

Antennal response to fragrance compounds in male orchid bees

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Summary. Male orchid bees (Euglossini) are attracted to floral and non-floral odours, which they collect and accumulate in hind tibial cavities for subsequent exposure during courtship. Fragrance preferences are species-specific, leading to relatively specialised pollination of euglossophilous plants. We tested the hypothesis that preferences for attractive compounds have led to species-specific sensory adaptations that are measurable by electroantennography (EAG). All of 16 synthetic fragrance compounds elicited significant responses on male bee antennae, with some difference of response spectra between individuals of *Euglossa* spp. and bumblebee (*Bombus terrestris*) controls, but no difference between three different species of *Euglossa*. There was no correspondence between a compounds' attractiveness in baiting assays and the size of its electrophysiological response. Our results strengthen the view that fragrance preferences are largely mediated by processes in higher nervous centres. Peripheral sensory tuning to single attractive odorants may be constrained by the need to detect and discriminate between many fragrances, including many that have repellent effects on male bees.

Key words. Bee olfaction – EAG – sensory specialisation – Euglossini – euglossine bees

Introduction

Male orchid bees are attracted to scents emitted by flowers of orchids and other plants as well as by non-floral sources, such as rotting logs or feces (Dressler 1982; Whitten *et al.* 1993). The males absorb the volatiles, mostly terpenoids and aromatics, with tarsal brushes of hairs and finally store them in pouches in the hind tibiae (Vogel 1966; Whitten *et al.* 1989; Eltz *et al.* 1999). The behaviour has given rise to an entire pollination syndrome, encompassing several hundreds of orchid species and many other euglossophilous plants (Williams 1982; Knudsen *et al.* 1999; Roubik & Hanson 2004). Males of different species of bees have different odour preferences, leading to relatively specialised plant-pollinator interactions (Ackerman 1983; Ackerman 1989). Although the ultimate causes of fragrance collection are still unclear, recent evidence suggests that the volatiles have a

role in euglossine courtship. Males of one species, *Euglossa cognata*, were shown to extract and expose substances from their hind legs when displaying at small mating territories (Eltz *et al.* in press).

Male bees forage for fragrances over much of their lives, finally acquiring substantial quantities of complex fragrance blends (Eltz *et al.* 1999). Comparison of hind leg extracts of *Euglossa imperialis*, *E. cognata*, and *E. tridentata* has shown that these bouquets possess species-specific qualities, even when males are sampled from distant and ecologically divergent localities (Eltz *et al.* 1999; Eltz *et al.* submitted). Natural euglossine fragrance sources occasionally emit single components (e.g., some non-floral odours; Whitten *et al.* 1993), but mostly relatively simple blends dominated by one or two major components (most euglossophilous orchids; Williams & Whitten 1983; Gerlach & Schill 1991). In many cases these dominant components are also attractive when presented in pure synthetic form during baiting assay. In a one-year study on Barro Colorado Island in Panama, Ackerman (1989) exposed 16 synthetic compounds, attracting thousands of individual males belonging to 44 species of bees. Although there was overlap of choices, most species had unique sets of chemical baits to which they were attracted. Thus, it seems clear that specific detection of fragrance sources is important to male orchid bees. Assuming that attractive fragrance sources are rare in the natural habitat, one might also expect strong selection for low detection thresholds concerning preferred odours. This might lead to differential sensory adaptations in different species of bees.

Here, we used Electroantennography (EAG) to investigate peripheral chemosensory specialisations in three species of *Euglossa*, testing a range of synthetic single fragrance compounds. EAG records sums of potentials from receptor neurons located in the antenna. It is used under the assumption that the amplitude of an EAG response is positively correlated with the sensitivity of the antenna to the presented odour compound. While the method seems generally suitable to detect antennal specialisations (Roelofs 1984), the neural causes of specialisations are not revealed: E.g., species-specific differences in EAG responses may arise either from differences in the number of receptors (or receptor types) responding to a given compound, or from alterations in their strength of response, or both. The method has previously been used to investigate odour detection in a wide variety of insects (Roelofs 1984; Schiestl & Marion-Poll 2002), including orchid bees (Schiestl & Roubik 2003).

