

Species-specific attraction to pheromonal analogues in orchid bees

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Abstract Male orchid bees (Euglossini) collect fragrances from flowers and other natural sources, a behaviour that has shaped the euglossine pollination syndrome. Males store such chemicals in hind leg pouches and later expose them during courtship display. In the present study, we show that complex bouquets of two sympatric species of *Eulaema*, *E. meriana* and *E. bombiformis*, are chemically distinct. When exposed during bioassays at display perches individual hind leg extracts rapidly and consistently attracted other males of the correct species, even if derived from males of disparate localities (French Guiana and Panama). Conspecific males as well as females of *E. bombiformis* arrived at natural perch sites only from downwind, and two copulations were observed. Our findings demonstrate that acquired odours mediate exclusive attraction within species and support the idea that such fragrances are pheromone analogues. Their role in acquiring matings and during male–male interaction is discussed.

Keywords Chemical communication · Odor signal · Attractant · Euglossini · Fragrance

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Introduction

Male neotropical orchid bees (Euglossini) have enlarged hind tibiae containing a fiber-filled pouch that the bees use for fragrance accumulation (Vogel 1966; Williams and Whitten 1983). During adult life, the male bees devote much time and energy to fill the pouch with volatiles collected at orchid flowers as well as other floral and non-floral sources. The specialised behaviour has given rise to a euglossine pollination syndrome, whereby males of 200+ bee species are pollinators of about 650 orchid species (Dressler 1982; Williams 1982). The evolutionary causes of fragrance collection are yet speculative (Roubik and Hanson 2004).

Male euglossines apply labial gland lipids—both solvent and carrier for volatiles (Whitten et al. 1989)—to fragrant substrates. Resulting blends are then collected with stereotypic movements incorporating all six legs (Vogel 1966; Kimsey 1984). Once stored inside the hind tibia the volatiles persist over long periods of time and eventually, as new fragrances are added, form a complex fragrance bouquet (Eltz et al. 1999). In *Euglossa*, blends are species-specific and remarkably independent of the locality and forest type (Eltz et al. 2005a). That fragrance blends should be characteristic of a given species is in agreement with one of the earliest and most straightforward hypotheses to explain fragrance collection: acquired volatiles have replaced endogenous pheromones for intraspecific communication during courtship (Vogel 1966). The present study examined this hypothesis quantitatively with behavioural experiments.

Male orchid bees occupy small non-resource-based territories in the forest understory, where they perch on stems or branches and where matings occur (Kimsey 1980; Eltz et al. 2003). Perching males perform a characteristic display behaviour that includes repeated hovering flights

(genus *Euglossa*) or wing buzzes (genus *Eulaema*) (Kimsey 1980; Stern 1991; Stern and Dudley 1991; Eltz et al. 2003). Fragrances are actively released during display by stereotypic leg movements, which promote transfer of hind tibial contents to tufts on the mid tibiae (Eltz et al. 2005b), where the volatiles are positioned to become dispersed by the wing strokes (Bembé 2004).

Eulaema meriana and *Eulaema bombiformis* are close relatives, the largest of all euglossines, and occur sympatrically in most of the Neotropics (Roubik and Hanson 2004). Males frequently display near hilltops or ridges (D. W. Roubik, T. Eltz, personal observation). On Barro Colorado Island, Panama, where most of the present study was carried out, perch sites are relatively easy to locate in old-growth forests on a plateau, which has attracted the attention of previous investigators (Kimsey 1980; Stern 1991). Here, male *E. bombiformis* prefer smaller perch trees (e.g. *Miconia*, *Gustavia*), whereas *E. meriana* displays on the lower trunks of large-canopy trees (Stern 1991). In both species, the display takes place predominantly during mid morning, with individual males perching at the same tree over hours and days. Conspecific males regularly approach and interact with perch holders (Stern 1991; D. W. Roubik, unpublished observation), and three copulations have been observed at perch sites of *E. meriana* (Kimsey 1980; Stern 1991). The role that fragrances may play in intraspecific communication has not previously been investigated.

In this paper, we combine field observations, chemical analyses of hind leg fragrances, and behavioural experiments to investigate the potential for fragrance signalling by perching male orchid bees. Bioassays with tibial extracts of both *Eulaema* were conducted at their known display sites, exploring the hypothesis of species-specific attraction. We tested whether tibial odours attracted males, or females, or both, and whether attraction depends on the display context.

Materials and methods

One part of the study took place on Barro Colorado Island (BCI), Panama, from February to May in 2005. During this period, we surveyed perch trees close to the new radio tower on the island plateau, near 160 m elevation, and observed displaying males of *E. meriana* and *E. bombiformis*. Standardised bioassays, herein “BCI 2005 bioassays”, were conducted using hind leg and head extracts prepared from local males collected at the time of the study, and the extracts were later subjected to chemical analysis (see below).

Between-locality bioassays were conducted in French Guiana (2003) and Panama (2004). These bioassays tested attractiveness of hind leg extracts of Panamanian males in French Guiana, and vice versa (see below).

Additionally, D. W. R. made 40 h of observations at three perch sites of *Eulaema bombiformis*, two in the radio tower area on BCI (2003–2004) and one at Santa Rita Ridge (2005), on a small forest trail, for a total of 20 days. These observations were made during the dry season (December–March), when the wind is light but steady from the north when the males are at their perch sites. In that study, compass directions of each bee approach were recorded.

Extracts

Male *E. meriana* and *E. bombiformis* were caught at display trees or lured to individual synthetic fragrance compounds 1,8-cineole, benzyl acetate, methyl salicylate, terpinene-4-ol or geraniol. Baits were screened with a fine nylon mesh to prevent chemical collection. Captured males were killed and pairs of hind legs and heads were removed and each extracted in 500 μ l of *n*-hexane (Aldrich, Germany).

BCI 2005 bioassays

Bioassays were conducted from 8:00 A.M. to 12:00 noon on bright days only. In a first series of bioassays, we tested species-specific attraction to hind leg extracts. Hind-leg extracts of both species as well as hexane controls were presented 25 times at display trees of *E. meriana* (11 different individual trees) and 25 times at display trees of *E. bombiformis* (three trees), for a total of 150 trials. The selected display trees were generally used by males at the time of the study (as confirmed by our surveys), but had been vacant for at least a few minutes before extract presentation. Individual hind leg extracts for bioassays (nine of *E. meriana*, five of *E. bombiformis*) were selected without a priori knowledge of their contents, but we did not employ those that had little or no smell to us. For each presentation, 30 μ l of a given extract was pipetted on filter paper (Whatman 1, 2.5 cm), the solvent was allowed to evaporate, and the filter paper was attached to the stem of the display tree with a stainless steel pin. The tree was observed for 10 min.

