Comparison of orchid bee (Hymenoptera: Apidae) species composition collected with four chemical attractants

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Abstract

Orchid bees (Hymenoptera: Apidae: Euglossini) are a diverse and important group of Neotropical pollinators. Numerous chemicals have been used in sampling orchid bees, and species-specific attraction, particularly of males, to these chemicals is well known. However, there have been few studies that have quantified differences in the species composition of orchid bees attracted to particular chemicals. In this study, we compared the abundance and species composition of orchid bees collected with 4 commonly used attractants: benzyl acetate, eucalyptol (or cineole), eugenol, and methyl salicylate. Eucalyptol collected the greatest abundance and species richness of orchid bees. Indicator species analysis revealed that 3 species, *Euglossa imperialis* Cockerell, *Euglossa obtusa* Dressler, and *Eufriesea mexicana* (Mocsáry), were significantly associated with eucalyptol, and 1, *Eulaema marcii* Nemésio, with benzyl acetate. The multi-response permutation procedure revealed relatively large differences in species composition of orchid bees collected with eucalyptol vs. benzyl acetate and eucalyptol vs. eugenol. Our results showed that eucalyptol and benzyl acetate were the most effective and complimentary attractants, but even less effective attractants such as eugenol may attract novel species.

Key Words: chemical ecology; Mesoamerican euglossines; benzyl acetate; eucalyptol; cineole; pollinator biodiversity; eugenol; methyl salicylate

Resumen

Las abejas de orquídeas (Hymenoptera: Apidae: Euglossini) son un grupo diverso e importante de polinizadores neotropicales. Numerosas sustancias químicas se han utilizado en el muestreo de las abejas de orquídeas, y la atracción específica de la especie, particularmente de los machos, a estos productos químicos es bien conocida. Sin embargo, ha habido pocos estudios que han cuantificado las diferencias en la composición de especies de abejas de orquídeas atraídas por cada químico en particular. En este estudio, comparamos la abundancia y composición de especies de abejas de orquídeas recolectados con 4 atrayentes comúnmente utilizados: acetato de bencilo, eucaliptol (o cineol), eugenol y salicilato de metilo. El eucaliptol recolectó la mayor abundancia y riqueza de especies de abejas de orquídeas. El análisis de especies indicadoras reveló que 3 especies, *Euglossa imperialis* Cockerell, *Euglossa obtusa* Dressler y *Eufriesea mexicana* (Mocsáry), se asociaron significativamente con el eucaliptol, y una, *Eulaema marcii* Nemésio, con el acetato de bencilo. El procedimiento de permutación multi-respuesta reveló diferencias relativamente grandes en la composición de especies de abejas de orquídeas recolectadas con el eucaliptol frente al acetato de bencilo y el eucaliptol vs. eugenol. Nuestros resultados mostraron que el eucaliptol y el acetato de bencilo fueron los atrayentes más eficaces y complementarios, pero incluso atractores menos efectivos como el eugenol pueden atraer otras especies.

Palabras Clave: ecología química; euglossinas mesoamericanas; acetato de bencilo; eucaliptol; cineol; biodiversidad de polinizadores; eugenol; salicilato de metilo

Orchid bees (Hymenoptera: Apidae: Euglossini) comprise a diverse and important group of New World pollinators. There are over 200 known species, and the tribe is widespread throughout the Neotropics (Roubik & Hanson 2004). Orchid bees pollinate roughly 650 species of orchids, as well as other plant species in about 30 different families, including some economically important crops such as Brazil nuts and rubber trees (Dressler 1982; Ackerman 1983; Roubik & Hanson 2004; Ackerman & Roubik 2012; Briggs et al. 2013). Males visit flowers to collect aromatic compounds or associated chemicals (Dodson et al. 1969; Roubik & Hanson 2004) that appear to be associated with species recognition, competition, and mate choice (Zimmermann et al. 2009). This behavior makes it possible to sample orchid bee males with aromatic compounds. Use of these compounds in conjunction with bait stations and insect nets, or in baited traps, is the most commonly used method of collecting orchid bees for scientific research.

