Report

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Function of environment-derived male perfumes in orchid bees

Highlights

- Perfume loads of male orchid bees were manipulated in dual-choice mating experiments
- Perfume-supplemented males lured more females for mating and sired more offspring
- Male display was stereotypic and its frequency unaffected by perfume supplementation
- Perfumes are sexual signals that evolve by sexual selection through female choice

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In brief

Henske et al. reveal the function of perfume signaling in orchid bees (*Euglossini*), an enigmatic chemical communication system in which males combine volatiles from environmental sources to concoct complex perfume blends, showing that perfumes are sexual signals that lure females for mating and increase male mating success through female choice.

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Function of environment-derived male perfumes in orchid bees

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SUMMARY

Perfume making in male orchid bees is a unique behavior that has given rise to an entire pollination syndrome in the neotropics.[1,2] Male orchid bees concoct and store species-specific perfume mixtures in specialized hind-leg pockets[3] using volatiles acquired from multiple environmental sources, including orchid flowers.[4,5] However, the function and the ultimate causes of this behavior have remained elusive.[2,6] Although previous observations suggested that male perfumes serve as chemical signals, the attractiveness for females has not be shown.[7,8] Here, we demonstrate that the possession of perfume increases male mating success and paternity in *Euglossa dilemma*, a species of orchid bees recently naturalized in Florida. We supplemented males reared from trap-nests with perfume loads harvested from wild conspecifics. In dual-choice experiments, males supplemented with perfumes mated with more females, and sired more offspring, than untreated, equal-aged, control males. Although perfume supplementation had little effect on the intensity of male courtship display, it changed the dynamics of male-male interactions. Our results demonstrate that male-acquired perfumes are sexual signals that stimulate females for mating and suggest that sexual selection is key in shaping the evolution of perfume communication in orchid bees.

RESULTS AND DISCUSSION

Ever since the discovery of the phenomenon,[1,6] the collection and accumulation of environmental volatiles by male orchid bees has intrigued scientists and naturalists, stimulating a large number of studies in pollination biology and bee ecology.[4,9–14] However, the central question has remained unanswered: what is the function of the acquired substances?

There is evidence that the complex perfumes are actively emitted during courtship display performed by males at vertical stems (perches) in the forest understory[7] (Figure 2A), suggesting that they serve to transfer chemical information to conspecifics. Additionally, comparative studies have shown that perfumes have evolved exceptionally fast[8] into species-specific chemical blends that may facilitate mate recognition.[15] Males display on the downwind side of the perch,[16] and females have been observed to approach the male from downwind and land at or near the perch, where mating takes place.[6,16–20] In accordance with these observations, perfumes have been hypothesized to function as inter-sexual signals that mediate mate recognition and/or indicate the genetic quality of males.[21,22] However, experimental evidence of female attraction to perfumes is lacking, and bioassays with perfume extracts only attracted conspecific males.[20] When a male bee displays and holds a display territory, other conspecific males are often attracted to the area and—after a brief interaction—only one competitor retains the perch.[17,19] These observations raise the possibility that perfumes may also transmit intra-sexual information about the competitive abilities of a male, thus resolving conflict either through capture or defense of courtship territories.[17,19] Equivalent signals have evolved across animals whereby both inter-sexual and intra-sexual communication is transmitted.[23–25] For example, the ''skraa calls'' of bower birds first evolved for aggressive display during male contests and were later co-opted for courtship and female choice.[26] Similarly, orchid bee perfumes may play a role in both inter-sexual and intra-sexual communication, mediating mate choice and establishing dominance hierarchies among males.

To elucidate the function of male perfumes in mate choice and male interaction behavior, we conducted cage experiments with the orchid bee *Euglossa dilemma*, a Mesoamerican species that has recently become naturalized in South Florida.[27,28] We obtained freshly emerged bees using trap-nests and released individually marked males and females into two large flight cages equipped with perch sites for male display as well as floral resources and nesting materials. Females built brood cells inside wooden boxes that they provisioned with pollen for larval consumption (Figures 2B and 2C); males and females fed on nectar flowers. Two males were present per cage at any time. We supplemented one male (supplemented male) with 1 μL of perfume harvested from wild conspecifics before introduction to the

