Blown by the wind: the ecology of male courtship display behavior in orchid bees

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Abstract. Many insects rely on chemical signals to transmit precise information on the location, identity, and quality of potential mates. Chemical signals are often broadcasted at sites with physical properties that maximize signal propagation and signal transmission. Male neotropical orchid bees (Euglossini) perch and display on vertical branches and tree trunks in the forest to expose volatile blends (perfumes) that they previously collected from their environment. Previous studies have shown that the chemical composition of perfume blends is highly differentiated even between closely related species. However, variation in behavioral components of perfume exposure and male display remain poorly understood. We conducted a four-year study on orchid bee display sites (8 species) in pacific Costa Rica, using field observations along with chemical analysis and cage experiments to assess display niche partitioning among sympatric species. We evaluated the influence of physical factors (terrain, wind, light) on the distribution of perch sites and on display behavior, and tested a prediction of the sex pheromone-analogue hypothesis, i.e., that displaying males have above-average quantities or qualities of acquired perfumes. Males of different species displayed in the same general area and sometimes in close proximity to each other, but partitioned the display niche by selecting different perch diameters, display heights, and by displaying at different times of the day. Most perch sites were located inside the forest on elevated ground, especially along ridges, where stronger winds may help disperse perfume signals. Furthermore, the angular position of displaying males on perches was narrowly determined by wind direction, with males being positioned on the downwind side of the perch, where they would be most conspicuous to conspecifics approaching on an odor trail. Although our results generally support the hypothesis that perfumes serve as pheromone analogues, we did not find differences in the perfume composition of males caught at display perches and males captured at chemical baits. This suggests that, while chemical communication is an integral part of orchid bee display, male display activity is not determined by the history of, and success in, volatile acquisition.

Key words: chemical communication; chemical ecology; Euglossini; fragrance; hilltopping; lek; perfume; pheromone; sexual display.

INTRODUCTION

Finding and acquiring a mate presents a crucial task for many animals, especially in species that do not aggregate at nest sites or at food sources. In most cases, males invest more time and energy into finding mates than do females, and obtaining access to females can be achieved through a number of different strategies. In many insect species, the sexes meet at non-resource based rendezvous localities such as hilltops or other landmarks, where males typically perch on or patrol specific sites while waiting for females (Thornhill and Alcock 1983, Alcock 1987, Ayasse et al. 2001). In such situations, long-range attraction of potential mates can be mediated and enhanced through the use of visual (Ohba 2004, Lewis and Cratsley 2008), acoustic (Bailey 1991) or chemical signals (Wyatt 2003). In addition, these mating signals may play a role in mediating pre-mating reproductive isolation among related species. In such cases, the species-specificity of the signal itself is often accompanied by temporal or spatial separation of signalling sites (Hödl 1977, Zimmermann et al. 2006, Schmidt et al. 2013). Finally, signal perceptibility can be optimized in relation to environmental or interspecific "noise" by adjusting either signal quality or the background against which it is perceived (Gomez and Thery 2004, Uy and Endler 2004, Schmidt and Balakrishnan 2015, Weber et al. 2016).

Chemically mediated mate attraction through sex pheromones, i.e., intraspecific chemical signals that advertise the sender's availability as a mate, is particularly widespread in insects (Karlson and Butenandt 1959, Wyatt

2003), where sex pheromones are usually synthesized de novo by the sender, sometimes using complex dietary precursors. In some exceptional cases, exogenous compounds may also be used without further modification (Tan and Nishida 2000, Eltz et al. 2007). Orchid bees (Euglossini, Apidae, Hymenoptera), a tribe of Neotropical corbiculate bees, have elaborated the use of exogenous compounds, compiling highly complex mixtures of volatiles acquired from their environment. These volatiles are not ingested through the diet. Instead, the males employ specialized external structures on the legs to collect volatile compounds from sources such as flowers, resin, sap, rotten wood or feces (Vogel 1966, Whitten et al. 1993). The chemical composition of individual "perfumes" is less variable within species than among them, with substantial differentiation between closely related species (Zimmermann et al. 2009). Perfumes are stored in pouches located in the hind tibia of males and are later released through stereotypical display behavior at the sites where mating occurs (Eltz et al. 2005b). Display behavior usually takes place around a central perch tree at which the male bee assumes a typical posture (Kimsey 1980, Stern 1991, Eltz et al. 2003; and see Fig. 1). The exposure of perfume then takes place during brief hovering flights while the male remains in close proximity to and facing the perch. Stereotypical leg movements leading to perfume exposure from the hind leg pouches have been documented in detail in one species, *Euglossa cognata* (Eltz et al. 2005b), but similar behaviors take place in other species and genera (see also *Results*). Aside from the characteristic perfume exposure, displaying male orchid bees perform more extensive 'patrolling' flights of several to a few dozen meters at intervals, after which the bee returns to its perch (Kimsey 1980, Eltz et al. 2003). During male display, conspecific males may arrive and engage in interactions with the resident male, and all of the few matings observed to date were at or near a perch tree (Dodson 1966, Kimsey 1980, Stern 1991, Eltz et al. 2003, Zimmermann et al. 2006). However, while all current evidence points to the perfumes being sex pheromone analogues (Eltz et al. 2005b, Weber et al. 2016), female attraction to perfumes has yet to be demonstrated, and the importance of chemical information for euglossine pre-mating communication remains essentially unknown.

In the present study, we evaluated the importance of chemical and visual communication during display by investigating how display behavior is integrated in and affected by the physical environment (terrain, wind, light).

FIG. 1. Male orchid bees showing the typical display perching position. Only the front and hind legs are in contact with the perch, the middle legs are held up and close to the body. The hind legs are nearly stretched and the head is in close proximity to the perch tree. Clockwise from top left to bottom left: *Euglossa flammea*, *Euglossa imperialis*, *Exaerete smaragdina*, *Eulaema speciosa*. Scale bar = 1 cm. [Colour figure can be viewed at wileyonlinelibrary.com]

We present results from eight sympatric species of orchid bees. We characterized display perches as well as the timing and spatial fine-tuning of display behavior. Specifically, we characterized perch sites and perching activity of different species to test for partitioning of the display niche, mapped and analyzed the spatial distribution of displaying males across habitat variables, and investigated whether displaying males position themselves on the perch in a predictable way in relation to wind and light. Finally, we tested a prediction of the sex pheromone-analogue hypothesis, i.e., that displaying male bees exhibit greater quantities of more complex perfumes than their non-displaying conspecifics. This prediction is based on the assumption that collecting perfumes and displaying them are time-consuming, costly activities. Only males that have attained the required signal criteria, e.g., a certain threshold intensity or complexity, would be expected to display.