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For comparison, we also tested male bumblebees, *Bombus terrestris*, on the same compounds. Together with orchid bees (Euglossini), honey bees (Apini), and stingless bee (Meliponini), bumblebees (Bombini) form the monophyletic clade of corbiculate Apinae. We used male bumblebees as an outgroup in order to get a general idea of the variation of antennal responses within Apinae, and to facilitate interpretation of the variation found among *Euglossa* species.

Materials and methods

Bees

Males of *Euglossa imperialis*, *E. cognata*, and *E. tridentata* were captured at chemical baits in forests surrounding the village Gamboa in Central Panama and consecutively transferred to Düsseldorf, Germany, where EAG was conducted. At the University of Düsseldorf Botanical Gardens the bees were introduced into 50 × 50 × 60 cm mesh cages placed in an air conditioned greenhouse (25–30 °C, 70–90 % relative humidity) where they learned to drink honey-water from artificial flowers. Individuals were subjected to EAG over the course of four weeks (N = 8 in *E. cognata* and *E. tridentata*, N = 9 in *E. imperialis*). Male *Bombus terrestris* (N = 7) were reared from a commercial laboratory colony.

Test substances

We tested the following 16 synthetic compounds: (1S)-(-)- α -pinene, (1S)-(-)- β -pinene, 1,8-cineole, methyl salicylate, benzyl cinnamate, hexahydrofarnesyl acetone, (E)-ocimene, benzyl benzoate, nerolidol, p-dimethoxy benzene, 2,3-epoxygeranyl acetate, benzyl acetate, (E)-methyl cinnamate, eugenol, 2-phenylethanol, and p-cresyl acetate. These are either known attractants for male orchid bees (Ackerman 1989; Ramirez *et al.* 2002) and/or feature prominently in the males' tibial fragrances (Eltz *et al.* 1999; Eltz *et al.* submitted; see Fig. 1). Hexane solutions (100 mg/ml) were prepared for each compound.

Antennal preparation, stimulation, and recording

Single antennae cut at the tip and at the third antennal segment were mounted between two glass pipettes filled with insect Ringer solution and connected to silver electrodes. Preparations were viable for 45 to 60 minutes, providing near-to-constant response levels over much of this time. During the test series, all substances were applied once per antenna and in constant order (synthetics as in Fig. 1, starting with methyl salicylate; solvent controls were applied twice on each antenna, defining the beginning and end of the test series). For every stimulus, 5 μ l of the test solution was pipetted on a fresh 2 × 10 mm strip of filter paper. The solvent was allowed to evaporate before the strip was placed in a clean pipette tip. For stimulation, 200 μ l of air was puffed over the filter paper and injected into a purified and moistened air stream blowing over the antenna. EAG responses were amplified and amplitudes were recorded (in mV) using Syntech (Hilversum) electrode holders, IDAC-232 acquisition controller, and EAG recording software.

Data analysis

To analyze whether a species' response to a given compound was greater than that elicited by the averaged respective solvent blanks, we calculated rank-based Wilcoxon-Matched-Pairs tests for all compounds, separately in each species. We then visualized differences in response spectra between individuals by non-metric Multidimensional Scaling (MDS), based on the Bray-Curtis similarity index (Legendre & Legendre 1998; Clarke & Gorley

