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SPECIES-SPECIFIC ANTENNAL RESPONSES TO TIBIAL FRAGRANCES BY MALE ORCHID BEES

THOMAS ELTZ,[1,*] MANFRED AYASSE,[2] and KLAUS LUNAU[1]

[1]*Department of Neurobiology, Sensory Ecology Group, University of Düsseldorf, Universitätsstr. 1, 40225 Düsseldorf, Germany*

[2]*Department of Experimental Ecology, Chemical Ecology Group, University of Ulm, Albert-Einstein-Allee 11, 89069 Ulm, Germany*

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Abstract—Male neotropical orchid bees (Euglossini) collect odoriferous substances from orchids and other sources and store them in tibial pouches, accumulating complex and species-specific bouquets. These fragrances are later exposed at display sites, presumably to attract females or conspecific males or both. We hypothesized that the necessity to detect and recognize specific fragrance bouquets has led to peripheral chemosensory specializations in different species of orchid bees. To test this, excised male antennae of four species of *Euglossa* were stimulated with complete tibial extracts of the same four species in a crosswise experiment. In the majority of the tested extracts, the amplitude of the electroantennogram (EAG) response was significantly different between species and always maximal in males of the extracted species. This effect did not appear to result from a given species' increased sensitivity toward certain attractive components: gas chromatography with electroantennographic detection (GC-EAD) of one extract of *Euglossa tridentata* evoked similar and generalized response patterns in all four species, encompassing a total of 34 peaks that elicited antennal responses. Therefore, the species effect in EAG responses to complete extracts likely resulted from species-specific interactions of compounds at the receptor level. Antennal specialization to conspecific bouquets adds additional strength to the argument that specificity is an important evolutionary aspect of euglossine tibial fragrances.

Key Words—Sensory specialization, olfaction, EAG, GC-EAD, odor bouquet, Euglossini.

* To whom correspondence should be addressed. E-mail: eltz@uni-duesseldorf.de

INTRODUCTION

Male orchid bees are uniquely adapted for collecting and storing volatile chemicals, which they seek at flowering orchids, aroids, palms, and other plants, as well as at nonfloral sources such as rotting wood and feces (Vogel, 1966; Dressler, 1982). The fragrances are dissolved in lipoid labial gland secretions and are transferred to spacious cavities in the bees' hind tibiae. Here, complex mixtures of terpenoids and aromatics accumulate over weeks or months (Whitten et al., 1989; Eltz et al., 1999). Mounting evidence suggests that males release the substances during bouts of courtship display, for which they defend small territories around perch trees in the forest understory (Bembé 2004; Eltz et al., 2005a). On rare occasions, females visit these territories to mate, but it is unknown whether their attraction is mediated by the fragrances or indeed by any other chemical stimulus (Kimsey, 1980; Eltz et al., 2003).

Chemically, the tibial contents seem to be species-specific in a rather broad sense: males of *Euglossa* vary substantially in qualitative and quantitative aspects of their bouquets, but much less so within than between species. Notably, each species has a characteristic set of fragrance compounds (~6–15) that is shared with high probability even by individuals from distant and ecologically divergent localities (Eltz et al., 1999, 2005b). Field experiments suggest that this chemical profile is sufficient to promote specific long-range attraction. When hexane extracts of *Eulaema meriana* and *Eulaema bombiformis* hind legs were applied to filter paper and exposed at known male display sites in the forest understory, these extracts quickly and reliably attracted other males of the correct species (Zimmermann, Roubik and Eltz, submitted). Thus, it seems that fragrance perception is important to male euglossines in two rather different contexts. First, males must find remote fragrance sources, which emit mostly either single components (e.g., some nonfloral odors (Whitten et al., 1989, 1993; Eltz et al., 1999) or simple blends of 2 to 10 components (most euglossophilous orchids; Williams and Whitten, 1983; Gerlach and Schill, 1991). Second, fragrance perception is important in the context of territorial behavior, where males encounter the much more complex blends previously accumulated by their peers. In either context, one might expect strong selection pressures leading to sensory specialization in favor of certain odor qualities and divergent sensory tuning in different species of bees.