A second series of bioassays was conducted (1) to further explore the importance of context on chemical attraction, and (2) to evaluate the potential of cephalic gland secretions alone to mediate attraction. To test for context effects, we presented *E. meriana* hind leg extracts at active display trees of *E. meriana* as well as at non-display trees of similar size located within 100 m ($N=39$). To test for attraction to cephalic gland secretions, we presented *E. meriana* head extracts at active display trees of *E. meriana* ($N=39$).

Chemical analysis

Extracts prepared for the BCI 2005 bioassays were transferred to the Department of Neurobiology, Düsseldorf, and

analysed by Gas Chromatography/Mass Spectrometry (GC/MS) using a HP 5890 II GC fitted with a 30-m non-polar DB-5 column and a HP 5972 mass selective detector. Injection was splitless, the oven programmed from 60 to 300°C at 3°C/min with automatic pressure programming. Peaks were cross-referenced with entries of the local user library (T. Eltz, unpublished), based on comparisons of mass spectra and associated retention times. New components were added to the library as the analysis progressed. Compound identifications were made by comparison with synthetic standards, by matching spectra and retention time with those in Adams (Adams 2001), and by R. Kaiser (Givaudan, Zürich) who analysed representative samples. Compounds were assigned to three groups: (1) exogenous fragrances, typically terpenoids or aromatics of low molecular weight, not present in head extracts, (2) endogenous long-chain lipids also present in head extracts [probably synthesised by the bees' large cephalic labial glands and transported to the hind tibiae during fragrance collection (Whitten et al. 1989, 1993)], and (3) other high-molecular-weight compounds, some potentially from plant sources, but not considered volatiles (e.g. phytosterols).

Similarity analysis

The composition of hind tibial contents was analysed with non-metric Multidimensional Scaling (MDS) and associated techniques (Legendre and Legendre 1998). MDS makes almost no assumptions about the form of the data and is flexible concerning the similarity metric used (Clarke and Warwick 2001). Absolute peak areas (integrated ion currents) were square-root transformed and standardised to represent relative peak contributions to individual samples (in %). From the data matrix, we derived a triangular similarity matrix based on the Bray–Curtis index. Similarities were ordinated in three dimensions using the MDS algorithm in PRIMER v5 (Clarke and Gorley 2001). Ideal MDS plots have interpoint distances that exactly match the rank order of dissimilarities between samples in the underlying matrix. Deviations from this match are expressed in terms of 'stress', with stress values <0.15 indicating a good fit concerning the overall structure of the plot (Clarke and Warwick 2001).

We tested the null hypothesis that the factor species had no effect on the rank order of between-individuals similarities using analysis of similarities (ANOSIM) permutation tests with one-way design (Clarke and Green 1988). Finally, we identified compounds that were responsible for creating the observed similarity patterns using the PRIMER SIMPER algorithm. This routine calculates percent contributions of each compound to the overall similarity within species or dissimilarity among them. The index weighs both the extent and consistency by which each compound contributes to the overall pattern (Clarke and Warwick 2001).

Between-locality bioassays

These bioassays were conducted by saturating a small (1 cm²) piece of Whatman 1 filter paper with hexane extract and placing this on the surface of a tree, held in place with a stainless steel pin. Presentations were made between 8:00 A.M. to 12:00 noon on sunny days. Extracts made from individual males of both species from Panama were placed at non-perch trees on a hilltop at 200 m in a forest at Montagne des Signes, French Guiana, during dry season (September) of 2003 ($N=1$ each; synchronous exposure). Reciprocal exposures of hind leg extracts taken from each species at Montagne Tortue, French Guiana, were made at non-perch sites on hilltops at Ancon Hill, Pipeline Road, and BCI, Panama, in January 2004 ($N=3$ exposures with *E. bombyiformis* extracts, $N=3$ exposures with *E. meriana* extracts). The presentations were made for 30 min at a site.

Results

Male display, wind and mating

In 2005, a total of 21 perch trees of *E. meriana* and four perch trees of *E. bombyiformis* were located on BCI. Males of both species displayed predominantly in the morning between 8:00 and 11:30. Displaying individuals perched and regularly buzzed their wings as previously described for *E. meriana* (Kimsey 1980; Stern 1991; Stern and Dudley 1991). In both species, other males regularly approached perch holders, but only non-aggressive interactions took place until one or both males left. No copulations or other encounters with females were observed on BCI in 2005.

Forty hours of observations at perch sites on BCI (2003–2004) and at Santa Rita Ridge (2005) witnessed two copulations of *E. bombyiformis*, one in each locality, at 9:51 a.m. on BCI on 18 December 2003, and at 9:48 a.m. on 23 March 2005, at SRR. In both cases, the female landed next to the perching male after direct and rapid approach from the south, which was downwind from the persistent dry season breeze. The pairs flew in copula, then disengaged when near the ground, after which the female left and the male landed at the perch. Both matings lasted 7 s. Approaches by conspecific males occurred also consistently from the southern downwind direction.