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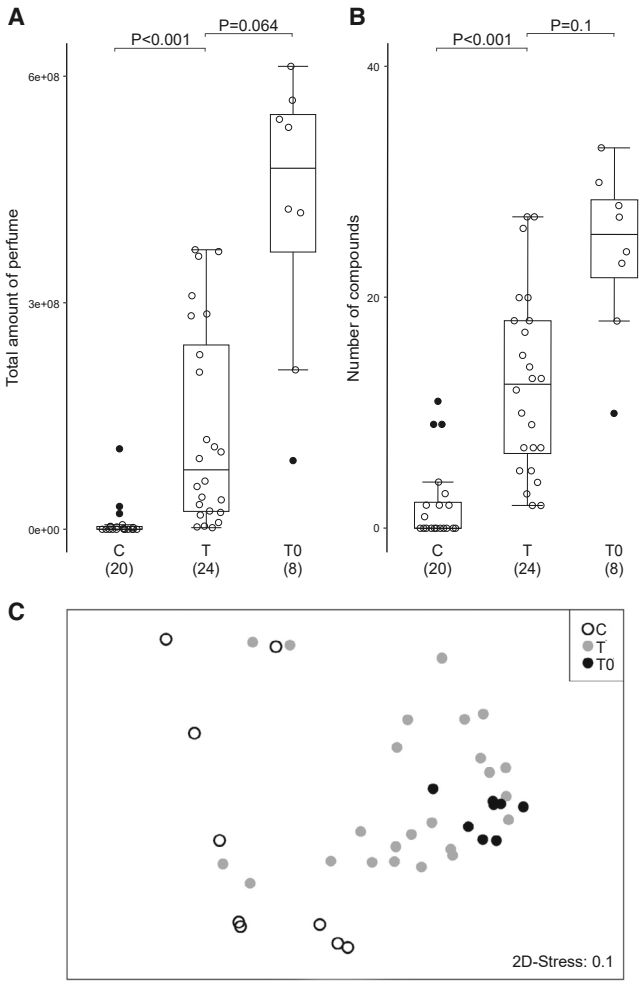

Figure 1. Effect of perfume supplementation on perfume loads of experimental bees

(A and B) Total amount of perfume (A) and number of perfume compounds (B) found in male hind-leg extracts.

(C) 2D-multidimensional scaling representation of Bray-Curtis dissimilarity of perfume compositions based on square-root transformed standardized peak areas.

(A–C) C = experimental control bees without perfume supplementation sampled at the end of the trial; T = experimental bees supplemented with species-specific perfume sampled at the end of the trial, T0 = perfume-supplemented bees sampled 1 day after supplementation. Freshly emerged bees without perfume supplement (n = 9) sampled after 1 day did not contain any perfume and are not shown. (A and B) Numbers in brackets indicate sample size. Boxplots show median (center line), upper and lower quartile (box limits), 1.5× interquartile range (whiskers), individual data points (unfilled dots), and outliers (black dots). p values were calculated using Kruskal-Wallis test followed by Bonferroni-corrected post hoc test. (C) All groups were statistically different from each other (pairwise PERMANOVA, C-T: $F = 6.646$, $R^2 = 0.215$, $p = 0.003$, T-T0: $F = 3.945$, $R^2 = 0.116$, $p = 0.018$, C-T0: $F = 11.477$, $R^2 = 0.433$, $p = 0.006$).

See also Figure S1.

cage, while the other male (control male) was handled in the same way but received no perfume supplement. In dual-choice experiments, we tested the effect of perfume supplementation on male display activity, on the initiation and outcome of male-male interactions, and on male mating success (using genetic paternity analysis, see STAR Methods).