Here, we used electroantennography (EAG) to quantify antennal responses to conspecific and heterospecific tibial extracts in four species of *Euglossa*. EAG records the sums of receptor potentials from the entirety of olfactory neurons located in the antenna, and the amplitude of the recorded signal is assumed to reflect the strength of stimulation evoked by an odor. The method has previously been used to investigate odor detection in a wide variety of insects (Roelofs, 1984; Schiestl and Marion-Poll, 2002), including orchid bees (Schiestl and Roubik, 2003). In addition to recording EAG responses to

complete extracts, we used gas chromatography coupled with electroantennographic detection (GC-EAD) to screen tibial extract components for their ability to elicit responses from antennae of male bees.

METHODS AND MATERIALS

Bees. Males of *Euglossa imperialis* (imp), *E. cognata* (cog), *E. mixta* (mix), and *E. tridentata* (trid) were captured during the first 2 wk of March 2005 in forests of the Barro Colorado National Monument, Panama. Individuals were captured upon arrival at screened 1,8-cineole (imp, cog, trid), methyl salicylate (imp, cog, mix), *p*-dimethoxybenzene (cog, trid, mix), and skatole (mix) baits, and were later transferred to Düsseldorf, Germany, in individual vials. The bees were introduced into 50 × 50 × 60 cm mesh cages placed in a greenhouse (25–30°C, 70–90% relative humidity) where they learned to drink honey-water from artificial flowers. Individuals were subjected to EAG (Düsseldorf) and GC-EAD (Ulm) over the course of 4 wk.

Test Extracts. Hexane extracts were prepared from males of all four species collected at the same time at the same Panamanian locality (see above). Individual heads and pairs of hind legs were extracted separately in 0.5 ml of p.a. grade hexane (Merck). Hind-leg extracts were screened by GC-MS for fragrance content, and three rich extracts of each species were selected as EAG stimuli. This screening for tibial content was necessary because many individual male *Euglossa* contain only tiny amounts of fragrances (Eltz et al., 1999). The selected extracts were likely derived from relatively old males that had accumulated substantial quantities. The hind-leg extracts contained a large quantity and variety of lower molecular weight (<300 Da) terpenoids and aromatics [the exogenous fragrances; see Eltz et al., (1999)], a range of long chain saturated and unsaturated hydrocarbons, alcohols, acids, acetates, diacetates, and esters ("lipids," mostly products of the bees' labial glands, added in the process of fragrance collection; Williams and Whitten, 1983), and a few high molecular weight compounds presumably derived from plant surfaces (e.g., triterpenoids) (see Appendix; Available online at www.springerlink.com; Search for DOI: 10.1007/s10886-006-9352-0). To test whether labial gland lipids alone stimulated antennae, we prepared and tested one head extract of each of the species. Head extracts contained roughly similar amounts of labial gland lipids as did the hind-leg extracts, but lacked fragrance compounds (see Appendix; Available online at www.springerlink.com; Search for DOI: 10.1007/s10886-006-9352-0).

EAG with Complete Extracts. 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. All extracts were tested once on each antenna. During the test series, one stimulus was applied every 2 min, with the

individual extracts arranged in blocks of four (one of each species per block, randomized within-block sequence), interspersed by solvent blanks. For every stimulus, 5 μl of the test solution were pipetted onto 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 μl of air were puffed over the filter paper and injected into a purified and moistened air stream blowing over the antenna. EAG responses were amplified and recorded (in mV) using Syntech (Hilversum, The Netherlands) electrode holders, IDAC-232 acquisition controller, and EAG recording software. The amplitude of the negative baseline deflection was used as a measure of response. To analyze whether a species' response to an extract was greater than that elicited by the averaged hexane blanks, we calculated rank-based Wilcoxon matched-pairs tests for all extracts, separately in each species. We then standardized responses to extracts across antennae to control for variations in the size of bee and the quality of preparation. Single-factor ANOVA was calculated to test for the overall effect of the factor *species* on the response to a given extract.