MATERIALS AND METHODS

Study site

Display sites were investigated in the lowland forests near the La Gamba field station, Puntarenas, Golfo Dulce region, Costa Rica, which is located adjoining the Parque Nacional Piedras Blancas in the pacific south of the country. The climate is characterized by high precipitation (5,900 mm/yr) and steadily high temperatures (on average 28°C, Huber et al. 2008). The forest harbors a species-rich euglossine community (>30 species from four genera, T. Pokorny and T. Eltz, *personal observation*). The area can be accessed via a trail system leading from the field station (situated in a small valley) into the nearby forest and along a ridge on a number of paths (Fig. 2).

Display/perch characteristics and male angular orientation

During the early wet season (March, April, and May) in the years 2012–2014, the trails were walked in search of perching male orchid bees almost daily between 06:30 and 12:30 hours (with up to four investigators covering different trails simultaneously during March of each of the years, while April and May were done by one experimenter, and only in the years 2013 and 2014). Displaying male orchid bees were recognized as such based on their specific perching posture (Fig. 1), and could in most cases
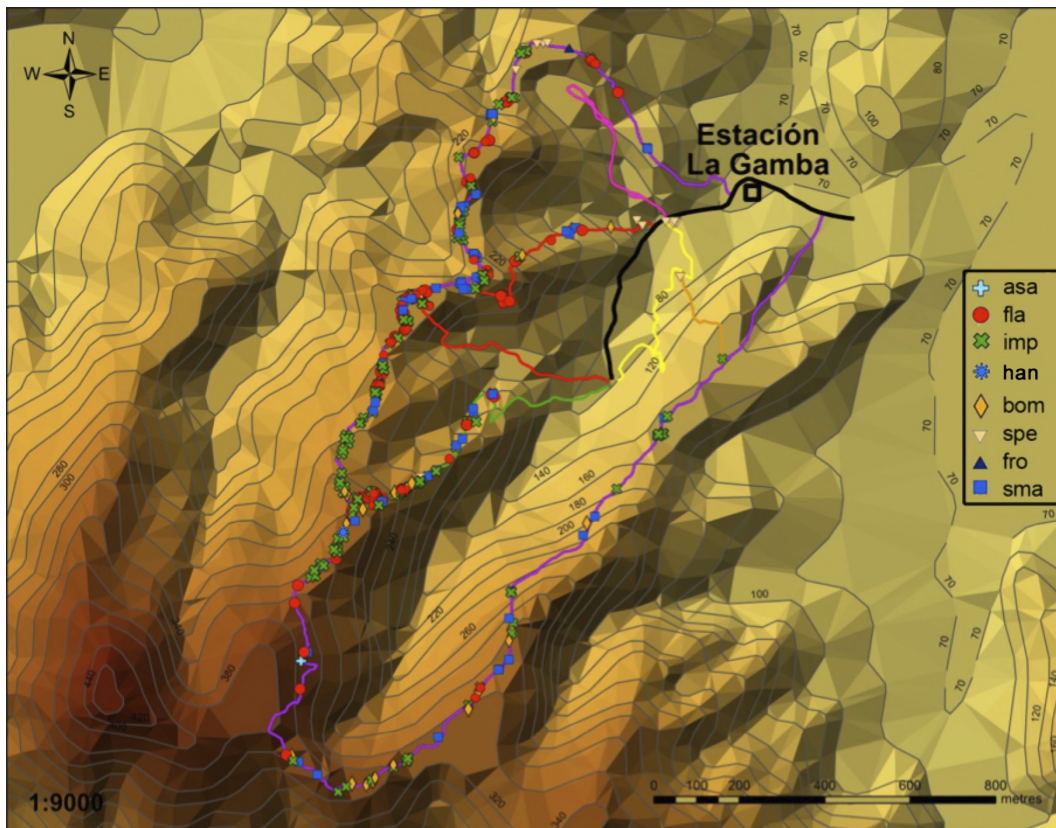

FIG. 2. Trail system around the La Gamba Field station, Puntarenas, Costa Rica showing perch sites encountered during 2012–2014. The Fila trail used for spatial autocorrelation analyses is purple. Species abbreviations: fla = *Euglossa flammea*, imp = *Euglossa imperialis*, han = *Euglossa hansoni*, bom = *Eulaema bombiformis*, spe = *Eulaema speciosa*, sma = *Exaerete smaragdina*, asa = *Euglossa asarophora*, fro = *Exaerete frontalis*.

be easily assigned to the correct species by size, proboscis length, coloration, and behavior. GPS location of the perch tree was determined using handheld tracking devices (Garmin, Schaffhausen, Switzerland), and the time was noted for each observation. Angular orientation of the perching bee was recorded and assigned to one of 24 angular segments (15°-steps) on a handheld compass. Wind direction was measured likewise by using a plume of either smoke (freshly extinguished match, 2013) or talcum powder (2014), noting the angular direction into which the plume was blown by the wind (downwind direction). Both methods yielded results even with the very low wind speeds that were usually encountered. When no displacement of the plume took place, this was interpreted as calm and no direction was assigned. The perching height of the bee was memorized before approaching the perch, and measured or, if it exceeded 2 m, estimated. Perch tree trunk circumference was measured at a height of 1 m, and diameter was calculated. Perch trees were marked using aluminum tags nailed or tied around the trunk well above or below the height at which the male bees displayed. The brightest canopy segment around the perch tree, i.e., the one in which the sky was least obscured by branches and leaves, was determined by hemispherical canopy photography using a Canon PowerShot G11 (Canon, Tokio, Japan) equipped with a Nikon fc-e9 fisheye lens (Nikon, Tokio, Japan, see Appendix S1 for details). When multiple observations were conducted for the same species in the same perch site, we only used the first measurement of the perch for the analyses of diameter and direction of the brightest canopy segment. However, if a perch was used by different species, we re-measured the perch for each species.

For the wind direction analysis, perching orientation, and perching height, we only included the first observation for each individual male bee. We assumed that consecutive observations at a specific perch site are repeated sightings of the same individual unless (1) the bee belonged to a different species than the first one observed, or (2) we could confirm that it was a different individual of the same species because we had either captured the previously perching male for perfume analysis or made the observations in different years. To analyze the species-specific timing of display we considered all observations for which the time had been recorded, including observations of possibly the same individual on different days. Individual bees for which all three parameters (perch diameter, perching height and time of display) had been recorded were incorporated into the dataset for multi-dimensional analysis. One of us (PJ) identified each perch tree to species, genus or family in April 2015 based on leaf, stem and, if present, floral characters.