To validate the effect of perfume supplementation, we collected and analyzed hind-leg extracts from freshly emerged males, males that had been supplemented with perfume on the day before, and experimental males (supplemented and control) after they spent 10 ± 3.6 (mean ± SD) days in the cage. Hind-leg pouches of freshly emerged bees did not contain any volatiles (n = 9). There was significant variation of quantity, complexity (number of compounds), and chemical composition of perfume among the three remaining groups (Figure 1A, quantity: n = 52, H = 34.097, p < 0.001; Figure 1B, complexity: n = 52, H = 32.572, p < 0.001; Figure 1C, composition: PERMANOVA, n = 52, $F = 6.725$, $R^2 = 0.261$, p < 0.001). Males supplemented with perfume the day before (n = 8) had the largest and most complex perfume loads, followed by experimental supplemented (n = 24) males (quantity: n = 32, H = −14.167, Z = −2.301, p = 0.064; complexity: n = 32, H = −13.083, Z = −2.127, p = 0.1). Males of the experimental control group (n = 20) were not always completely empty, but managed to acquire some volatiles in the cage, possibly from leaves of food plants (J.H., unpublished data) or from microbes associated with living or decaying plant parts.[5] However, the quantity and complexity of their volatiles was substantially lower than that of supplemented males (Figure 1A, quantity: n = 44, H = −19.533, Z = −4.277, p < 0.001; Figure 1B, complexity: n = 44, H = −19.479, Z = −4.269, p < 0.001) and different in chemical composition compared with those of supplemented males (pairwise PERMANOVA: $F = 6.646$, $R^2 = 0.215$, $p = 0.003$; Figures 1C and S1). Thus, it appears that volatile compounds available in the cages did not reflect the diversity of compounds available to wild males and, consequently, the composition of perfume given to supplemented males. The analytical results show that perfume supplementation was effective in raising quantity and complexity of hind-leg volatiles in most supplemented males for the entire duration of each trial, consistent with (1) hind-leg pouches being efficient volatile storage containers[29] and (2) moderate loss of perfume contents during display by experimental males.[7]

Males started to exhibit display behavior 3.1 ± 2.1 days after being released into the cage with some individuals displaying on the first day. There was no difference between experimental groups in either the onset of display behavior (n = 50, U = 272, Z = −0.802, p = 0.422) or display activity (n = 50, U = 392, Z = 1.543, p = 0.123; Figure 2E; see STAR Methods), suggesting that display behavior is highly stereotypic and does not require previous collection or possession of volatiles.

The possession of perfume loads had a strong influence on the initiation and the outcome of male-male interactions that regularly took place during display bouts. To the observer, these interactions appear competitive in nature, with males engaging in ritualized zigzag or sustained circling flights near the perch. These interactions rarely involve physical contact and usually end with one of the males leaving the site and the other resuming display at the perch site where the encounter took place[6,17–20] (J.H., unpublished data). Male *E. dilemma* regularly interacted in this manner during display in the experimental cages. Interactions took place more often at the perch of the supplemented male (n = 33, U = 270, Z = 4.902, p < 0.001; Figure 2F). However, the possession of perfume did not increase the likelihood that a

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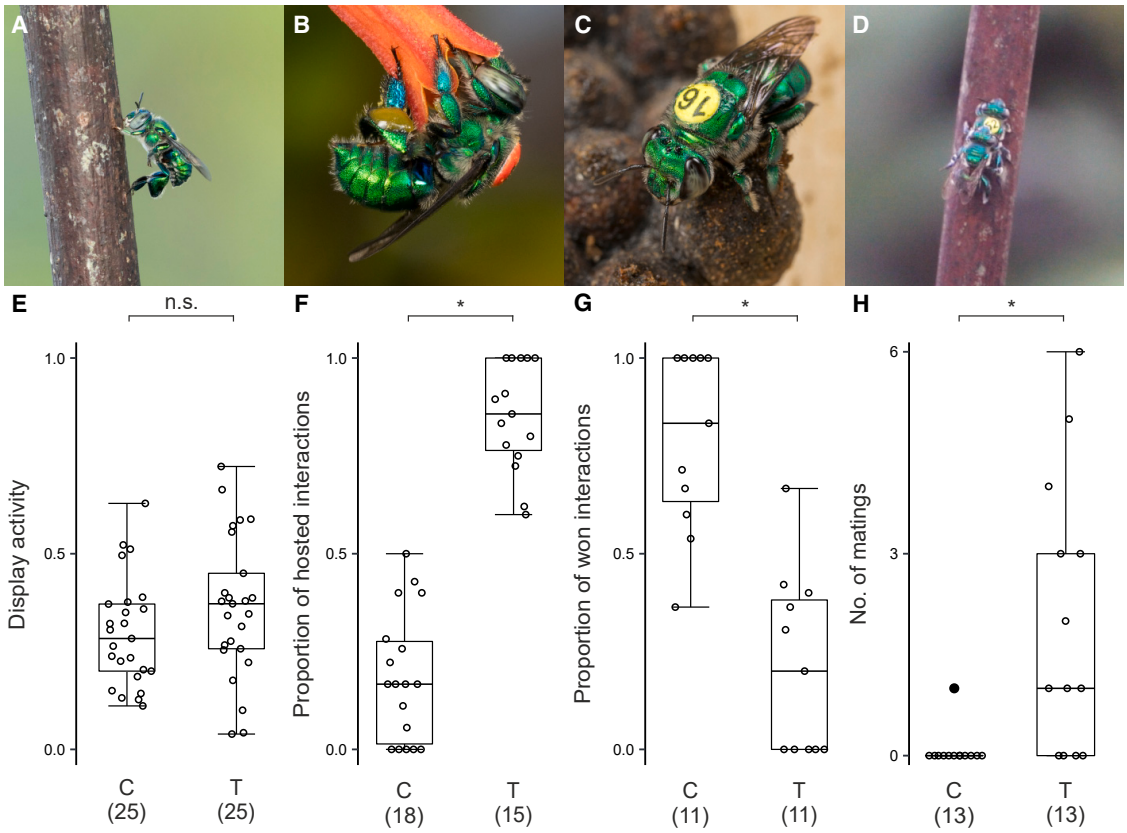