Chemical composition of tibial extracts

A complete list of the compounds detected in BCI (2005) hind leg extracts is given in the Appendix. Individual extracts of both species contained a wide range of fragrance compounds (Table 1), with individual bouquets consisting of 3 to 31 from a total of 106 compounds (median=14 in

Table 1 Most frequently detected fragrance compounds in hind-leg extracts of male *E. meriana* and *E. bombiformis* from BCI, Panama

RT (min)	Compound	Number of individuals with compound			Contribution to within species-similarity (%)		Contribution to between-species dissimilarity (%)
		<i>E. bombiformis</i> N=7	<i>E. meriana</i> N=41	Both N=48	<i>E. bombiformis</i>	<i>E. meriana</i>	
5.52	Pinene, alpha	–	14	14	–	0.41	0.40
6.56	Sabinene	–	34	34	–	5.74	2.44
7.07	Myrcene	4	4	8	0.77	0.02	0.66
8.29	Cymene, para- (ortho-?)	–	27	27	–	3.20	1.62
8.54	Benzyl alcohol	5	7	12	2.59	0.55	2.09
8.58	Cineole, 1,8-	–	27	27	–	4.19	1.98
9.06	Ocimene, (E) -beta-	7	–	7	10.54	–	4.84
9.50	Terpinene, gamma-	–	22	22	–	2.02	1.22
9.93	Sabinene hydrate, cis- (rel. to OH)	–	21	21	4.04	2.79	
9.99	?	–	17	17	–	2.89	2.12
11.10	Sabinene hydrate, trans-?	–	12	12	–	0.67	0.72
11.15	?	–	13	13	0.49	0.45	
11.35	?	–	11	11	–	0.40	0.48
11.58	Phenylethyl alcohol, 2-	5	–	5	5.78	–	3.96
12.71	Limonene oxide, trans-?	1	15	16	–	1.15	0.90
12.86	Ipsdienol	7	–	7	16.41	–	6.59
13.71	Benzyl acetate	5	8	13	6.15	0.48	4.05
14.58	Terpinen-4-ol	–	40	40	–	31.62	8.74
14.95	Methyl salicylate	–	22	22	–	2.30	1.59
17.31	Carvone	3	27	30	0.60	4.76	1.79
17.71	Phenylethyl acetate, 2-	4	–	4	4.67	–	4.23
18.00	Carvone epoxide, cis-	5	38	43	5.57	27.93	6.82
19.35	Indole	3	–	3	–	1.87	
20.99	?	2	4	6	0.15	0.05	0.47
21.16	trans-(trans-Carveol) epoxide?	–	12	12	–	0.69	0.67
23.06	?	7	–	7	6.72	–	2.99
23.27	Geranyl acetate	2	–	2	0.16	–	0.38
28.48	Bulnesene, alpha-?	2	–	2	0.18	–	0.47
30.39	?	7	20	27	12.53	2.15	4.22
34.38	?	7	–	7	14.89	–	6.29
34.56	Methoxynaphthol	7	–	7	10.71	–	5.11
36.68	Bergamotol, (Z) -alpha-, trans-	–	15	15	–	1.34	1.33

Contribution of compounds to within-species similarity as well as between-species dissimilarity of the fragrance bouquets is given. Selection of compounds for this table was based on the frequency of occurrence in individual samples (corrected for differences in sample size between the two species). A complete list of chemical compounds in hind-leg extracts, including data on relative abundance, is given in the [Appendix](#)

E. bombiformis and 13 in *E. meriana*). There was no difference in the number of components present in extracts between the two species ($N=48$; Mann–Whitney $U=148.5$; $p=0.97$). MDS produced non-overlapping species-specific clusters of individuals (Fig. 1a), and the factor *species* had a significant effect on fragrance composition (ANOSIM: $N=48$; $R=0.93$; $p<0.001$). Within *E. bombiformis*, average similarity of fragrance composition was 60.2%, within *E. meriana* 44.4% (based on the PRIMER SIMPER algorithm). Between individuals of different species, the average similarity was only 11.08%. In both species, ten major components characterised the respective bouquets by

contributing ~90% to the within-species similarity. Of those, a minority was abundant in one species but absent in the other. Those were ipsdienol, an unidentified compound at 34.38-min retention time, and (*E*)-beta-ocimene in *E. bombiformis*, and terpinen-4-ol, sabinene and 1,8-cineole in *E. meriana*. Other major compounds, e. g. benzyl alcohol, myrcene and carvone, were shared by both species but differed in relative quantity.

The composition of labial gland lipids in hind leg extracts also differed between species (ANOSIM: $R=0.74$; $p<0.001$), but there was greater overlap in comparison to fragrance composition (Fig. 1b). All major lipid compounds were

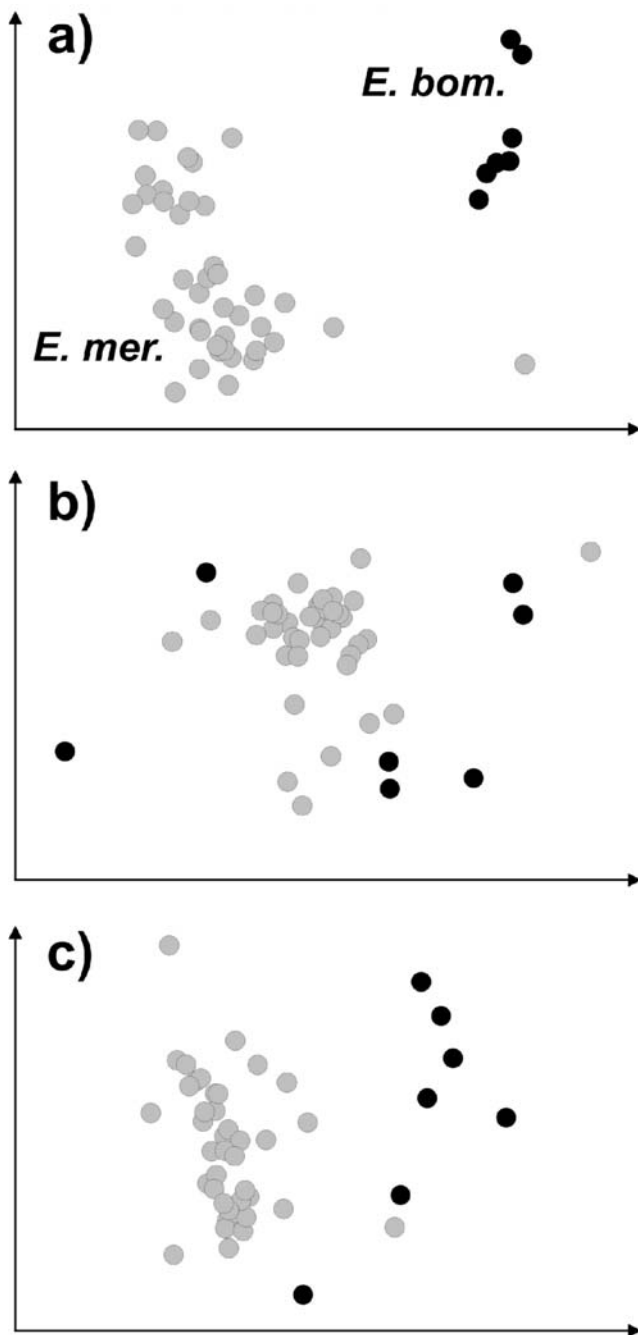


Fig. 1 Chemical similarity of hind tibial contents of individuals of *E. meriana* and *E. bombiformis* illustrated as two-dimensional MDS plots derived from Bray–Curtis similarities, **a** based on fragrance compounds only (106 compounds; stress=0.12), **b** based on compounds that were also detected in head extracts (presumed labial gland lipids; 31 compounds; stress=0.11), and **c** based on all detected compounds (stress=0.13)