GC-EAD Screening. The tibial extract *trid 3* was selected to screen for the electrophysiological effect of its single components. Antennal preparations were identical to those described above. GC-EAD was done with an HP 6890 gas chromatograph fitted with a DB-5 column (J&W Scientific, Folsom California, 30 m × 0.25 mm I.D.). A variable outlet splitter (SGE) was used to divide the effluent 3:1, and the larger fraction was directed onto the bee antennae. Nitrogen was used as a makeup gas. Flame ionization detector and EAD signals were recorded with Syntech EAD recording software. For each run, 1 μl of the extract was injected splitless, testing antennae of males of all four species. The GC oven was programmed from 50 to 300°C at 10°C/min. A peak was classified as "GC-EAD active" when it coincided with a negative EAD baseline deflection in all analyzed runs. Each antenna was tested only once, and all scored antennae originated from separate individuals.

RESULTS

EAG Responses to Complete Extracts. All tested hind-leg extracts elicited substantially larger antennal responses than did solvent blanks in each of the species [Wilcoxon matched-pairs tests: $P < 0.01$ in all 48 comparisons (12 extracts × 4 species); Figure 1]. Irrespective of the perceiving species, the response level varied between extracts, with *E. tridentata* extracts eliciting relatively large and *E. imperialis* extracts relatively small responses (Figure 1). This variation corresponded to the overall concentration of fragrances in the different extracts (see Appendix; Available online at www.springerlink.com; Search for DOI: 10.1007/s10886-006-9352-0). In 9 of the 12 hind-leg extracts, there was a significant effect of the perceiving species on the magnitude of the

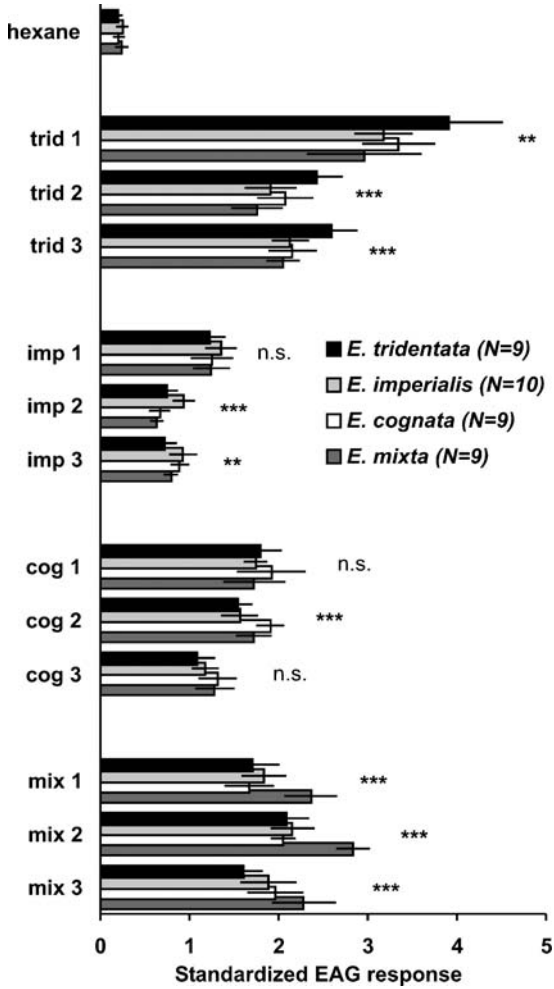

FIG. 1. Standardized electroantennogram response (mean and standard deviation) of males of four species of *Euglossa* to hind-leg extracts taken from males of the same four species captured in Central Panama. Three extracts of each species were tested (trid = *E. tridentata*, mix = *E. mixta*, imp = *E. imperialis*, cog = *E. cognata*). Significance levels of the factor "perceiving species" on response amplitude are given (single-factor analysis of variance: $N = 37$; $^{**}P < 0.01$; $^{***}P < 0.001$).

response (see Figure 1), with the correct (extracted) species showing the largest response in all cases. The species effect was particularly pronounced in the case of *E. tridentata* and *E. mixta*. Here, the extracted species showed a response that was roughly 20% larger than that of the other species.