Figure 2. Reproductive success and perfume-dependent behaviors of *Euglossa dilemma* during flight cage experiments

(A) Male showing characteristic perching behavior during display.

(B) Marked female with pollen loads drinking nectar from *Hamelia patens* flower.

(C) Marked female on brood cells constructed in wooden nest box.

(D) Copulation event during experiment after female (with tag) landed on the perch (image captured from video sequence; see Video S1A).

(E) Male display activity (proportion of intervals where display behavior was observed) did not vary with perfume supplementation (T).

(F and G) (F) Perfume-supplemented males received more visits by control (C) males (i.e., hosted more interactions) at their perch site, whereas (G) control males showed a higher proportion of won interactions.

(H) Successful mating events were achieved almost exclusively by perfume-supplemented males.

(E–H) Numbers in brackets indicate sample size, C = control bees with no perfume, T = perfume-supplemented males. Asterisks (*) indicate significant differences at $p < 0.003$, Mann-Whitney U test. Boxplots show median (center line), upper and lower quartile (box limits), 1.5× interquartile range (whiskers), individual data points (unfilled dots), and outliers (black dots).

See also Figure S2 and Video S1A.

male retained his perch. To the contrary, the control male more frequently resumed display at the interaction perch ($n = 22$, $U = 6$, $Z = -3.622$, $p < 0.001$; Figure 2G). These results refute the hypothesis that perfumes function as signals of competitive prowess in male-male competition for perch sites but suggest that control males were attracted by the perfume stimuli released by supplemented males. This is congruent with field observations where male orchid bees (1) are attracted to conspecific perfumes in bioassays and (2) usually approach conspecific display sites from downwind.[20] Our observations are consistent with the idea that males visit the display sites of other males as an opportunistic strategy for sneaking copulations,[17] a low-cost strategy for males that have not yet acquired sufficient perfume to attract mates on their own.[17] Our observations are ambiguous with regard to the hypothesis of male orchid bees forming leks.[19] Leks are non-resource-based cooperative aggregations formed by males engaging in joint courtship to entice females.[30] In the natural habitat orchid bee display sites are sometimes clumped in space, e.g., around treefall clearings or on top of hills and ridges, possibly representing an "exploded" lek.[16,19] That supplemented males were more frequently visited by the control male is in general agreement with the idea of leks. Thus, perfumes could serve as a social signal among males to congregate, increasing the individual chance for copulation.