detected in both species (see Appendix). When MDS was done based on all compounds detected in hind leg extracts (including fragrance compounds and labial gland lipids), the strength of the separation between species (reflected in the ANOSIM R value) was intermediate between the two previous analyses (Fig. 1c; $R=0.87$, $p<0.001$).

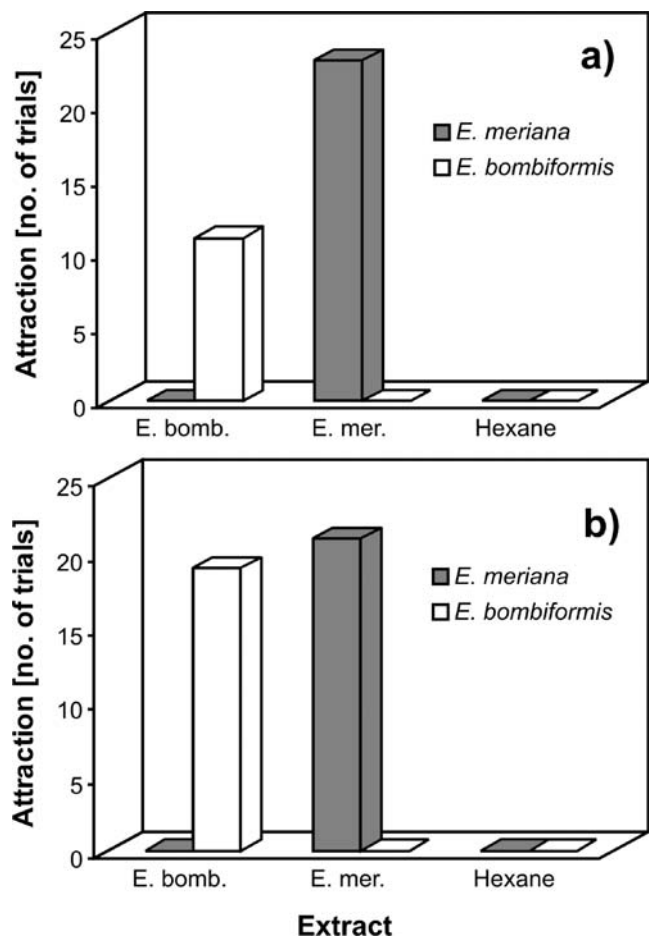


Fig. 2 Attraction of male *Eulaema* to conspecific and heterospecific hind-leg extracts as well as to solvent controls presented on filter paper at display trees of *E. meriana* (**a**) and *E. bombiformis* (**b**). Twenty-five presentations (trials) were done with each type of extract at each species' display trees

BCI 2005 bioassays

Hind-leg extracts only attracted males of the extracted (correct) species (Fig. 2). Females were never seen. Solvent alone attracted no bees. *E. bombiformis* males were more frequently attracted to hind leg extracts when these were exposed at the *E. bombiformis* display trees (Fisher's exact test: $N=50$; $p<0.05$). *E. meriana* came in equal frequency to *E. bombiformis* perches (Fisher's exact test: $N=50$; $p<0.05$), which were all very near the perches of *E. meriana* (sometimes less than 10 m, never more than 50 m away). Arrival of the first male took place on average 2.88 min (*E. meriana*) and 3.72 min (*E. bombiformis*) after extract presentation. In 50% of the cases, more than one male (up to four) was attracted. Males attracted to hind leg extracts first came to within about 3 m, then halted and hovered in the air in the same way as if approaching a perching conspecific male. Most males then closed in on the filter paper, hovered 20 to 50 cm from it and often made repeated

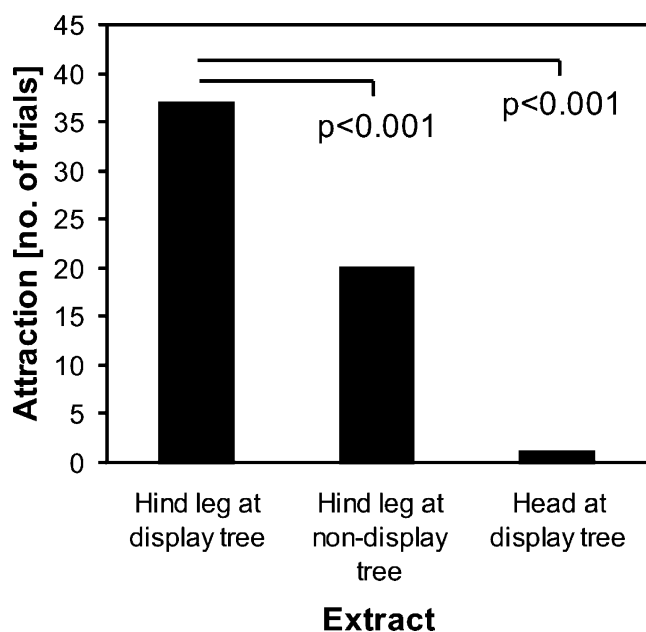


Fig. 3 Attraction of male *Eulaema meriana* to conspecific hind-leg extracts presented at display trees of *E. meriana* and at nearby non-display trees, and likelihood of attraction to conspecific head extracts. Thirty-nine presentations (trials) were done in each category. Significance values refer to Fisher's exact tests (see text for details)

darting moves against it (see below). Thirty-one percent of attracted *E. meriana* and 30% of *E. bombiformis* landed on the filter paper and performed collecting behaviour. If more than one individual was present, the males usually circled around each other before one or both left, but few aggressive interactions were observed.