In contrast to hind-leg extracts, the head extracts elicited only weak responses that were not significantly different from averaged solvent blanks in most comparisons (data not shown here). The following responses were marginally significant (Wilcoxon matched-pairs tests; $P < 0.05$): *E. cognata* to *E. tridentata*, *E. tridentata* to *E. tridentata*, and *E. imperialis* to *E. cognata*, *E. imperialis*, and *E. tridentata*.

GC-EAD Screening. The tibial extract *trid 3* was selected to screen for physiologically active components. We analyzed a total of 17 GC-EAD runs: 5 with *E. tridentata*, 6 with *E. cognata*, 3 with *E. imperialis*, and 3 with *E. mixta* antennae. A total of 33 GC peaks elicited reproducible negative baseline deflections in all species, and 1 additional peak, an uncharacterized trace compound (28 in Figure 2), did so only in *E. tridentata*. Most of the dominant fragrance components elicited EAD responses, frequently causing strong negative deflections. Reproducible deflections also were caused by several minor fragrance components and by eicos-9-enyl-1,20-diacetate (34), the major component of *Euglossa* labial gland secretions. A few peaks were not active in spite of their relatively large size. These included the moderate peaks of α-pinene and β-pinene and the massive peak of (*E*)-nerolidol. The overall response pattern was similar between species, although some late eluting fragrance compounds seemed to cause somewhat larger responses in *E. tridentata* (29 and 30 in Figure 2).

DISCUSSION

Tibial fragrance bouquets of male *Euglossa* bees elicited larger EAG responses from conspecific than heterospecific antennae. This suggests that peripheral olfactory specialization has occurred in the different species of orchid bees, presumably because of different olfactory cues being important in their respective life cycles.

The various species of euglossine bees differ in the type of fragrances they prefer to collect. This is evident in the range of synthetic chemicals that can be used to lure males of different species during baiting assays (Ackerman, 1989). In a previous study, we tested a broad range of these pure compounds, but did not find a species-specific difference in the EAG responses, although the tested species had quite distinct chemical preferences on the behavioral level (Eltz and Lunau, 2005). These findings are broadly confirmed by the GC-EAD screening of extract components in the present investigation: response patterns to bait chemicals such as 1,8-cineole, eugenol, *p*-dimethoxybenzene, 2,3-epoxygeranyl acetate, or benzyl benzoate were surprisingly similar across species.