Roubik & Hanson (2004) list nearly 50 chemicals that have been used as orchid bee attractants, and they also give species-specific lists of effective attractants in their orchid bee species accounts. However, while there is good qualitative knowledge of the species-specific effectiveness of many orchid bee attractants, there have been few studies that have quantitatively compared the species composition of orchid bees attracted by these chemicals.

Mesoamerica is one of the world's biodiversity hotspots (Mittermeier et al. 1999). Cusuco National Park, in northwest Honduras, has been designated a Key Biodiversity Area, but is threatened by human population growth and associated land cover changes (Green et al.

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2012). The park contains a diverse assemblage of at least 24 species of orchid bees (McCravy et al. 2016). As part of an ongoing assessment of orchid bee diversity within the park, we did a study comparing the species composition of orchid bees attracted to 4 commonly used chemical attractants.

Materials and Methods

The study was done from 15 Jun to 30 Jul 2013 at Cusuco National Park (15.5333°N, 88.2500°W), about 30 km west of San Pedro Sula in the state of Cortés. Cusuco National Park has an area of about 23,400 ha and ranges in elevation from just above sea level to 2425 m. It is primarily cloud forest; habitat types include disturbed and undisturbed mature broadleaf forest, secondary broadleaf forest, disturbed pine–broadleaf forest, mature pine forest, open disturbed grassland, open disturbed logged areas, and open coffee plantations.

Orchid bees were collected with insect nets at bait stations that consisted of a cotton ball containing a chemical attractant suspended approximately 1.7 m above the ground by a string. Five bait stations, approximately 5 m apart, were established during each collection period. For each collection period, 1 of 4 chemical attractants was used. Attractants were purchased from Sigma Aldrich, Inc. (St. Louis, MO, USA) and included benzyl acetate (product # W213500; ≥ 99% purity), eucalyptol (# W246506; ≥ 99% purity), eugenol (# W246700; ≥ 98% purity), and methyl salicylate (# W274518; ≥ 98% purity). Product numbers and purity values were obtained from http://www.sigmaaldrich.com/united-states.html (accessed 4 Mar 2017). For each collection period, 1 of the 4 attractants was applied to all 5 bait stations. Each cotton ball received 30 drops initially; re-application with 20 additional drops was done every 30 min. Each collection period was from 9 AM to 11 AM. Sampling was done at 6 sites within the park (Table 1). Each site was sampled on 8 different dates, with each chemical being used twice at each site. Chemicals were randomly assigned to the first 4 collection dates at each site, then again to the last 4 collection dates. For each chemical, the 2 samples per site were pooled for analyses. Collected orchid bees were identified based on reference specimens and Roubik & Hanson (2004).

Because orchid bee species vary in their altitudinal distributions (Roubik & Hanson 2004), we compared species composition of each pair of sampling sites using the Morisita–Horn index of similarity (Morisita 1959; Horn 1966). We then used regression analysis to measure the potential association between Morisita–Horn values and altitudinal differences. EstimateS version 9.1 (Colwell 2013) was used to calculate Morisita–Horn values, and regression analysis was done in SigmaPlot 13.0.

The Chao1 richness estimator (Chao et al. 2009) was used to estimate asymptotic orchid bee species richness overall and for each attractant. This analysis estimates the minimum species richness present, based on the relative numbers of singletons (1 individual of a species collected) and doubletons (2 individuals of a species collected). The Chao calculator (Ecological Archives E090-073-S1, Chao et al. 2009) was used to calculate Chao1 estimates, as well as probabilities that an additional individual sampled would represent a previously undetected species (q_0, or the proportion of singletons in the sample, f_1 / n). For each Chao1 estimate, the proportion of singletons was less than 50% (i.e., f_1 / n < 0.5), as recommended by Anne Chao (cited in Colwell 2013).