In the second series of bioassays, hind leg extracts of *E. meriana* attracted significantly fewer conspecific males when exposed at non-display trees, compared to display trees (Fisher's exact test: $N=78$; $p<0.0001$) (Fig. 3). Although hind leg extracts attracted males in more cases than did the synthetic benzyl acetate (Fisher's exact test: $N=78$; $p=0.025$), comparatively fewer males collected at the hind leg extracts (Fisher's exact test: $N=198$; $p<0.01$; 31% of males vs 71% at benzyl acetate). The behaviour of males arriving at hind leg extracts was different from that of males arriving at benzyl acetate. The darting moves against the filter paper described above were significantly more frequent when approaching hind leg extracts (Fisher's exact test: $N=66$; $p=0.001$). During 39 10-min exposures, only a single male *E. meriana* arrived at a head extract (Fig. 3). It briefly landed on the filter paper and then left the area.

Between-locality bioassays

Males of the correct species came within 5 min to all extracts presented on hilltops in French Guiana and Panama, and these included simultaneous exposure of the extracts of both

species, separated by less than 10 m, at the French Guiana site. Individual placement of extracts of *E. meriana* at Ancon Hill (two sites) attracted a male within 5 min on three exposures (3 days), while those of *E. bombiformis* at BCI and Pipeline Road (three exposures, 3 days) each attracted one to three males, and never females.

Discussion

Tibial odours of male euglossines induced species-specific long-range attraction of other males to display sites, supporting the idea that the accumulated fragrances are pheromone analogues (Vogel 1966). Although it is assumed that fragrance signals primarily target females for the purpose of mating, no females were attracted during bioassays. This lack of attraction corresponds with the general rarity of observed matings in euglossine bees (Roubik and Hanson 2004). In a previous study on BCI, D. L. Stern carried out 5 months of observations of *E. meriana* display and witnessed only one copulation (Stern 1991). Only two more have been reported in all the euglossine literature (Kimsey 1980). This study is the first to report matings of *E. bombiformis*, a species that is conspicuous and displays at accessible sites. The rarity of observed euglossine matings may result from low mating frequency of females paired with the unusually long life of both sexes, combined with the fact that rarely more than one displaying male can be observed at a time. Male *Eulaema* have adult life spans of up to 5 months (Ackerman and Montalvo 1985), potentially occupying display sites over much of their life. In two observed mating encounters, *E. bombiformis* females approached from downwind, landed on the perch, achieved copula, flew briefly in the air and separated after approximately 7 s. However, more observations and successful bioassays are required to prove female anemotaxis to male volatiles.

Why were male *Eulaema* attracted to hind-leg odours? With the exception of *E. meriana* in the first series of bioassays, male approaches were always more likely when extracts were exposed at the correct perch trees. This and the rapidity of male arrival from downwind suggest that attraction took place from relatively nearby and in a territorial display context, not simply for the purpose of fragrance collection (Eberhard 1997; Roubik 1998). Behavioural components not usually observed when collecting at fragrance baits (darting moves) and the low frequency of collecting at the hind leg extracts also suggest that fragrance collection was not the primary purpose of the males' approaches. Attracted males approached extracts from downwind, in the same way as they approached perching conspecifics. One interpretation is that fragrance signals are used by males in ritualised contests for perch sites, previously called 'jousting', in which interacting males of *E. bombi-*

formis fly by each other in tight circular patterns, passing head by head many times (Roubik and Hanson 2004).

In contrast to fighting, the exposure of fragrances may allow mutual assessment of phenotypic quality without incurring the risk of injury. Aside from being species-specific badges, fragrances may communicate their owners' physical strength, manoeuvrability, cognitive capacity, or age (Schemske and Lande 1984; Eltz et al. 1999). Quality or quantity of individual fragrances may decide whether males occupy perches or adopt transient behaviour (Stern 1991).

Alternatively, or additionally, males attracted to hind leg extracts may have expected to sneak copulations at their competitors' perch sites. The parasitism of male fragrance signals might be a profitable alternative for males if costs of fragrance collection are high. Especially young males, which possess smaller amounts of fragrances in *Euglossa* (Eltz et al. 1999), could benefit from such opportunistic behaviour.

Male attraction to pheromones of other males is known in several bees (Ayasse et al. 2001), and is common in bumblebees (*Bombus* spp.), where males use labial gland secretions to mark territories or flight paths (Free 1987; O'Neill et al. 1991; Kindl et al. 1999). Chemical marking may be employed in attracting virgin queens for mating, but this has yet to be demonstrated. Instead, conspecific males are regularly attracted during bioassays (Free 1987). One effect of the deposited scent marks is that males converge on a common flight path, sometimes leading to "streams" of patrolling males (Kullenberg 1973; Goulson 2003). Similarly, fragrance communication may be involved in the formation of euglossine display hot spots. For example, on BCI displaying *Eulaema* males are predictably abundant on the northeastern top of the island, where male perch sites are scattered in approximately 20–30 ha of old-growth forest (unpublished data).

While our data demonstrate that specific attraction took place in response to complex blends of tibial odours, we cannot entirely rule out that endogenous compounds contributed to this effect. Cephalic labial gland lipids, which are constantly added to the tibial bouquet during fragrance collection, did not elicit attraction when exposed as head extracts, in agreement with their suggested function as a fragrance carrier (Whitten et al. 1989, 1993). However, some volatile components may well be perceptible to the bees and modify effects of the tibial odour. Additionally, one might argue that males secrete volatile compounds directly into the tibial pouch and that these compounds are essential for specific attraction. Some evidence exists that this is not normally the case: First, all lipid compounds that occurred in a large number of individuals (which would be expected from pheromonal compounds) were also detected in head extracts, suggesting that they were produced by the cephalic labial glands and not by glandular tissue associated with the hind tibial pouch. In addition, caged male *Euglossa*

imperialis show no systematic change in fragrance composition over 2 weeks in captivity, which suggests tibial pouches are for storage alone. Finally, most terpenoids and aromatics detected in *Eulaema* hind legs are known from euglossophilous orchids or other external sources (Hills et al. 1972; Whitten et al. 1986, 1988; Gerlach and Schill 1991; Whitten and Williams 1992).