Female orchid bees mate only once in their lifetime, resulting in functional monogamy.[31] Thus, male access to females is very limited and copulations are difficult to observe,[31] even in a flight cage (but see below). To test whether male mating success depends on the possession of perfume, we genotyped diploid female offspring using microsatellite markers. Each female mother and her corresponding larvae offspring were sampled after females had completed at least four brood cells. Dissections of

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spermathecae revealed sperm in 51.9% of females, showing that they had mated in the cages. Paternity analysis unambiguously found single mating in those females, confirming a previous study,[31] and revealed that the brood of 26 out of 27 inseminated females was sired by a supplemented male ($n = 27$, $T = 26$, $Z = 4.619$, $p < 0.001$). Copulations were achieved by a greater portion of supplemented than of control males ($n = 26$, $\chi^2 = 10.4$, $Z = 3.162$, $p = 0.005$, $\varphi = 0.632$), and individual supplemented males inseminated more females than control males ($n = 26$, $U = 139.5$, $Z = 3.227$, $p = 0.003$; Figure 2H) and sired more offspring ($n = 26$, $U = 138$, $Z = 3.133$, $p = 0.005$). These results constitute the first direct evidence that male perfume affects the mating preference of female orchid bees. In fact, only one female mated with a control male in our experiment. Notably, this control male was exceptional in possessing a perfume similar in composition (Figure S2) and amount to the corresponding supplemented male. It is known that male orchid bees occasionally collect perfume from the hind tibial surface of other conspecific males,[32] dead or alive, especially in captive conditions (J.H., unpublished data). Although we cannot rule out the possibility that the control male obtained the perfume after mating, it is likely that perfume theft facilitated this mating event.

In addition to inferring paternity via genotyping, we directly observed eight copulation events during the course of the experiment; in all cases, the female approached the male perch from downwind in a zigzag-flight pattern in accordance with anemotactic tracking of a perfume plume.[33] In most cases, females quickly landed on the display perch underneath the male and mating took place, lasting only a few seconds (see Video S1A). In one case, the female flew back and forth between two perch sites, closely inspecting the control and the supplemented male, but finally making a choice to copulate with the supplemented male. These observations further corroborate the hypothesis that perfumes transfer information during pre-mating behavior.

Conclusions

In this study, we demonstrate that female orchid bees select mates based on the males' possession of environment-derived perfume. Our results show that perfume directly affects male mating success through female choice, therefore supporting the hypothesis that perfumes are primarily inter-sexual signals, i.e., sex pheromone analogs. In addition, we showed that perfumes released by displaying males attract other males and facilitate the location of conspecific courtship territories.

Sex pheromones have been shown to mediate species recognition in a diverse array of insect lineages, including moths and beetles,[34–38] and natural selection is thought to have optimized their recognition function.[39–41] In fact, previous comparative studies of perfume phenotypic diversity support the view that natural selection has shaped chemical specificity and divergence across lineages of orchid bees.[8,15,21] However, recent studies revealed substantial heritable variation in pheromone traits, especially in male-calling systems, suggesting that sex pheromones can also evolve by sexual selection.[42–47] With perfume volatiles being scarce and unpredictable in natural habitats,[48–50] the process of concocting perfume is certainly costly to male orchid bees, which provides an opportunity for perfumes to evolve as honest indicators of survival/age, foraging success, cognitive skill and/or competitive strength.[21,48] Under this scenario, females that respond to perfume stimuli before mating are effectively selecting males that express such fitness components, underlining the importance of sexual selection in shaping perfume signals. What exactly constitutes an attractive perfume needs to be addressed in future choice experiments. Aside from manipulating parameters like perfume chemical composition and complexity, it may be possible to see how the presence of certain specific major compounds, such as 2-hydroxy-6-nona-1,3-dienyl-benzaldehyde (HNDB) in *E. dilemma*,[51] contribute to perfume attractiveness. Mutations in genes of the olfactory system are known to affect HNDB perception in males,[52] but they likely also affect perception in females, possibly resulting in concerted evolution of male signals and female preferences.[51] Finally, by revealing female mate choice as the driver of perfume collection, our findings have clarified the evolutionary force behind the male euglossine pollination syndrome (or perfume-flower syndrome) that has mystified generations of pollination biologists, including Charles Darwin.[53]

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2023.03.060.