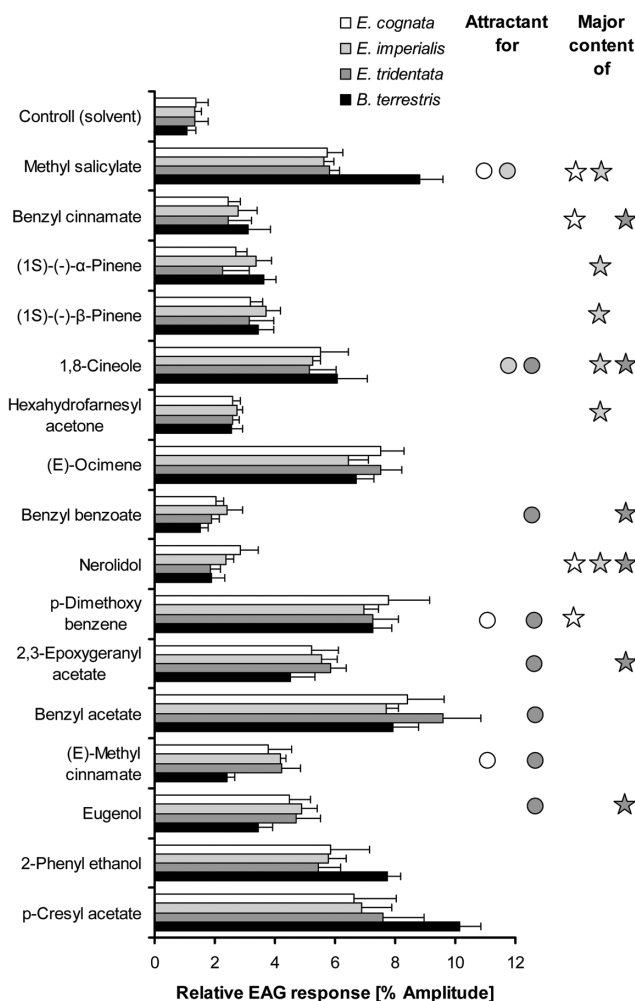


Fig. 1 Relative EAG responses (mean and standard deviation) of male *Euglossa* and *Bombus terrestris* to 16 synthetic fragrance compounds. Relative responses to solvent controls are also given. Filled circles indicate attractiveness to males in bioassays (Ackerman 1989; G. Gerlach, pers. comm.; T. Eltz, pers. obs.), stars denote compounds that are major components of tibial fragrances in Central Panama (Eltz *et al.* 1999; Eltz *et al.* submitted). Shades of bars and symbols indicate species affiliation

2001). In order to control for variations in size of bee and quality of preparation, we calculated the similarity index on standardized data. For this we transformed the absolute responses (amplitudes in mV) to relative responses (in % of the sum of responses per individual, i.e., the responses of an individual antenna to all tested compounds add up to 100 %; see also Fig. 1). Multidimensional Scaling (MDS) was done using the Software PRIMER (Clarke & Gorley 2001). In a perfect MDS plot, the rank order of distances between any two individuals correspond exactly to the ranked similarities in the underlying triangular similarity matrix (deviations from an exact match are represented in terms of stress, with values below 0.15 indicating a meaningful representation).

Results

All tested compounds elicited significantly larger absolute responses than did solvent blanks in each of the species

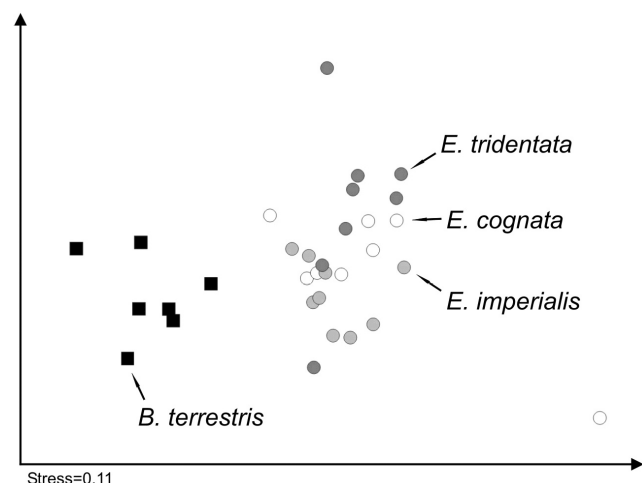


Fig. 2 Two-dimensional MDS representation of individual bees' responses to 16 synthetic fragrance compounds, based on Bray-Curtis similarities. Different symbols/shades are used for the different species. Note distinct cluster of male *B. terrestris*. Stress = 0.11