Insect semiochemicals are normally synthesised de novo or modified from dietary precursors (Roelofs 1995). In the case of orchid bees, fragrance bouquets are harvested in dynamic natural environments. Thus, every fragrance source, e.g. a given species of orchid, provides only part of the final tibial bouquet. This is illustrated by *Eulaema meriana*, which is known to collect fragrances from more than 40 different floral sources (Ramírez et al. 2002) including some with strikingly different scent compositions (Hills et al. 1972; Whitten et al. 1986; Gerlach and Schill 1991; Whitten and Williams 1992). The accumulation of distinct tibial odours is achieved by innate preferences for certain odour qualities combined with learned avoidance of those that have already been collected in substantial amounts (Eltz et al. 2005a).

Our behavioural experiments with *Eulaema* show that chemical distinctness translates into a characteristic sensory perception and elicits species-specific attraction. Which aspects of the bouquets are essential in that respect remains for future study. Electrophysiological investigations in three species of *Euglossa* have found larger antennal responses to the correct (conspecific) hind leg extracts (Eltz et al. 2006), but have found no corresponding difference in regard to characteristic single components (Eltz and Lunau 2005). This finding stresses the importance of non-additive effects between multiple components in creating a specific chemosensory impression.

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Appendix

Incidence and relative abundance of chemical compounds detected in hexane extracts of hind legs of *Eulaema meriana* and *E. bombiformis*. Abundances are the average of a given compound's (peak) contribution to the individual GC/MS ion chromatogram (in % of the total ion current). Compounds are indexed as exogenous fragrances (F), labial gland lipids also found in head extracts (H), and other high molecular weight non-fragrance compounds (NF).

RT (min)	Compound	Number of individuals with compound			Average rel. abundance (% of total ion current)		
		Class	<i>E. bombiformis</i> N=7	<i>E. meriana</i> N=41	Both N=48	<i>E. bombiformis</i>	<i>E. meriana</i>
5.32	Thujene, alpha-	F	0	4	4	0.00	0.01
5.52	Pinene, alpha-	F	0	14	14	0.00	0.04
6.24	Benzaldehyde	F	0	8	8	0.00	0.07
6.56	Sabinene	F	0	34	34	0.00	0.73
6.83	Pinene, beta-	F	0	11	11	0.00	0.05
7.07	Myrcene	F	4	4	8	0.11	0.01
7.47	Menthene, 3-para- ?	F	0	6	6	0.00	0.02
7.75	cyclic monoterpene	F	0	2	2	0.00	0.01
8.02	Terpinene, alpha-	F	0	2	2	0.00	0.00
8.29	Cymene, para- (ortho- ?)	F	0	27	27	0.00	0.32
8.46	Limonene	F	0	10	10	0.00	0.04
8.51	Phellandrene, beta	F	0	3	3	0.00	0.01
8.54	Benzyl alcohol	F	5	7	12	0.50	0.51
8.58	Cineole, 1,8-	F	0	27	27	0.00	0.59
8.66	Ocimene, (Z)-beta-	F	0	2	2	0.00	0.02
9.06	Ocimene, (E)-beta-	F	7	0	7	1.55	0.00
9.50	Terpinene, gamma-	F	0	22	22	0.00	0.23
9.93	Sabinene hydrate, cis- (rel. to OH)	F	0	21	21	0.00	0.68
9.99	?	F	0	17	17	0.00	0.84
10.53	Terpinolene	F	0	7	7	0.00	0.03
11.10	Sabinene hydrate, trans-?	F	0	12	12	0.00	0.05
11.13	?	F	1	0	1	0.04	0.00
11.15	?	F	0	13	13	0.00	0.07
11.35	?	F	0	11	11	0.00	0.02
11.58	Phenylethyl alcohol, 2-	F	5	0	5	2.98	0.00
12.71	Limonene oxide, trans- ?	F	1	15	16	0.03	0.32
12.86	Ipsdienol	F	7	0	7	3.65	0.00
13.71	Benzyl acetate	F	5	8	13	2.74	0.99
14.58	Terpinen-4-ol	F	0	40	40	0.00	4.80
14.95	Methyl salicylate	F	0	22	22	0.00	0.27
15.17	Terpineol, alpha-	F	0	2	2	0.00	0.01
15.36	Dihydro carvone, cis- ?	F	0	1	1	0.00	0.00
15.60	Dihydro carvone, trans-	F	0	9	9	0.00	0.08
16.25	aromatic compound	F	0	1	1	0.00	0.01
16.93	?	F	1	0	1	0.18	0.00
17.31	Carvone	F	3	27	30	0.27	0.73
17.65	?	F	1	0	1	0.22	0.00
17.71	Phenylethyl acetate, 2-	F	4	0	4	4.27	0.00
17.90	?	F	1	0	1	2.44	0.00
18.00	Carvone epoxide, cis-	F	5	38	4	3.47	12.92
18.49	Geranial	F	1	0	1	0.09	0.00
18.82	Carvone oxide, trans-	F	0	1	1	0.00	0.00
18.97	?	F	0	8	8	0.00	0.12
19.35	Indole	F	3	0	3	0.22	0.00
20.12	?	F	0	5	5	0.00	0.01
20.99	?	F	2	4	6	0.09	0.04
21.16	trans-(trans-Carveol) epoxide?	F	0	12	12	0.00	0.23
21.26	?	F	0	1	1	0.00	0.01
21.47	?	F	0	1	1	0.00	0.00
21.58	?	F	0	1	1	0.00	0.00
21.76	?	F	1	0	1	0.04	0.00
22.22	Eugenol	F	0	1	1	0.00	0.00
23.06	?	F	7	0	7	0.64	0.00
23.21	Copaene, alpha-	F	1	0	1	0.05	0.00