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AUTHOR CONTRIBUTIONS

Conceptualization, T.E. and S.R.R.; methodology, J.H., N.W.S., S.R.R., and T.E.; investigation, J.H. and N.W.S.; formal analysis, J.H.; visualization, J.H.;

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Euglossa dilemma	This paper	N/A
Chemicals, peptides, and recombinant proteins		
p-dimethoxybenzene	Sigma Aldrich	Cat#D121350-100G
n-hexane	Carl Roth	Cat#3907.1
Critical commercial assays		
DNeasy Blood and Tissue kit (DNA extraction)	QIAGEN	Cat#69506
GoTaq (PCR Mastermix)	Promega	Cat#M7132
Hi-Di Formamid (Applied Biosystems)	ThermoFisher	Cat#4311320
GeneScan500LIZ Size Standard (Applied Biosystems)	ThermoFisher	Cat#15843570
GeneScan600LIZ Size Standard (Applied Biosystems)	ThermoFisher	Cat#15849846
Deposited data		
Raw Data (Behavioral observations)	This paper	https://doi.org/10.6084/m9.figshare.21640868
Raw Data (Paternity Analysis)	This paper	https://doi.org/10.6084/m9.figshare.21640868
Rawa Data (GC/MS Analysis)	This paper	https://doi.org/10.6084/m9.figshare.21640868
Experimental models: Organisms/strains		
Euglossa dilemma	This paper	N/A
Oligonucleotides		
see Table S1 for primer sequences	metabion	N/A
Software and algorithms		
SPSS (v.12.0.0.0)	IBM	https://www.ibm.com/de-de/spss
R (v. 4.1)	N/A	https://cran.r-project.org/
Primer (v.6)	N/A	https://www.primer-e.com/
Geneious Prime	Dotmatics	https://www.geneious.com/prime/
GeneMarker	Softgenetics	https://softgenetics.com/products/genemarker/
ChemStation	Agilent	N/A
Other		
Insectaria (40 x 40 x 60 cm)	Bioform	Cat#AerM
Stingless Bee Cerumen	*Melipona panamica*	Self-collected
2μL-microcapillary tubes (Hirschmann)	Carl Roth	Cat#L919.2
1.2μL-CTC-syringe (Hamilton)	Sigma Aldrich	Cat#28617-U
Hand net	Bioform	Cat#A4a
Wooden nest box (8.9 x 6.4 x 3.7 cm)	Darice	N/A
Food plants	Nurseries	N/A
Opalith-tags	Holtermann	Cat#4766
Binoculars (Pentax Papilio)	Pentax	N/A
ABI 3730XL DNA analyzer	Applied Biosystems	Cat#A41046
HP5890II gas chromatograph	Hewlett-Packard	N/A
HP5972 mass spectrometer	Hewlett-Packard	N/A
DB-5ms column	Agilent	Cat#122-5532E

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RESOURCE AVAILABILITY

Lead contact

Further information and requests of resources and reagents should be directed to and will be fulfilled by the lead contact, Jonas Henske (jonas.henske@rub.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Raw data on behavioral observations, paternity, and perfume analysis have been deposited at figshare (figshare: https://doi.org/10.6084/m9.figshare.21640868) and are publicly available as of the date of publication. DOIs are listed in the key resources table. This study does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Experiments took place in 2018, 2019 and 2021 (August – December) on the Campus of the University of Florida (UF) Ft. Lauderdale Research and Education Center in Davie, Florida. We used one cage in 2018 and 2019 (15 x 15 x 4 m, see Methods S1) and two cages in 2021 (15 x 15 x 4 m; 9 x 9 x 3 m). Experimental flight cages were made from shade cloth to provide forest understory conditions. We obtained plants from local nurseries, mostly: *Hamelia patens* (nectar), *Penta sp.* (nectar) and *Senna alata* (pollen). We used stingless bee cerumen obtained from stingless bee colonies (*Melipona panamica*) in Panama as a main resin source. Wooden nest boxes (8.9 x 6.4 x 3.7 cm; Darice) were placed in the cages to facilitate nest building of females. We obtained experimental bees (*Euglossa dilemma*) by trap-nesting bees with wooden boxes placed around buildings on the campus of the UF Fort Lauderdale Research and Education Center, at Fern Forest Nature Center, Flamingo Gardens Wildlife Sanctuary and at private residences. The trap-nests were collected and stored in small insectaria (40 x 40 x 60 cm). We checked nests daily for newly emerged bees to use for experimentation.