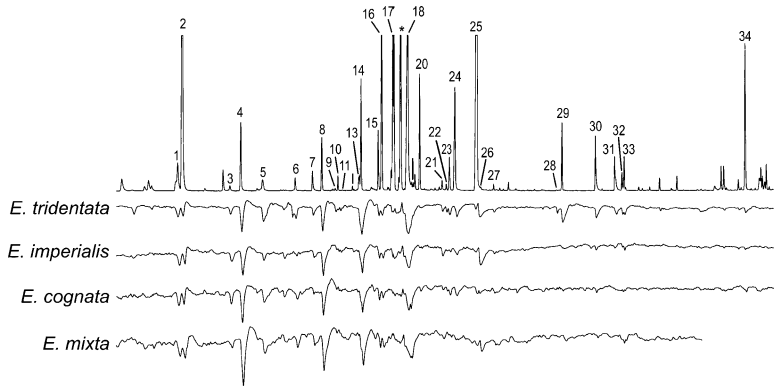

FIG. 2. Antennal responses of four male *Euglossa* spp. to components of a hind-leg extract of *E. tridentata* (*trid 3* in Figure 1) eluting from the gas chromatograph. The flame ionization detector chromatogram is shown along with one representative electroantennographic detection (EAD) signal for each of the study species. Numbered peaks correspond to reproducible negative deflections of the baseline in all four species, except peak (28), which was active in *E. tridentata* only. Peaks (12) and (19) are marked but not numbered on the chromatogram because of limited space. (1) Limonene + 1,8-cineole; (2) (*E*)-β-ocimene; (3) ipsdienol; (4) *p*-dimethoxybenzene; (5) ?; (6) trace; (7) 2,3-epoxygeraniol; (8) eugenol; (9) (*E*)-methyl cinnamate; (10) β-elemene, ?; (11) trace; (12) (*E*)-caryophyllene; (13) (*E*)-isoeugenol; (14) 2,3-epoxygeranyl acetate; (15) (*E*,*E*)-α-farnesene; (16) β-bisabolene; (17) sesquiterpene alcohol; (18) *p*-methoxycinnamic alcohol; (19) methoxyeugenol; (20) (*E*)-farnesene epoxide; (21) (*E*)-methyl *p*-methoxycinnamate; (22) hedycaryol; (23) similar methoxyeugenol; (24) (*E*)-*p*-methoxycinnamyl acetate; (25) benzyl benzoate; (26) trace; (27) hexahydrofarnesyl acetone; (28) trace; (29) ?; (30) 3,7,11,15-tetramethyl-hexadeca-2,6,10,14-tetraen-1-ol, ?; (31) diterpene alcohol; (32) + (33) long-chain unsaturated acids; (34) eicos-9-enyl-1,20-diacetate; (35, not shown) long-chain acetate. *Peak of (*E*)-nerolidol, the only major component that did not elicit an EAD response. See text for further specifications.

Why should complete tibial bouquets elicit species-specifically different EAG responses if critical components do not? Natural fragrance cues encountered by male bees are in fact mixtures, either the tibial fragrances themselves (as exposed by displaying males) or the more simple blends of the floral sources from which they are derived. Detecting and discriminating among mixtures of odorants is certainly important to the bees and may be a challenging task given that more than 30 species of euglossines may coexist in neotropical forests. Antennal specializations to certain odorant combinations might facilitate fragrance resource partitioning among competing males of different species as well as benefit fragrance-based chemical communication within species (e.g., facilitate species recognition).

On the mechanistic level, species-specific differences in EAG responses to complete extracts could have accumulated from small species-specific differences in sensitivity to certain components. Each of these differences may have been too small to be detected when components were tested individually (as in Eltz and Lunau, 2005), but when combined in complex blends, they might produce a significant overall effect. Furthermore, with mixtures of odorants, antennal responses may also be influenced by how different components interact on the level of olfactory receptor neurons (Akers and Getz, 1993; Cromarty and Derby, 1997). Recent work on *Drosophila* suggests that individual receptor proteins can confer either excitatory or inhibitory effects on an olfactory neuron, depending on precisely which odorant is binding to them (Hallem et al., 2004). In the case of complex mixtures, a number of different odorants could bind simultaneously to any given neuron, with relative concentrations determining the outcome of the neuron-level response. If tested as mixtures, inhibitory effects of certain components may have substantial effects on the overall antennal response. For orchid bees, Schiestl and Roubik (2003) have argued that inhibitory interaction of compounds at the receptor level may be responsible for decreased EAG responses to certain synthetic fragrance mixtures. We hypothesize that species-specific inhibitory effects were responsible or at least contributed to differential responses to tibial bouquets. This view has interesting implications for the orchid–euglossine bee coevolution. Normally, a given euglossophilous orchid attracts only one or a few species of euglossine bees (Roubik and Hanson, 2004), although its scent may be dominated by a broadly attractive component. In such a case, selectivity appears to be achieved by minor modifying compounds that render a given bouquet unattractive to all but the pollinating species (Williams and Dodson, 1972). The inhibitory effects of these modifiers may already be operating at the antennal level. Future studies should attempt to imitate naturally occurring orchid fragrances by mixing synthetic components in the correct blends. Using stringent comparative EAG testing with pollinating and nonpollinating bees might help to elucidate the sensory basis of specialized orchid–bee interactions.

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