23.27	Geranyl acetate	F	2	0	2	0.11	0.00
23.58	Methyl cinnamate, (E)-	F	0	8	8	0.00	0.07
23.74	Cubebene, beta-	F	0	1	1	0.00	0.01
23.75	?	F	0	2	2	0.00	0.04
23.89	?	F	0	3	3	0.00	0.04
24.05	?	F	0	2	2	0.00	0.01
24.75	?	F	0	7	7	0.00	0.03
24.95	?	F	0	5	5	0.00	0.04
24.96	?	F	0	1	1	0.00	0.01
24.99	Caryophyllene, (Z)-	F	0	1	1	0.00	0.01
25.02	?	F	0	2	2	0.00	0.02
25.67	Bergamotene, (E)-alpha-	F	0	2	2	0.00	0.02
25.71	?	F	1	0	1	0.04	0.00
25.72	?	F	1	4	5	0.05	0.02
26.52	?	F	0	1	1	0.00	0.00
26.55	Farnesene, (E)-beta-	F	0	1	1	0.00	0.01
26.61	?	F	1	0	1	0.08	0.00
26.70	?	F	0	1	1	0.00	0.01
27.51	?	F	0	1	1	0.00	0.00
27.53	Dodecanol	H	0	1	1	0.00	0.00
27.72	Germacrene D	F	1	3	4	0.25	0.11
27.83	aff. Farnesene	F	0	1	1	0.00	0.01
28.25	Bicyclogermacrene ?	F	0	2	2	0.00	0.07
28.48	Bulnesene, alpha- ?	F	2	0	2	0.07	0.00
28.55	?	F	0	1	1	0.00	0.01
28.78	Farnesene	F	0	4	4	0.00	0.02
29.00	?	F	0	1	1	0.00	0.01
30.39	?	F	7	20	27	2.86	0.23
31.10	nerolidol, (E)-	F	0	1	1	0.00	0.00
31.65	Spathulenol ?	F	0	1	1	0.00	0.00
32.90	Dodecyl acetate	H	2	12	14	0.19	0.21
33.25	Farnesene epoxide, (E)-alpha-	F	0	6	6	0.00	0.03
33.28	?	F	0	1	1	0.00	0.01
33.31	?	F	0	3	3	0.00	0.04
33.31	Tetradecanal (=Myristylaldehyde)	H	1	5	6	0.35	0.01
34.38	?	F	7	0	7	2.94	0.00
34.56	Methoxynaphthol	F	7	0	7	1.55	0.00
35.28	2-Propenoic acid, 3-[4-Methoxyphenyl]-	F	0	3	3	0.00	0.01
35.65	Tetradecanol	H	1	1	2	0.06	0.00
35.98	?	F	0	2	2	0.00	0.01
36.48	?	F	0	1	1	0.00	0.00
36.68	Bergamotol, (Z)-alpha-, trans-	F	0	15	15	0.00	0.13
38.87	Benzyl benzoate	F	0	6	6	0.00	0.04
38.89	?	F	0	2	2	0.00	0.01
39.24	Tetradecanoic acid	H	0	1	1	0.00	0.00
40.62	Tetradecyl acetate	H	3	19	2	6.66	1.16
41.08	Hexadecanal	F	1	0	1	0.15	0.00
41.50	?	F	0	1	1	0.00	0.01
41.67	Hexahydrofarnesyl acetone	F	0	10	10	0.00	0.04
42.70	Benzyl salicylate	F	0	1	1	0.00	0.00
43.21	Hexadecanol	H	1	4	5	0.10	0.02
43.41	?	F	0	1	1	0.00	0.00
45.10	Cyclohexadecanolid ?	NF	0	1	1	0.00	0.01
45.70	aromatic compound	F	0	1	1	0.00	0.00
45.97	Hexadecanoic acid?	H	0	2	2	0.00	0.03
47.47	Hexadecyl acetate	H	3	22	25	4.10	1.84
47.53	?	NF	0	2	2	0.00	0.01
47.86	?	NF	2	5	7	0.06	0.02
47.91	?	F	0	6	6	0.00	0.03
48.36	?	F	0	1	1	0.00	0.04

48.37	Falcarinol, (Z)-	F	0	3	3	0.00	0.05
49.38	?	H	0	2	2	0.00	0.02
49.50	?	H	1	36	37	0.02	0.46
49.91	?	H	1	2	3	0.02	0.01
50.44	Heneicosane	H	1	1	2	0.34	0.02
50.62	?	F	0	1	1	0.00	0.03
51.82	?	H	1	6	7	0.50	0.37
53.06	?	F	1	1	2	0.24	0.03
53.76	Octadecanol acetate	H	3	8	11	1.07	0.48
55.58	Tricosene	H	6	40	46	23.83	20.49
55.59	?	H	1	2	3	7.75	1.00
56.47	Tricosane	H	6	37	43	2.71	2.62
58.86	Octadecanoic acid, butyl ester ?	NF	1	1	2	0.20	0.03
61.12	?	NF	0	1	1	0.00	0.04
61.20	Pentacosene	H	1	34	35	0.17	2.28
62.06	Pentacosane	H	7	39	46	3.75	3.16
64.05	?	NF	0	7	7	0.00	0.11
65.96	?	H	1	12	13	0.89	0.42
66.45	?	H	0	1	1	0.00	0.25
66.56	?	H	3	40	43	3.97	6.23
66.91	?	H	1	0	1	0.19	0.00
67.21	Heptacosane	H	2	14	16	0.62	0.36
69.07	?	H	1	9	10	0.18	0.18
70.77	Nonacosadiene ?	H	1	39	40	1.51	6.20
71.03	?	NF	1	10	11	0.08	0.19
71.19	?	H	5	40	45	6.84	10.06
71.95	Nonacosane	NF	2	14	16	0.28	0.30
72.64	?	NF	0	2	2	0.00	0.04
73.19	?	NF	0	3	3	0.00	0.03
74.89	?	H	1	38	39	0.43	4.30
75.38	Untriacontadien ?	NF	0	3	3	0.00	0.07
75.75	?	H	3	19	22	1.09	1.29
76.25	Hentriacontane	H	1	7	8	0.10	0.13
79.49	Tritriacontadiene ?	NF	0	2	2	0.00	0.12
79.51	?	NF	0	14	14	0.00	0.92
80.67	Phytosterole ?	NF	0	6	6	0.00	0.18
81.31	similar beta-Amyrin	NF	0	32	32	0.00	2.50
81.59	similar alpha-Amyrin	NF	0	3	3	0.00	0.17
81.62	Phytosterole	NF	0	13	13	0.00	0.61
82.39	similar alpha-Amyrin	NF	0	38	38	0.00	4.84