Mean numbers of orchid bees collected were compared among attractants by 1-way ANOVA; pairwise multiple comparisons were done with the Holm–Sidak method. Data were square root transformed to satisfy the assumption of equal variances. Orchid bee species composition was compared among attractants by the multi-response permutation procedure (MRPP). The MRPP *A* value is a measure of the extent to which species composition of different groups diverge. *A* values <0.1 are common, and values >0.3 are relatively high (McCune & Grace 2002). To further examine differences in attractants, paired MRPP analyses were done. Holm's step-down procedure (Holm 1979) was used to control experiment-wise error rate for MRPP paired comparisons. In this procedure, the n^{th} smallest *P* value is compared with 0.05 / (number of comparisons + 1 – *n*). Indicator species analysis (Dufrêne & Legendre 1997), or ISA, was used to identify particular orchid bee species that were strongly associated with a particular attractant. ISA produces indicator values, ranging from 0 to 100, with the latter representing perfect association of that species with a particular attractant, ie., consistently associated with that attractant among different trials, and never associated with any other attractant. ANOVA was done in SigmaPlot, Version 13.0. MRPP and ISA were done by PC-Ord, Version 4.25.

Table 1. Locations and altitudes of orchid bee sampling sites within Cusuco National Park, Honduras.

Latitude	Longitude	Altitude (m)
15.5114°N	88.1855°W	999
15.4964°N	88.1899°W	1202
15.5005°N	88.2144°W	1631
15.5021°N	88.2046°W	1403
15.4944°N	88.2139°W	1600
15.4971°N	88.2211°W	1685

Results

A total of 649 orchid bees representing 17 species and 3 genera were collected (Table 2). *Euglossa imperialis* Cockerell and *Eulaema marcii* Nemésio were the most abundant species collected, with 411 and 111 individuals, respectively. There was no significant relationship between Morisita–Horn index values and altitudinal differences between sampling sites (F = 0.011; df = 1, 13; P = 0.918), indicating that altitudinal differences among the sites did not affect orchid bee species composition in this study. There was a significant overall difference in the numbers of orchid bees collected with the different attractants (F = 11.702; df = 3, 20; P < 0.001), with pairwise comparisons showing that eucalyptol collected significantly greater numbers than each of the other 3 attractants (P < 0.05; Table 2). Eucalyptol also collected the greatest species richness (15 species), with the other attractants ranging from 5 to 7. The Chao1 estimate of overall species richness was 17.40, and 15.29 for eucalyptol. Each of the other 3 attractants had an estimated species richness of 7.0 (Table 2).

Four orchid bee species were significantly associated with a particular attractant, based on ISA (Table 2). Three of these, *E. imperialis*, *Euglossa obtusa* Dressler, and *Eufriesea mexicana* (Mocsáry), were associated with eucalyptol, and 1, *E. marcii*, with benzyl acetate. Large proportions of 2 other *Eulaema* species, *Eulaema luteola* Moure and *Eulaema meriana* (Olivier), were collected in equal numbers with benzyl acetate and methyl salicylate. *Euglossa dilemma* Bembé & Eltz, on the other hand, was similar in abundance in eucalyptol and eugenol collections. Of 154 total *Eulaema* collected, 118 (76.6%) were collected with benzyl acetate, whereas 400 of 489 (81.8%) *Euglossa* were collected with eucalyptol. All 6 *Eufriesea* individuals were collected with eucalyptol.

The MRPP revealed significant overall differences in orchid bee species composition collected with the 4 attractants (*A* = 0.30, *P* <

Table 2. Numbers of orchid bees collected (with mean ± SE) using 4 chemical attractants (BA = benzyl acetate, EUC = eucalyptol, EUG = eugenol, MS = methyl salicylate) at Cusuco National Park, Honduras, in Jun to Jul 2013, with observed and estimated species richness values, q_0 values, and results of indicator species analysis (ISA).