Display angular orientation experiment

We conducted cage experiments in March 2014 in a large flight cage (9 × 9 × 3.5 m) at the La Gamba field station. The cage contained nectar (*Calathea lutea*, *Stachytarpheta* sp.) and pollen (*Senna alata*) plants as well as cut stems positioned upright to offer perching opportunities (*n* = 21, diameter 3.1–5.4 cm). Fifteen males of each *Euglossa imperialis* and *Euglossa flammea* were caught at chemical baits and transferred to the cage. They quickly learned to drink from artificial feeders containing a sugar water solution (30–40% sugar). After a few days, the first males began displaying in the cage, using stems or branches of live plants or cut stems as display perches. Throughout the experiment, seven perches were regularly used by *E. imperialis* and three by *E. flammea*. Experiments were conducted between 06:30 and 12:00 hours as this was the time window with the highest display activity. Wind direction was manipulated using a household standing fan, with the propeller box covered by mosquito mesh to reduce air flow. The fan was positioned either to the NW, NE, SW or SE of the respective display perch, facing towards it. Speed (levels 1–3) or distance from the perch were adjusted so that the air flow at the perch was between 0.3 and 0.5 m/s as measured by a handheld electronic aerometer (Windmaster2, Kaindl Electronics, Rohrbach, Germany). All four wind directions were tested in random order on each of the seven (*E. imperialis*) and three (*E. flammea*) perches. Control (fan off) observations were made intermittently, and natural wind direction during these controls was measured using talcum powder. In the case of both experimental and control observations, the first perching bee was observed for 1 min and the perching orientation was assigned to 1 of 20 angular segments (18°-steps) around the perch tree. Orientation was documented for each "perching event" during this time, defined as landing in the typical perching position (Fig. 1) between the short hovering or patrolling flights that are part of the display behavior. For each observation under either natural or manipulated wind direction in cage experiments, we conducted statistical analysis on the median angle of bee position during 1-min observation intervals.

Factors influencing perch distribution along the trails

We mapped trails and display sites and analyzed the topographical features of our study site using a 3D-landscape model in ArcGIS 10.1 (ESRI, Redlands, California, USA) (Fig. 2) that included elevational data derived from an aerial survey and the associated topographical map of the Servicio Geodésico Interamericano (Edicion 1 IGCR 1964, courtesy Estación Tropical La Gamba). We searched for orchid bee perching sites by walking repeatedly through an established trail system, which we mapped using a handheld GPS device; tracks were averaged to draw the reference trails (Fig. 2). We interposed the GPS position of orchid bee perching sites and partitioned the trails into 10-m segments. We estimated canopy cover in the field for each of these segments. The left and right side of the trail segment were each assigned to one of six categories (0- only low herbaceous

vegetation dominated by ferns (<3 m of height); 1- low woody vegetation of shrubs and small trees (<3 m); 2- low woody vegetation of shrubs and small trees interspersed with single taller trees; 3- disturbed forest with canopy cover up to 40%; 4- high forest with canopy cover of 40–80%; 5- high forest with canopy cover of 80–100%), and a mean value for each segment was used for further analysis. We determined the elevation above sea level of the middle of each 10-m trail segment based on the topographical map in ArcGIS. Additionally, we inferred the small-scale topographical "exposedness" of each trail segment by subtracting its elevation from the mean elevation of two points situated at 10 m distance orthogonally to the left and to the right of the trail. We estimated global solar radiation (WH/m^2) for each trail segment using the "Area solar radiation" tool in ArcGIS, selecting 4/15/2014 as a representative date, and 7–11 a.m. as the display-relevant time of day.

Perfume samples and chemical analyses

We captured displaying male orchid bees between 06:30 and 12:00 h on March–May 2013, March–May 2014, April–June 2015, and September 2013 using hand nets. We placed captured males in a clean, labeled Eppendorf vial with an air hole for transport. In addition, for each displaying male captured, a conspecific (control) male was captured at chemical baits positioned 50–100 m distant from the display site, usually on the same day (and rarely the following day), resulting in a paired-design statistical analysis (see *Statistical analyses*). After returning to the field station, bees were killed by freezing at −20°C, after which both hind legs were cut off with a pair of clean scissors and placed in a vial containing 500 μL of hexane for perfume extraction. Each individual sample was then stored at −20°C until chemical analysis in Bochum, Germany. Samples were analysed using a HP5890II gas chromatograph coupled to a HP5972 mass spectrometer (GC-MS, Hewlett-Packard, Palo Alto, California, USA), equipped with a DB-5MS column (30 m, 0.25 μm film thickness, 0.25 mm diameter), with splitless injection (1 μL). The GC oven was programmed from 60 to 300°C at 10°C/min followed by 15 min isothermal at 300°C. Further analyses were based on all compounds eluting before *n*-tricosane (i.e., volatile and semi-volatile compounds) and excluded hydrocarbons as well as alcohols and acetates known to originate from the bees' labial glands (see Eltz et al. 2005*a*, Pokorny et al. 2014). We analyzed these subsets of compounds to estimate perfume complexity (number of compounds) and total amount of perfume (summed peak area of all compounds). Additionally, we measured head width (i.e., an indicator for body size) and wing damage (number of notches in fore wing edge, as age correlate) of each individual male. For a subset of males of the two most common species (*E. imperialis* and *E. flammea*) that we captured between April and June 2015, we additionally analyzed perfume composition in detail. Chromatographic peaks were characterized by retention time and mass

spectrum and added to a user-built spectral library in the Agilent ChemStation Software (Agilent Technologies, Santa Clara, California, USA) for reference. We calculated the relative abundance (%) of each entry/compound relative to the total amount of perfume present per individual. These standardized values were used for numerical analysis of perfume similarity (see *Statistical analyses*). We inferred compound structure by comparing spectra and retention times (indices) to entries in commercial spectral libraries or to those of reference samples (see also Eltz et al. 2005*a*, Zimmermann et al. 2009).

Statistical analyses

We tested for differences in perfume complexity, perfume concentration, head width and wing damage between paired samples of displaying and non-displaying (control) bees using the Wilcoxon signed rank test for paired data, as implemented in the base ("stats") package of R v.3.0.2 (R Core Team 2015). We used the same software to test for differences in perch tree diameter and perching height globally between species using the Kruskal–Wallis tests and between pairs of species using the Bonferroni-corrected post hoc tests (package pgirmess). The multidimensional dataset encompassing perch diameter, perching height and time of display was analyzed using one-way analysis of similarities (ANOSIM) in Primer v.6 (Clarke 1993, Clarke and Gorley 2001). ANOSIM is a non-parametric permutation statistic that tests whether a factor (here "species") affects the rank order of pairwise similarities in a similarity matrix (here Bray–Curtis similarities in measurements of display parameters between individual bees). The resulting R value ($-1 < R < 1$) indicates the degree of separation between species (complete separation of species: $R = 1$, no separation of species: $R = 0$). Using the same software, we characterized the the similarity of perfume composition between individual bees as pairwise Bray–Curtis similarity based on standardized (percentage) chemical data, and used ANOSIM and non-metric Multidimensional Scaling (n-MDS; Primer v.6) to test for and visualize differences between displaying and bait-captured males. Circular-circular correlations of angular bee position with wind direction and brightness around the perch tree and tests of between-species differences in the time of display (Watson's U^2 test) were done using Oriana v.4 (Kovach Computing Services, Anglesey, UK).