References

- Ackerman JD, Montalvo AM (1985) Longevity of euglossine bees. *Biotropica* 17:79–81
- Adams RP (2001) Identification of essential oil components by gas chromatography/quadrupole mass spectroscopy. Allured Publishing, Carol Stream, USA
- Ayasse M, Paxton RJ, Tengo J (2001) Mating behavior and chemical communication in the order Hymenoptera. *Annu Rev Entomol* 46:31–78
- Bembé B (2004) Functional morphology in male euglossine bees and their ability to spray fragrances (Hymenoptera, Apidae, Euglossini). *Apidologie* 35:283–291
- Clarke KR, Green RH (1988) Statistical design and analysis for a ‘biological effects’ study. *Mar Ecol Prog Ser* 46:213–226
- Clarke KR, Gorley RN (2001) PRIMER v5: user manual/tutorial. Primer-E, Plymouth
- Clarke KR, Warwick RM (2001) Change in marine communities: an approach to statistical analysis and interpretation, 2nd edn. Natural Environment Research Council, UK
- Dressler RL (1982) Biology of the orchid bees (Euglossini). *Ann Rev Ecol Syst* 13:373–394
- Eberhard WG (1997) Graverobbing by male *Eulaema sebrai* bees (Hymenoptera: Apidae). *J Kans Entomol Soc* 70:66
- Eltz T, Lunau K (2005) Antennal response to fragrance compounds in male orchid bees. *Chemoecology* 15:135–138
- Eltz T, Whitten WM, Roubik DW, Linsenmair KE (1999) Fragrance collection, storage, and accumulation by individual male orchid bees. *J Chem Ecol* 25:157–176
- Eltz T, Roubik DW, Whitten WM (2003) Fragrances, male display and mating behaviour of *Euglossa hemichlora*—a flight cage experiment. *Phys Entomol* 28:251–260
- Eltz T, Roubik DW, Lunau K (2005a) Experience-dependent choices ensure species-specific fragrance accumulation in male orchid bees. *Behav Ecol Sociobiol* 59:149–156

- Eltz T, Sager A, Lunau K (2005b) Juggling with volatiles: exposure of perfumes by displaying male orchid bees. *J Comp Physiol A* 191:575–581
- Eltz T, Ayasse M, Lunau K (2006) Species-specific antennal response to tibial fragrances in male orchid bees. *J Chem Ecol* 32:71–79
- Free JB (1987) Pheromones of social bees. Chapman and Hall, London
- Gerlach G, Schill R (1991) Composition of orchid scents attracting euglossine bees. *Bot Acta* 104:379–391
- Goulson D (2003) Bumblebees—behavior and ecology. Oxford University Press
- Hills HG, Williams NH, Dodson CH (1972) Floral fragrances and isolating mechanisms in the genus *Catasetum* (Orchidaceae). *Biotropica* 4:61–76
- Kimsey LS (1980) The behaviour of male orchid bees (Apidae, Hymenoptera, Insecta) and the question of leks. *Anim Behav* 28:996–1004
- Kimsey LS (1984) The behavioural and structural aspects of grooming and related activities in euglossine bees (Hymenoptera: Apidae). *J Zool* 204:541–550
- Kindl J, Hovorka O, Urbanova K, Valterova I (1999) Scent marking in male pre-mating behavior of *Bombus confusus*. *J Chem Ecol* 25:1489–1500
- Kullenberg B (1973) Field experiments with chemical sexual attractants in Aculeate Hymenoptera males II. *Zoon Suppl* 1:31–42
- Legendre P, Legendre L (1998) Numerical ecology, 2nd edn. Elsevier, Amsterdam
- O'Neill KM, Evans EJ, Bjostad LB (1991) Territorial behaviour in males of three North American species of bumblebees (Hymenoptera: Apidae, *Bombus*). *Can J Zool* 69:604–613
- Ramírez S, Dressler RL, Ospina M (2002) Abejas euglosinas (Hymenoptera: Apidae) de la región Neotropical: listado de especies con notas sobre su biología. *Biota Colombiana* 3:7–118
- Roelofs WL (1995) The chemistry of sex attraction. In: Meinwald J (ed) Chemical ecology. National Academy, Washington, DC, pp 103–118
- Roubik DW (1998) Grave-robbing by male *Eulaema* (Hymenoptera, Apidae): implications for euglossine biology. *J Kans Entomol Soc* 71:188–191
- Roubik DW, Hanson PE (2004) Orchid bees of tropical America: biology and field guide. Instituto Nacional de Biodiversidad (INBio), Heredia, Costa Rica
- Schemske DW, Lande R (1984) Fragrance collection and territorial display by male orchid bees. *Anim Behav* 32:935–937
- Stern DL (1991) Male territoriality and alternative male behaviors in the euglossine bee, *Eulaema meriana* (Hymenoptera: Apidae). *J Kans Entomol Soc* 64:421–437
- Stern DL, Dudley R (1991) Wing buzzing by male orchid bees, *Eulaema meriana* (Hymenoptera: Apidae). *J Kans Entomol Soc* 64:88–94
- Vogel S (1966) Parfümsammelnde Bienen als Bestäuber von Orchidaceen und *Gloxinia*. *Österr Botan Zeit* 113:302–361
- Whitten WM, Williams NH (1992) Floral fragrances of *Stanhopea* (Orchidaceae). *Lindleyana* 7:130–153
- Whitten WM, Williams NH, Armbruster WS, Battiste MA, Strekowski L, Lindquist N (1986) Carvone oxide: an example of convergent evolution in euglossine pollinated plants. *Syst Bot* 11:222–228
- Whitten WM, Hills HG, Williams NH (1988) Occurrence of ipsdienol in floral fragrances. *Phytochemistry* 27:2759–2760
- Whitten WM, Young AM, Williams NH (1989) Function of glandular secretions in fragrance collection by male euglossine bees. *J Chem Ecol* 15:1285–1295
- Whitten WM, Young AM, Stern DL (1993) Nonfloral sources of chemicals that attract male euglossine bees (Apidae: Euglossini). *J Chem Ecol* 19:3017–3027
- Williams NH (1982) The biology of orchids and euglossine bees. In: Arditti J (ed) *Orchid biology: reviews and perspectives*. Cornell University Press, Ithaca, NY, pp 119–171
- Williams NH, Whitten WM (1983) Orchid floral fragrances and male euglossine bees: methods and advances in the last sesquidecade. *Biol Bull* 164:355–395