(Wilcoxon Matched-Pairs tests: $p < 0.05$ in all pairwise comparisons), with only responses to benzyl benzoate and nerolidol verging on the marginal. Fig. 1 shows the mean relative responses to synthetic compounds separately for each species. Different compounds varied strongly in their effects on bee antennae, with benzyl acetate eliciting the strongest relative responses in euglossines. The strength of the response was not closely related to a compounds' attractiveness in bioassays. E.g., methyl salicylate is an attractant for *E. imperialis* and *E. cognata*, but *E. tridentata* are never seen at this chemical. Nevertheless, the antennal responses were basically identical in all three species. Similarly, strong responses to p-dimethoxy benzene were also observed in *E. imperialis*, which is never attracted to that compound. Also, antennal responses did not correspond to the representation of the respective compounds in the species' tibial bouquets (Fig. 1).

Generally, across all synthetic compounds, the response spectra were very similar in the three species of *Euglossa*. Compared to that, male *Bombus terrestris* showed rather strong deviations, which is visualized by the two-dimensional MDS representation (Fig. 2).

Discussion

The tested synthetic fragrance compounds elicited differential but highly predictable responses on male bee antennae, with some difference of response spectra between bee genera (*Euglossa/Bombus*) but no difference between the different species of *Euglossa*. Also, the strength of an EAG response in the three orchid bee species was not related to the attractiveness of the tested compound in bioassays. These findings suggest that specific chemical bait preferences are not mediated by peripheral sensory adaptations. This interpretation should be regarded with some caution, however, because of the relative crudeness of the EAG method. Electroantennography

records sums of potentials produced by the whole of the antenna, but is essentially blind to the number and response profiles of olfactory receptors that contribute to that sum. Thus, an increase of a stimulus-specific response in a given receptor may remain undetected because it is cancelled out by compensatory effects, e.g., decreases in responsiveness in other receptors. In theory, similar EAGs could be generated by quite different sets of receptor types that have converged in their response profiles. More sophisticated methods such as single sensillum recording (SSR) or calcium imaging of glomerular responses in the antennal lobe would be necessary to single out such effects (Galizia *et al.* 2004).

Along with Schiestl and Roubik (2003) and another unpublished study by C. Skov (pers. communication) our results emphasize the generalized nature of peripheral fragrance perception in euglossine bees. In contrast to insect pheromone perception, which is frequently characterized by outstanding peripheral specialisation to single compounds, perception of floral odours involves multiple types of broadly tuned olfactory receptors, each responding to a wide range of relatively different chemical compounds (Akers & Getz 1992; Smith & Getz 1994). Odour recognition and discrimination is believed to depend largely on the interaction of multiple sets of receptors in higher nervous centres (Galizia *et al.* 1999; Galizia & Menzel 2000). In such a system, a given type of receptor that is particularly responsive to a certain compound (e.g., a major attractant) will not evolve independently from the rest of the sensorium. Increasing sensitivity to particular compounds may impose critical costs, e.g., concerning general discrimination ability. Such constraints may have counteracted peripheral sensory tuning to single compounds in the three species of *Euglossa*. Additionally, increasing sensitivity towards certain attractive compounds likely reduces sensitivity to others, including repellent ones. This may also be costly, because accumulation of species-specific odour blends in male euglossines may depend heavily on avoidance of certain odours. In accordance with this view, male euglossines refuse to collect their favourite compounds once these are blended with small amounts of unattractive modifiers (Williams & Dodson 1972). Alternatively, it might be argued that insufficient time was available for selection to act on peripheral sensory traits in the three *Euglossa*. Unfortunately, euglossine phylogenies are incomplete and data on species divergence times preliminary. In morphology *Euglossa imperialis* (subgenus *Glossura*) is quite distinct from *Euglossa cognata* and *E. tridentata* (subgenus *Euglossa* s.s.) (Dressler 1978), suggesting that divergence occurred several million years ago. This interpretation is backed by a molecular clock analysis based on COI sequence data, which suggests that even very closely related *Euglossa* (e.g., within a subgenus) have diverged more than 1.3 million years ago (C. Dick, unpublished data). Thus, lack of time alone is unlikely to explain the very similar responses to synthetic compounds in the study species.

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