We used the generalized linear model (GLM) module in Statistica v12 (Statsoft, Tulsa, Oklahoma, USA) to test the influence of elevation, small scale topographical "exposedness", canopy cover, and solar radiation on the number of perches located in a given 10 m-trail segment. The dependent variable was specified as Poisson distributed, with log as the link function.

To test whether perch sites exhibit spatial clustering that is independent of physical factors (i.e., interactions of the bees themselves) we calculated spatial autocorrelations along the trails with R using the commands acf and ccf. To

this end, the number of perch sites in each of the (equidistant and equally sized) 10 m-segments along the FILA trail were treated as a sequence of data. All these analyses were confined to the FILA trail, which is the longest trail and accounts for approximately 71% of all perch sites (Fig. 2). By not including the other (substantially shorter) trails, we allow the data to be treated as a linear sequence ordered according to spatial positions which would not be the case if bifurcations were included. We calculated spatial autocorrelations for perch numbers of all species combined and for perch numbers of each of the two most common species, *E. imperialis* and *E. flammea*, separately. To control possible confounding effects of elevation on the number of perch sites, the analyses were performed separately for altitudinal windows of 100 m (100–200 m, 200–300 m, and above 300 m) or 50 m in size (125–175 m, 225–275 m and 325–375 m).

Results

Perch tree use

A total of 374 trees serving as display perches were documented for males of eight different species (Fig. 2; genera abbreviated as follows: *Euglossa* = *E*., *Eulaema* = *El*., *Exaerete* = *Ex*.): *E. asarophora* ($n = 2$), *E. flammea* ($n = 115$), *E. hansoni* ($n = 1$), *E. imperialis* ($n = 142$), *El. bombiformis* ($n = 40$), *El. speciosa* ($n = 18$), *Ex. frontalis* ($n = 1$) and *Ex. smaragdina* ($n = 69$). Most trees were only used as perch sites by males of one species, but 12 individual trees were used by males of two species, and a further one by males of three species. Perch use by different species was not simultaneous. One individual tree was occupied in the first and third year of the study, 29 trees were occupied in two consecutive years, and two perch trees were occupied by displaying males in 3 yr. In some cases, individual bees were observed to perch on two ($n = 20$) or even three ($n = 3$) neighboring trees, usually situated about 2–3 m apart from each other. Two hundred and thirty-six marked perch trees could be located in April 2015. Of these, 195 could be identified to species (81 tree species), 37 to genus (18 genera), and 4 trees only to plant family. In total, perch trees belonged to 76 genera in 43 plant families (see Appendix S2). A given orchid bee species used several tree species from a number of genera and plant families without obvious preferences for taxonomic affiliation. For example, for *E. imperialis* 88 perch trees belonging to 60 species were identified, while for *El. speciosa* the seven identified perch trees were all were from different species. Up to four bee species used the same tree species as display perch, while several tree species were documented only once for one euglossine species. Overall, male orchid bees were found to perch mostly on trees with relatively smooth stem surface, but no further general characteristics were noted.

The display behavior of five of the eight observed species had not previously been recorded in the literature (*E. asarophora*, *E. hansoni*, *El. speciosa*, and the two

species of *Exaerete*). Our observations corroborate that it is typical for males of the genus *Euglossa* to perform short, repetitive hovering flights facing their perches (see Kimsey 1980). In contrast, males of *Eulaema*, including *El. speciosa,* remained seated on the perch for most of the time but repeatedly buzzed their wings (see Stern 1991). The two species of *Exaerete* followed a similar display pattern as *Euglossa* spp. in that they performed hovering flights, albeit in lower frequency than *Euglossa*. Males of all species and all genera regularly patrolled the vicinity of their perches, and all species were seen performing leg crossing behavior (see Eltz et al. 2005*b*) during either hovering flights (*Euglossa*, *Exaerete*) or patrolling flights (*Eulaema*).

Display niche partitioning among species

Perch characteristics differed across species, both globally and in pairwise comparisons. Due to the low number of observations, *E. asarophora*, *E. hansoni* and *Ex. frontalis* (2, 3, and 1 observations, respectively) were excluded from pairwise species comparisons and post hoc tests in all analyses. Perch trees chosen by the different species differed in their diameters (Kruskal–Wallis test, total $n = 374$, $H = 205.23$; df = 7; $P < 0.001$, Fig. 3a). *Exaerete smaragdina* exhibited the least specificity in the choice of its perch, displaying not only on tree trunks but also on dead vegetation such as shed fern fronds ($n = 3$) or fallen twigs with leaves ($n = 2$; measurements not included in further perch tree analyses), or on rectangular wooden sign posts ($n = 2$). Males of different species also displayed at different heights on the perch tree (Kruskal–Wallis test, total $n = 409$, $H = 277.80$; df = 7; $P < 0.001$, Fig. 3b). Post hoc tests revealed that all pairs of species except *El. bombiformis* and *El. speciosa* differed in either perch tree diameter, display height, or both. Pairwise comparisons of daily display activity differed at the Bonferroni-corrected significance level in all cases (Watson's $U^2 > 0.30$, $P < 0.005$) except for the comparisons between *El. bombiformis* and *Ex. smaragdina* (Watson's $U^2 = 0.193$, $P < 0.05$) and between *E. flammea* and *E. imperialis* (Watson's $U^2 = 0.158$, $P > 0.05$). *Eulaema speciosa* was the earliest species to show display behavior (from 06:00 to about 08:00 h at the latest), while *E. imperialis* display could be observed from 07:00 to as late as 12:00 h. The ANOSIM of perching parameters (diameter of perch, height of perch site, time of perching, see also Appendix S3) clearly separated the five sufficiently sampled species (Global $R = 0.575$, $P < 0.001$), and all pairwise tests showed significant separation at the Bonferroni-corrected P level.