METHOD DETAILS

Experimental setup

We marked emerged females with numbered tags (Opalith-tags; Holtermann Imkereibedarf, Brockel, Germany) and introduced them to the experimental cages on their first day of adult life. We checked nest boxes in the cages every night and sampled females and their corresponding offspring after they had built and provisioned four brood cells. We immediately dissected spermathecae to examine mating status.[54] Females and their brood cells were transferred to 95%-ethanol for later DNA extraction. We marked freshly emerged males from collected trap-nests with scratch marks on the thorax.[55] Supplemented bees received perfume supplementation (see below). Control bees were handled in the same way but did not receive perfume. Both control and supplemented bees were kept individually for one night in small insectaria (40 x 40 x 60 cm) equipped with a nectar plant before releasing them into the experimental cages on the following day between 8 and 10 am. The timing of introduction of experimental males in the cages varied somewhat depending on the availability of freshly emerged individuals, which sometimes was a limiting factor. In such cases, males were consecutively introduced ensuring the presence of one control and one supplemented male in one cage at any given time. Males spent at least 6 days in the cage before sampling and introduction of a new male, which was variable due to the limited access to freshly emerged male bees. Hind-legs of males were sampled separately and stored in n-hexane (see GC/MS analysis) and the remaining body was transferred to 95%-ethanol. Since pollen is a limited resource for breeding females, we did not allow more than 20 females to be active in a cage at any given time, ensuring the availability of at least 3 unmated females during the experimental time of each male. In total 55 of 102 females were inseminated, of which we considered 27 for downstream paternity analysis. The remaining inseminated females had to be discarded because either their entire offspring consisted of haploid individuals (n=5) or associated experimental males were lost to unknown causes in the cage and could not be genotyped to ensure reliable paternity assignment between control and supplemented males (n=23). We emphasize that, in doing so, we did not discard any known copulations of control males. During the entire course of the study, 15 control males and 9 supplemented males were lost to unknown causes. A total number of 26 males (13 control and 13 supplemented males) were included in the paternity analysis. These males spent an average of 10.7±3 (m±sd) days in the cage with no difference between experimental groups (U=76.5; Z=-0.415; P=0.687). On average, 8.7 ± 4.3 females (m±sd) were available to control males and 8.9 females ± 4.5 were available to supplemented males (n=26; U=85; Z=0.026; P=1.000). For analysis of perfume content in male hind-legs (n=44) and behavioral differences (n=50; see below), all males were taken into account for which the respective information was available, including males that were excluded from paternity analysis.

Behavioral observations

We monitored male display and interaction behavior using standardized observation intervals during which we scanned all potential perch sites, i.e. vertical stems and structures, three times. One observation interval lasted 10 min and monitoring took place during 5 - 10 observation intervals per day between 7 am and 11:30 am, when bees were most active. We identified displaying males using

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binoculars (Pentax Papilio) to recognize specific scratch marks on the thoraces of the supplemented and control bees. For each male, we quantified overall display activity (proportion of intervals in which display behavior was observed), the frequency of hosting male interactions (received interactive visits from the other male) at display perches, and the outcome of such interactions. A hosted interaction was recorded when one male showed undisturbed display behavior at a perch site and was interrupted by the other male. An outcome of an interaction was only recorded when one male retreated from the interaction and the other one remained at the perch site resuming display. In other cases both males left the perch site or males continued to interact close to the perch site but were not observed to resume display. For interaction analyses we only considered males, which had participated in at least five interactions resulting in n=33 for the analysis of hosted interactions and n=22 for the analysis of won interactions.

Perfume supplementation

To supplement experimental males with species-specific perfume we attracted wild *Euglossa dilemma* at Fern Forest Nature Center using screened p-dimethoxybenzene baits. Bees were caught with a hand net and we harvested perfume by gently squeezing the hind-legs of the bees absorbing the perfume with 2μL-microcapillary tubes (Hirschmann Laborgeräte GmbH, Eberstadt, Germany; see Video S1B). The extracted perfumes were stored in glass-vials at -20°C. Each perfume we used to supplement experimental males consisted of extracted perfumes of 5 to 8 wild-caught individuals, depending on yield. For perfume supplementation 0.5μL of the harvested perfume were applied to each hind-leg pocket of supplemented males (for further technical information see Eltz et al.[56]). To apply harvested perfumes we used 2μL-microcapillary tubes in 2018 and 2019 and a 1.2μL-CTC-syringe (Hamilton, Inc, Reno, NV, USA) in 2021 (see Video S1C).