Species	BA	EUC	EUG	MS	Total	ISA *P* value[a]
Euglossa imperialis Cockerell	3 (0)	358 (**87**)	1 (0)	49 (10)	411	0.001
Eulaema marcii Nemésio	98 (**88**)	0 (0)	8 (2)	5 (2)	111	0.001
Euglossa dilemma Bembé & Eltz	1 (1)	11 (24)	14 (38)	5 (8)	31	0.141
Eulaema luteola Moure	13 (32)	1 (1)	0 (0)	13 (40)	27	0.074
Euglossa obtusa Dressler	1 (1)	13 (**81**)	2 (2)	0 (0)	16	0.001
Eulaema meriana (Olivier)	7 (29)	2 (4)	0 (0)	7 (29)	16	0.292
Euglossa mixta Friese	0 (0)	2 (3)	0 (0)	8 (40)	10	0.101
Euglossa heterosticta Moure	0 (0)	4 (25)	0 (0)	4 (17)	8	0.278
Euglossa tridentata Moure	0 (0)	4 (17)	0 (0)	0 (0)	4	1.000
Eufriesea mexicana (Mocsáry)	0 (0)	3 (**50**)	0 (0)	0 (0)	3	0.040
Eufriesea rugosa (Friese)	0 (0)	2 (33)	0 (0)	0 (0)	2	0.217
Euglossa cybelia Moure	0 (0)	2 (17)	0 (0)	0 (0)	2	1.000
Euglossa hansoni Moure	0 (0)	2 (17)	0 (0)	0 (0)	2	1.000
Euglossa purpurea Friese	0 (0)	2 (33)	0 (0)	0 (0)	2	0.231
Euglossa variabilis Friese	0 (0)	2 (17)	0 (0)	0 (0)	2	1.000
Eufriesea schmidtiana (Friese)	0 (0)	1 (17)	0 (0)	0 (0)	1	1.000
Euglossa obrima Hinojosa-Díaz, Melo & Engel	0 (0)	0 (0)	1 (17)	0 (0)	1	1.000
Total	123	409	26	91	649	
Mean number per site ± SE[b]	20.5 ± 5.4a	68.2 ± 16.7b	4.3 ± 1.9a	15.2 ± 4.7a	108.2 ± 20.0	
Observed species richness	6	15	5	7	17	
Estimated species richness	7.00	15.29	7.00	7.00	17.40	
Probability of an additional individual being a previously undetected species (q_0)	0.0163	0.0049	0.0769	0.0000	0.0031	

Numbers in parentheses are indicator values, with statistically significant indicator values in **bold**.
[a]*P* values represent the proportion of 1000 randomized trials with an indicator value equal to or exceeding the observed indicator value.
[b]Mean numbers of orchid bees collected followed by the same letter are not significantly different (ANOVA, Holm–Sidak multiple pairwise comparison method, $P > 0.05$).

0.001). Each of the 6 MRPP pairwise comparisons showed a significant difference in species composition between attractants (Table 3). Effect sizes (*A* values) varied substantially; they were relatively high for eucalyptol/benzyl acetate and eucalyptol/eugenol comparisons, and low for all comparisons involving methyl salicylate.

Discussion

Chao1 analyses suggested that the 4 attractants collected most of the species richness present, within the limits of the methodology used. Use of additional attractants in this study would have very likely yielded additional species. Incorporation of traps could also yield different species. Nemésio & Vasconcelos (2014) found that passive sampling with baited traps collected significantly different orchid bee species composition compared with active netting at bait stations, although all species collected by traps were also collected by active netting. However, they advise against relying on baited traps as the sole or main basis of orchid bee faunistic studies because of trap bias.

Table 3. Multi-response permutation procedure *A* values (with associated *P* values in parentheses) for paired comparisons of orchid bee species composition collected using 4 chemical attractants (BA = benzyl acetate, EUC = eucalyptol, EUG = eugenol, MS = methyl salicylate) at Cusuco National Park, Honduras, in Jun – Jul 2013. All paired comparison *A* values were significant after adjustment for multiple comparisons using Holm's step-down procedure.