Angular orientation at the perch tree

The orientation of bees around the display tree was weakly and negatively correlated with the direction of the brightest 15° segment (circular-circular correlation, $r = -0.04$, $P < 0.05$, Fig. 4a), while there was a strong
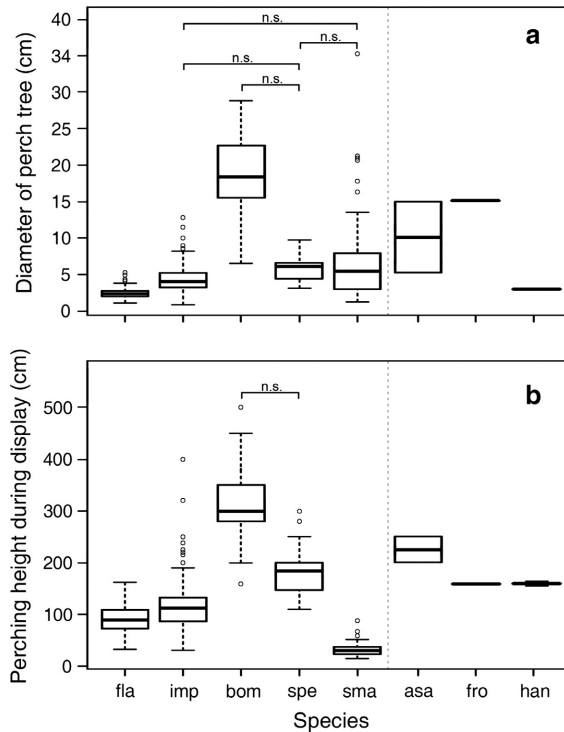
FIG. 3. Diameter of display perch trees (a) used by the studied species and height at which display was performed (b). Boxplots show median, quartiles and range. Outliers are indicated by open circles. Species to the right of the dashed line were excluded from pairwise comparisons due to low sample size; see text for statistics. Species abbreviations: fla = *Euglossa flammea*, imp = *Euglossa imperialis*, bom = *Eulaema bombiformis*, spe = *Eulaema speciosa*, sma = *Exaerete smaragdina*, asa = *Euglossa asarophora*, fro = *Exaerete frontalis*, han = *Euglossa hansoni*.

positive correlation with the direction into which the wind blew during display (downwind; circular-circular correlation, $r = 0.77$, $P < 0.05$, Fig. 4c). In fact, wind direction had high predictive power with regard to display direction (Fig. 4b, d). In some cases, the bees were observed changing their orientation around the perch, and subsequent re-measuring of the wind direction revealed that the wind had turned. In two cases, mating could be observed involving the perching male, and in both cases the female arrived from downwind (both cases *E. imperialis*, see *Discussion* and Appendix S4). The display orientation experiment clearly confirmed the importance of wind direction. For the seven *E. imperialis* and three *E. flammea* perches there was a clear correlation between the manipulated wind directions and the resulting orientation of the observed perching bee (circular-circular correlation, *E. imperialis*: $r = 0.661$, $P < 0.05$; *E. flammea*: $r = 0.412$, $P < 0.05$). Additionally, in all control measurements (without fan), bees changed their orientation according to the prevailing natural wind direction in the cage (circular-circular correlation, *E. imperialis*: $r = 0.586$, $P < 0.05$; *E. flammea*: $r = 0.771$, $P < 0.05$).

Distribution of display perches

We found 0–11 perch sites in a given 10-m trail segment, with the majority of segments (75.8%) containing 0 perches, and 13.8% containing one. Perch distribution along the trail system was non-random. Of the four factors included in the GLM, canopy cover and elevation had a significant positive effect on the number of display sites per segment (Table 1), with the vast majority of perch sites being situated in forested areas on ridges (Fig. 2). Elevation had the strongest overall effect, with only 0.09 perch sites/segment on average in the lowest quarter of the covered elevational range, and 0.67, 0.96, and 0.37 in the 2nd, 3rd, and 4th highest quarter, respectively. The effect of canopy cover was mostly based on the fact that there were no perch sites in segments without trees >3 m height. Among segments with trees, the highest average number of perch sites (0.82 per segment) was found in category 4 (40–80% canopy cover). Small scale exposedness and insolation had no significant effect.

Display perches were more aggregated along the FILA trail than expected by chance as shown by spatial autocorrelation analyses (Appendix S5), i.e., the number of perches in a 10-m trail segment was partially predictable from the number in neighboring segments, especially from that of directly adjoining segments (Ljung-Box test: $Q\_LB = 63.71$, df = 1, $P < 0.001$ for the analysis including all perches of all species). This result remained at least qualitatively unchanged when analyses were restricted to more narrow elevational ranges or to perches of either of the two most common single species, *E. imperialis* and *E. flammea*, see Appendix S5.

Display, perfumes, size and age

Perfume samples were obtained from 93 displaying and an equal number of control males (Fig. 5). The perfumes did not differ in their complexity (number of compounds) or their concentration (area sum of chromatographic peaks) between displaying and control males when analyzing all species together, and most single species (Wilcoxon signed rank tests for paired data, $P > 0.17$). The only exception we found was for *E. imperialis*, in which perfumes of displaying males contained a higher number of compounds (higher complexity) than those of control males (Wilcoxon signed rank test for paired data, $P < 0.05$). Chemical composition of perfumes differed strongly between males of *E. imperialis* and *E. flammea* (Fig. 6, ANOSIM $R = 0.89$, $P < 0.01$). However, we found no within-species difference when comparing males captured at display sites and males captured at chemical baits (*E. imperialis*: $R = 0.023$, $P = 0.1$; *E. flammea*: $R = 0.06$; $P = 0.13$). Notably, however, none of the outliers of species clusters in Fig. 6, neither of *E. imperialis* nor *E. flammea*, were displaying males.