Paternity analysis

New microsatellite markers were designed with Geneious Prime (Dotmatics, Boston, MA, USA) software using the genome of *Euglossa dilemma* (see Table S1).[57] We extracted DNA from sampled males, females and brood using DNeasy Blood and Tissue kit (QIAGEN, Inc, Valencia, CA, USA) following the manufacturer's protocol. Multiplex-PCR was conducted using GoTaq (Promega, Inc, Madison, WI, USA) and consisted of 30 cycles of 94° for 30s, 60°for 90s and 72° for 90s with an initial step of 95° for 2min and a final elongation step of 72° for 10min. Forward primers were labeled with fluorescent tags (6-FAM, ATTO532, ATTO550, ATTO565). Fluorescent PCR products were then diluted with water to a 1:10 ratio and combined with HiDi formamide and Liz 500 size (in 2021 Liz 600) standard. Fragment sizes were measured with an ABI 3730XL DNA analyzer (Applied Biosystems, Inc, Foster City, CA, USA) at Microsynth SeqLab in Switzerland. Alleles were identified using GeneMarker (Softgenetics LLC, State College, State College, PA, USA) software and we determined paternity by visually comparing alleles among parents and offspring taking advantage of the haplodiploid reproduction system of orchid bees (haploid males can be clearly assigned to diploid brood and females).

GC/MS analysis

We analyzed perfume volatiles in male hind-legs from the following males: freshly emerged males, males that had been supplemented with perfume on the day before, and experimental males of both supplemented and control groups at the end of their cage time. We transferred hind-legs to vials containing 500μL of n-hexane for GC/MS analysis. Samples were stored at -20°C until chemical analysis in Bochum, Germany. Samples were analyzed using a HP5890II gas chromatograph coupled to a HP5972 mass spectrometer (Hewlett Packard, Palo Alto, CA, USA), equipped with a DB- 5MS column (30 m, 0.25 μm film thickness, 0.25 mm diameter), with splitless injection (2μL). The GC oven was programmed from 60 to 300°C at 10°C/min followed by 15 min isothermal at 300°C. For further analysis cuticular hydrocarbons and long chain alcohols and acetates known to derive from bees' labial glands were excluded.[3,58,59] We analyzed perfume complexity (number of compounds) and perfume quantity (summed peak area of all compounds) using the software ChemStation (Agilent Technologies, Santa Clara, CA, USA). We identified compounds using commercial mass spectral libraries[60,61] in conjunction with our own user libraries. We calculated the relative abundance of each compound relative to the total amount of perfume for all bees.

QUANTIFICATION AND STATISTICAL ANALYSIS

We tested for differences in display activity, onset of display activity (number of days until males were first observe to display after introduction into the cages), time spent in the cages, the number of females available to the males, the frequency of hosting male interactions, the outcome of male interactions and individual male mating success (number of inseminated females and number of sired offspring) between control and supplemented males using two-tailed Mann-Whitney U tests as implemented in SPSS statistics v. 28.0.0.0 (IBM, Armonk, NY, USA). We used the same software to test for differences in perfume quantity and complexity between control males (C), supplemented males (T) and freshly emerged supplemented males (T0) using a Kruskal-Wallis test followed by Bonferroni-corrected post-hoc test. We further tested for differences in perfume composition between these groups using global and pairwise Permanova based on square-root transformed standardized data and visualized it using an nMDS plot (non-metric multidimensional scaling). Permanova was calculated in R (v.4.2.1) using the "vegan" package, nMDS was plotted using Primer-e (v.6). We used Yates modified two-tailed chi-square test to test for difference in the proportion of supplemented and control males contributing to copulations. To test whether inseminated females were more likely mated with a perfume-supplemented versus a control male we used a two-tailed binominal test.