., W.R. and M.F.S.-B. wrote the paper. All authors discussed the results, commented on the paper and agreed to the final version.

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Data availability

Data tables are accessible at a server of the University of Würzburg (archiv.rz.uni-wuerzburg.de). For access, please contact it-support@uni-wuerzburg.de.

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