Neither size (headwidth) nor age (wing damage) differed between displaying males and males captured at chemical baits as controls (Wilcoxon signed rank test for paired data, $P > 0.18$).
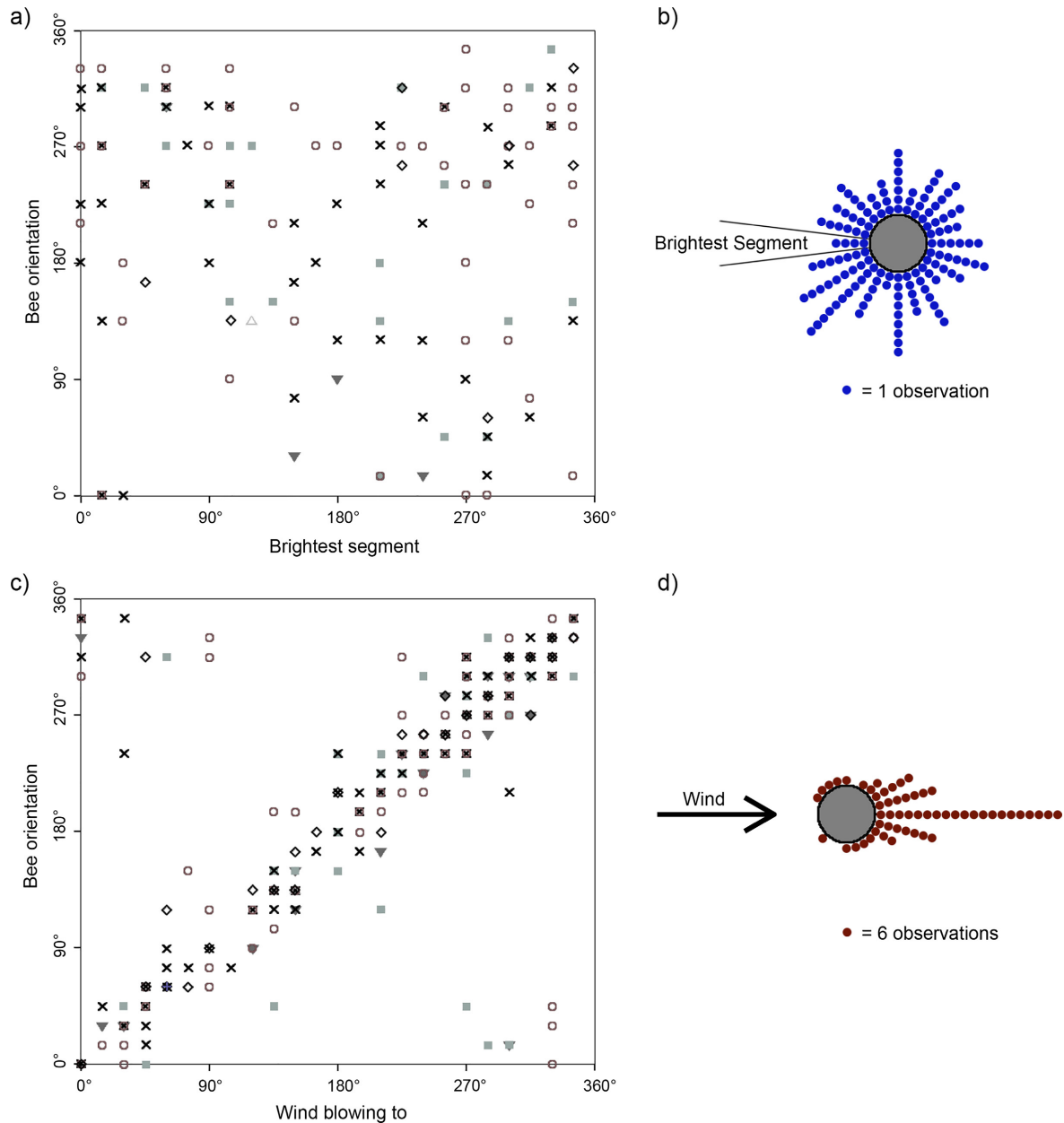
FIG. 4. Relationship between the angular position of the perching bee and the direction of the brightest canopy segment above the perch (a, b) or the direction where the wind was headed (c, d). See text for statistics. Note that these are circular data, i.e., the data points positioned in the top left and bottom right corners of the scatterplots in (a) and (c) are not outliers (360° = 0°). Different symbols are different species; open circles: *Euglossa flammea*, ×: *Euglossa imperialis*, open diamonds: *Eulaema bombiformis*, triangles: *Eulaema speciosa*, squares: *Exaerete smaragdina*, +: *Euglossa asarophora*, open triangle: *Exaerete frontalis*. Panels (b) and (d) are based on the same data as (a) and (c), respectively, but represent the orientation of all bees relative to (b) the direction of the brightest segment and (d) the direction where the wind was headed. The grey circle depicts the tree trunk, while observed relative bee positions are indicated by dots. [Colour figure can be viewed at wileyonlinelibrary.com]

DISCUSSION

During this study, we have recorded an unprecedented number of display perches from eight species of orchid bees, including five species for which no published information on display had been available before. This was possible in part due to the unusually high frequency of

orchid bee display behavior observed in the La Gamba trail system (T. Eltz, *personal observation*). The reasons for this high abundance of displaying males are probably complex, but in the light of the current evidence it appears that the topography of the area in general, and the trail system in particular, is an important factor. The study site is characterized by steep slopes, with trails often

TABLE 1.   Generalized linear model effects of physical factors on the distribution of perch sites among 10 m-segments ($N = 835$) of the trail system around the La Gamba research station, Puntarenas, Costa Rica.

	df	Wald statistic	P
Intercept	1	29.6	0.00000
Canopy cover	1	6.1	0.01316
Elevation	1	46.6	0.00000
Relative exposedness	1	0.8	0.37468
Solar radiation	1	0.4	0.51856

following the tops of ridges flanking a large central valley (Fig. 2). Along these trails, we found that orchid bees favored the more elevated sites for display, perched on trees of species-specific diameter at species-specific height, and showed a strikingly tight association with wind direction regarding their angular orientation on the perch. Overall, our data represent strong circumstantial support for perfume dispersion being the central function of male orchid bee display, and for male perfumes serving as sex pheromone analogues.

Choice and partitioning of perch sites

While male orchid bees clearly preferred certain sites along the trails for display, only a small fraction of perch trees was actually used for display in successive years. This suggests that males primarily select the general site and secondarily the specific tree. This is also supported by the complete lack of male preferences for specific tree taxa. However, within the general requirements that appear to apply for most species of orchid bees, such as the perch tree having a a relatively smooth surface (Kimsey 1980; T. Pokorny and T. Eltz, *personal observation*), there are clear species-specific preferences for certain perch tree diameters, perching heights, and perching times. These parameters, along with the chemical specificity of the perfume blends (Zimmermann et al. 2009, Weber et al. 2016), may be important in reducing signal interference among species and/or for promoting reproductive isolation (Hödl 1977, Schmidt et al. 2013). Display height and perch tree diameter have previously been shown to differ between orchid bee species displaying in the same habitat, including closely related species such as the sibling species *Eulaema bombiformis* and *Eulaema meriana* on Barro Colorado Island, Panama (Kimsey 1980, Stern 1991). Similarly, display perch separation between the closely related *Euglossa imperialis* and *Euglossa flammea*, also distinguished by coloration (green vs. bronzy) and perfume composition, was nearly complete in the present study. Though not all species in the present study differed in every niche parameter, multivariate analysis resulted in clear species distinction of the display niche. We note, however, that we did not observe the display behavior of the majority of the >30 orchid bee species present in our study site,
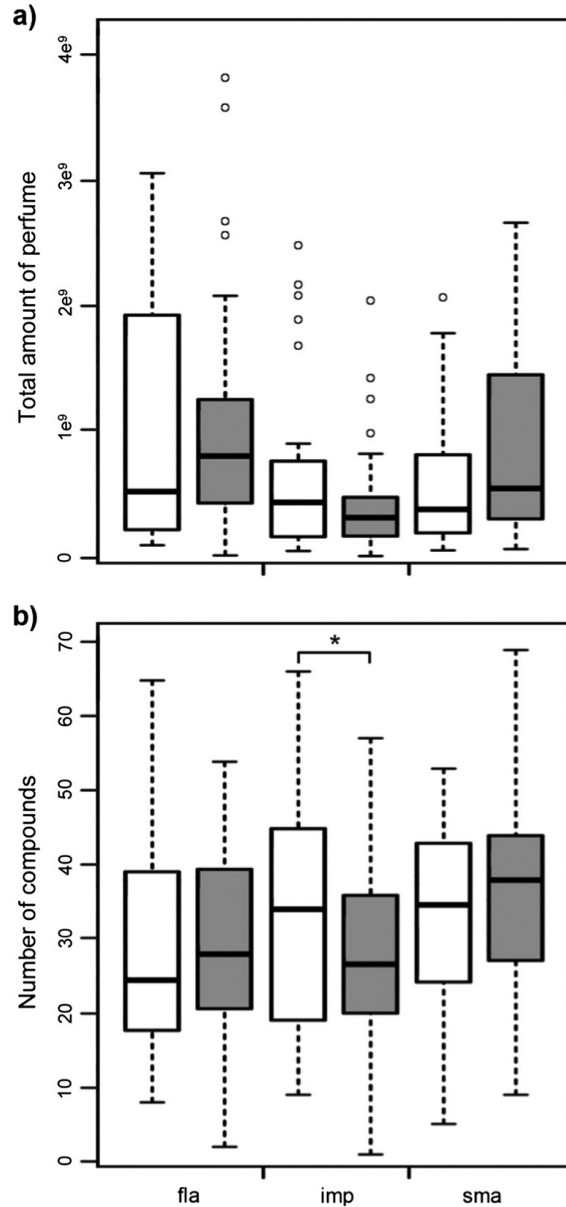