Comparison	*A* value (*P* value)
BA vs. EUC	0.35 (<0.001)
BA vs. EUG	0.21 (0.002)
BA vs. MS	0.19 (0.002)
EUC vs. EUG	0.27 (0.001)
EUC vs. MS	0.18 (0.002)
EUG vs. MS	0.17 (0.005)
Overall	0.30 (<0.001)

Overall, eucalyptol was the most effective attractant both in terms of numbers of individuals and species richness collected in our study, with *E. imperialis* being collected in greatest quantity (Table 2). But even rare species of *Euglossa* and *Eufriesea* were collected, with the exception of *Euglossa obrima* Hinojosa-Díaz, Melo & Engel. However, *Eulaema* were poorly represented in eucalyptol collections. Methyl salicylate and especially benzyl acetate were relatively effective in attracting *Eulaema* species, and the large eucalyptol vs. benzyl acetate *A* value (Table 3) suggests that these 2 attractants are highly complementary. But it should be noted that, of the paired attractant combinations, only eucalyptol and eugenol collected all 17 species, demonstrating that even an attractant that collects relatively few individuals can still be useful in species richness inventories.

In a study of orchid bees at a lowland site in Costa Rica, Janzen et al. (1982) used the same attractants that we used, plus a fifth attractant, methyl cinnamate. They also found eucalyptol (referred to by the alternate term "cineole" in their study) to be most effective in terms of orchid bee abundance and species richness collected among the 4 attractants (excluding methyl cinnamate). However, in that study, orchid bee abundance was more evenly distributed among the 4 attractants, with eucalyptol accounting for 38.7% (670 of 1731) of total captures vs. 63.0% in our study. Eugenol, on the other hand, collected 33.6% of total captures in the Janzen et al. (1982) study, vs. only 4.0% in our study, and benzyl acetate accounted for only 4.4% of captures in the Costa Rica study, vs. 19.0% in our study. These differences could be due to a variety of factors. There is evidence that the effectiveness of chemical attractants varies both geographically and temporally (Nemésio 2012), even within a species. This may be due to a variety of climatological or biotic factors. Perhaps eugenol volatilizes and disperses more readily in warmer lowland conditions than in the higher elevation cloud forest environment of our study. Of the 4 attractants we used, eugenol has the highest boiling point of 254 °C, vs. 212 °C, 176 to 177 °C, and 222 °C for benzyl acetate, eucalyptol, and methyl salicylate, respectively (http://www.sigmaaldrich.com/united-states.html; accessed 4 Mar 2017). Interestingly, there was an inverse relationship between attractant effectiveness and attractant boiling point in our study, suggesting that, in addition to inherent "attraction," volatility may play an important role in the effectiveness of orchid bee attractants. For comparison purposes, we used the chemicals in their "out of the bottle" state, but mixing with a volatile carrier such as ethyl alcohol might increase the effectiveness of some chemicals, such as eugenol. Factors such as volatility, climatic conditions, amount of attractant used, time of day that sampling is done, and many others may greatly affect results. Nemésio (2012) provides a thorough review of methodological concerns associated with factors such as these that could affect orchid bee sampling.

Of the 32 species of orchid bees Janzen et al. (1982) collected, only 10 overlapped with our study. In general, among the species in common to both studies, species–attractant relationships were similar. In the Janzen et al. (1982) study, eucalyptol and methyl salicylate accounted for almost all (68.3% and 31.4%, respectively) of *E. imperialis* captures, as was the case in our study (87.1% and 11.9%, respectively). Likewise, benzyl acetate and methyl salicylate accounted for the great majority of *E. meriana* collected in their study (90.4%) as well as ours (87.5%). All *E. mexicana* collected in both studies were attracted by eucalyptol, whereas *E. mixta* was collected primarily with methyl salicylate in both our study (80%) and the Janzen et al. (1982) study (100%). In our study, *Euglossa heterosticta* Moure was captured in equal numbers with eucalyptol and methyl salicylate, whereas in Janzen et al. (1982) all 4 individuals of this species were collected with eucalyptol.

Orchid bees are vital components of Neotropical forest ecosystems. Orchid bee conservation requires increased knowledge of their diversity and ecology, and much of this knowledge base depends on rigorous sampling methodology using chemical attractants. There are numerous chemicals available for use as orchid bee attractants, and these attractants vary widely in their overall and species-specific effectiveness. While there is substantial qualitative knowledge of orchid bee species attraction to specific chemicals, more detailed comparative information is needed on chemical attractants and the species assemblages they attract.

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