FIG. 5.   Total amount of perfume (a) and number of perfume compounds (b) of displaying (clear boxplots) and control group males (grey boxplots); see text for statistics. Species abbreviations: fla = *Euglossa flammea*, imp = *Euglossa imperialis*, sma = *Exaerete smaragdina*.

which may be explained by spatiotemporal separation of display sites, with the missing species displaying at higher positions, i.e., in the canopy, or at observation-averse times, i.e., near dusk or dawn.

Orientation on the perch: sender conspicuousness

The direction of light illuminating the perch had negligible influence on the bees' perching orientation, which was instead strongly linked to the current wind direction
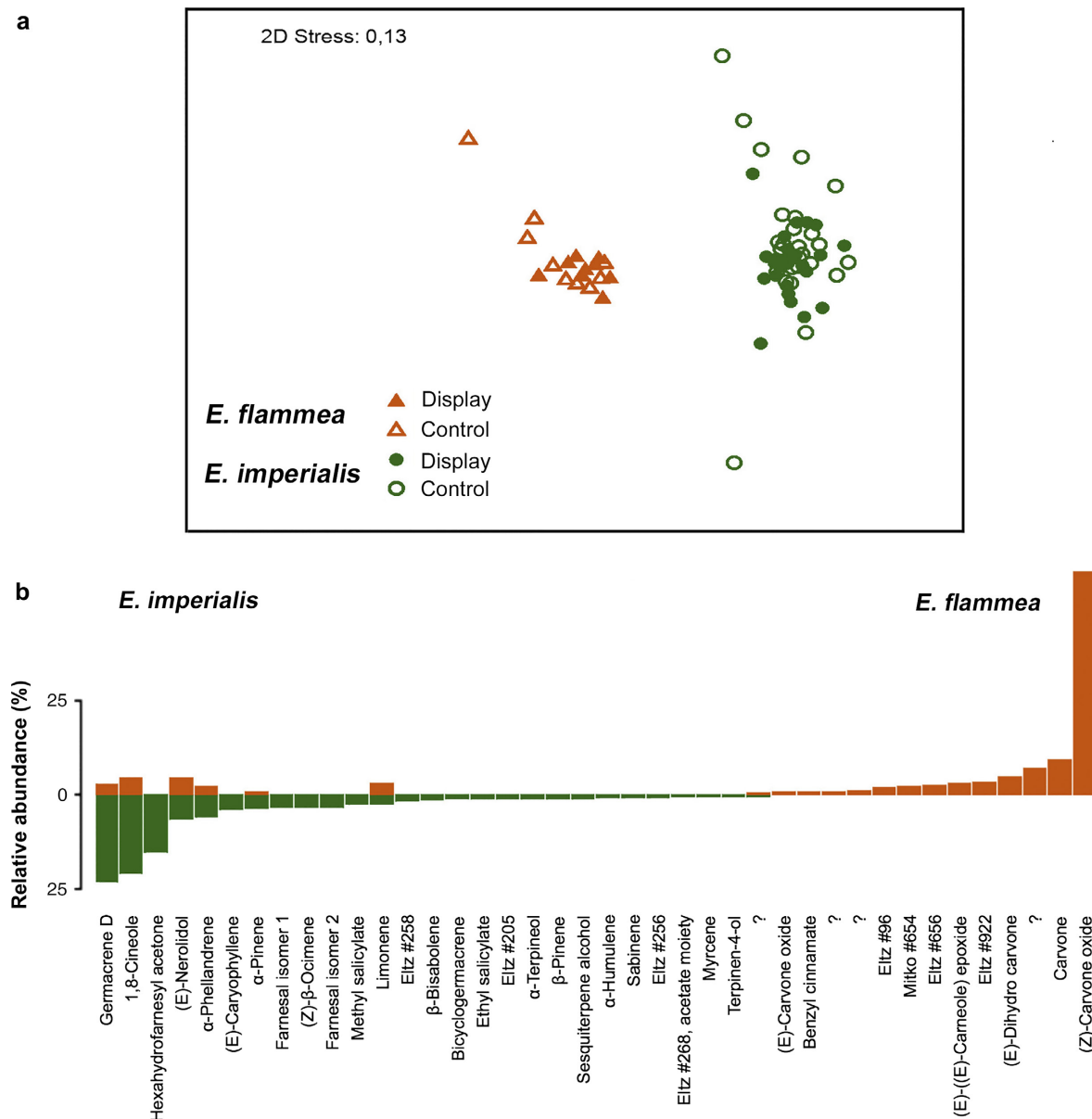
Fig. 6. Composition of tibial perfumes of male *Euglossa imperialis* and *Euglossa flammea*, two common and closely related sibling species of orchid bees in La Gamba, Costa Rica. (a) Two-dimensional Multidimensional Scaling representation of the similarity/dissimilarity of perfume mixtures of individual males caught either during display or at chemical baits (control). Based on Bray–Curtis distances of square-root transformed standardised data (stress 0.13). (b) Comparison of tibial perfume composition of the two species. The 40 most abundant compounds are shown, and bars represent untransformed relative abundances (average percentage contribution to total peak area). Compound names are given whenever available, or entry numbers in our unpublished spectral libraries. [Colour figure can be viewed at wileyonlinelibrary.com]

in all species. Bees were predominantly positioned on the leeward side of their perch tree in the field, and cage experiments unambiguously demonstrated the link between wind direction and bee orientation. Notably, bees responded to very weak air currents nearly imperceptible for human observers and showed hardly any delay in repositioning after wind directions had changed. We suspect that the displaying males use the intermittent patrolling flights not only to check their territory for

conspecific arrivals, but also to assess the wind direction. The more or less elliptical flight paths during patrolling flights (Kimsey 1980) would be ideally suited for this purpose. Corroborating this idea, repositioning by *Euglossa* spp. and *Exaerete* spp. usually took place after the patrolling flights, and only rarely during the sequences of short hovering flights (T. Pokorny, *personal observation*), where bees remain in close vicinity (1–2 cm) to the perch. In any case, downwind positioning on the perch

appears to be an integral aspect of male display. In the genus *Euglossa*, perfumes are relocated during hovering flights from the hind tibial pouch to hair patches on the mid-tibiae, from where they appear to be ventilated further by wing stroke (Bembé 2004, Eltz et al. 2005b). The downwind position may be important for producing a coherent odor plume that is not obstructed immediately by the perch. Additionally, the downwind position would maximize the visibility of a displaying male to conspecifics that approach from downwind. While the chemical signal is likely responsible for medium to long-range attraction, visual signals might play a role in close-range mate recognition. Many orchid bees exhibit conspicuous iridescent metallic colors (hues ranging from red over bronze and green to blue) or show prominent coloring on the metasoma (see Roubik and Hanson 2004). That male positioning was not strongly affected by the direction of the brightest sky segment does not rule out a role of visual signals in the context of display. Stern (1991) found that perch occupancy of *El. meriana* increased with the amount of diffuse light a perch received, but decreased with the amount of direct radiation, suggesting that males select display sites for maximum visibility but a low risk of overheating. Perches with mainly diffuse light are also expected to be more evenly illuminated from all sides, allowing males to conduct positional adjustments in response to the wind, which had an overruling effect on display orientation in our study. The two copulations of *E. imperialis* observed during the course of this study (Appendix S4) support the view that the downwind position of males at the perch facilitates mate location and recognition. In both cases, the female approached from downwind, and spent some time in the downwind vicinity before drawing nearer to the male on the perch. The final approach to the perch, during which one of the males was seen to ascend during hovering flight and repeatedly perform perfume exposition behavior (several "leg crossing" movements, see Eltz et al. 2005b), was swift.

Hilltopping and the question of leks

Display perches tended to be in places with medium to high canopy cover on elevated ground, i.e., ridge tops. One obvious factor favoring elevated ground may be the improved exposure to air currents leading to optimized perfume plume dispersion. Ridges and hilltops are generally good places for chemical baiting of orchid bees (males; Ricklefs et al. 1969; T. Eltz, *personal observation*), presumably because the slightly stronger winds dissipate bait odors to large areas in a short time. The same mechanisms may also favor dissipation of male perfume signals, and males may have adapted to select elevated display sites. In addition, being at elevated positions may generally facilitate mate finding. Hilltopping is a phenomenon well known from other insects such as butterflies, flies, wasps, and perhaps even bumblebees (see Goulson et al. 2011) whereby any individual seeking a mate ascends to the top of the nearest hill, leading to an aggregation of sexually active individuals and an increased chance of finding a mate (Alcock 1987, 2007). In the case of orchid bees, the placement of display sites on elevated ground may reduce the area that a virgin female orchid bee has to search for male perfume plumes.

Leks are congregations of males in certain areas formed for the purpose of mating, and the possibility of lek formation has been previously been discussed for male Euglossini by Kimsey (1980). In the present study, we found that display perches were aggregated in space along the trails, and this finding was retained in part when we controlled for elevation. This suggests that male orchid bees may use additional cues for perch site selection, e.g., the presence of other displaying males. Indeed, occasionally more than one male of a given species displayed at the same time at different perches in relative proximity (a few dozens of meters) to each other. Furthermore, males of the same species were frequently observed interacting at a given perch site, which was also noted previously by other observers (Kimsey 1980, Stern 1991, Zimmermann et al. 2006), so male euglossines appear to be aware of the presence of other displaying males in the vicinity and may in fact visit each other on a regular basis. It seems plausible that males initiate display in areas where other males engage in the same activity, possibly as a response to conspecific perfume signals. However, at present we cannot differentiate whether aggregations in space are caused by the presence of conspecific males or simply by favorable display conditions (see also Kimsey 1980).

Display and perfumes

It has been shown that the amount and complexity of individual perfume loads of male orchid bees increases with male age (as indicated by wing wear, see Eltz et al. 1999, 2015). If perfumes exposed during the orchid bees' territorial display function as sex pheromone analogues and as basis for female choice, it would be expected that only males that have accumulated a complete signal, i.e., older males, will engage in mating display. However, in our comparison of perfumes of displaying and control males there was no difference in the number of compounds or in the total perfume amount for most species. Only in *E. imperialis*, displaying and control males differed in the complexity (number of compounds) of their perfumes, however, we found no difference in the total amount of perfume. This observation, although unexpected, does not refute the hypothesis that perfumes function as pheromone analogues and/or provide a mechanism for female choice. Several factors may explain the lack of a difference. First, the controls, males captured at chemical baits, could have been confounded because they probably included many males that had themselves occupied display sites before. Also, there was no difference in size or age (wing wear) between the two groups in any species. It is well known that male orchid bees collect volatiles throughout much of their lives

(Stern 1991, Eltz et al. 1999), i.e., volatile collection and display are alternating, not mutually exclusive activities. Second, the onset of display behavior might not be determined by perfume load. If the only way for males to attain copulations is during display behavior, and the costs of display behavior are sufficiently low, it would be expected that every male, regardless of his age and perfume phenotype, should engage in display behavior. Early display may even benefit males in gaining experience for male-male interactions that might be necessary to gain access to particularly preferable display sites. Whether competition among males for preferred sites occurs, and how such preferred sites are characterized, is unknown. Lastly, it may not be intensity or complexity of male perfumes that matter for choosy females, but the overall composition (template match) or the possession of only one/a few critical compounds in certain ratios or amounts. In that case we may simply have not measured the correct chemical parameters. Future studies manipulating individual male perfume acquisition in controlled cage experiments are necessary to test effects of perfume phenotype on the phenology and intensity of male display behavior as well on male mating success.

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Supporting Information

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