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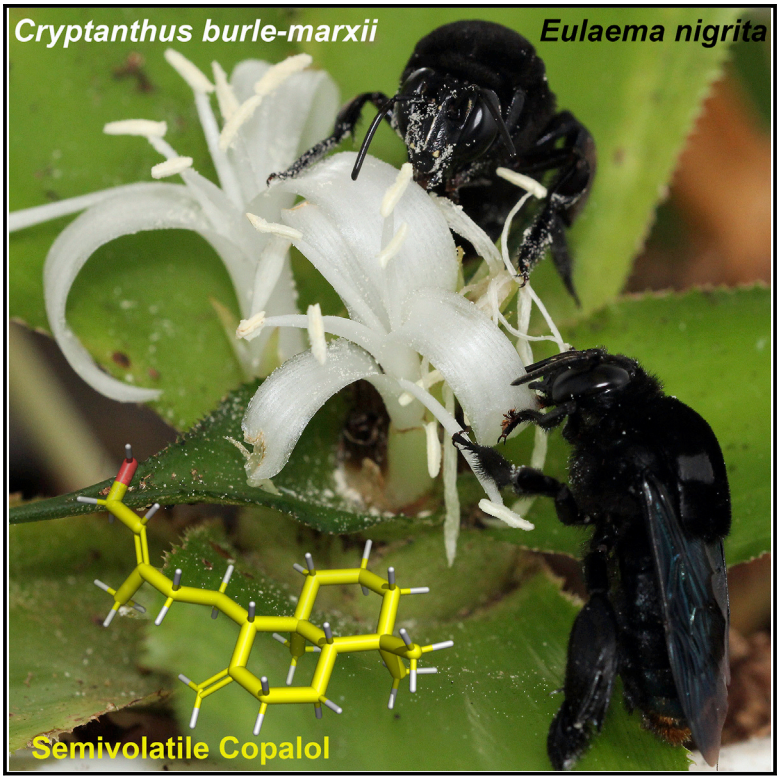
A Semivolatile Floral Scent Marks the Shift to a Novel Pollination System in Bromeliads

Graphical Abstract



Highlights

- Novel report of pollination by scent-seeking male euglossine bees among bromeliads
- Pollinator shift *in statu nascendi* from hummingbirds to male euglossine bees
- Copalol, a semivolatile floral scent compound, is a scent reward for pollinators
- Semivolatile-mediated pollinator attraction seems to be largely underestimated

Authors

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In Brief

Milet-Pinheiro et al. decode the chemical communication of the unusual mutualistic association between an apparently scentless bromeliad and scent-seeking male euglossine bees. Copalol, a semivolatile floral scent compound, mediates the interaction and is hereby the heaviest known attractant for these specialized pollinators.

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A Semivolatile Floral Scent Marks the Shift to a Novel Pollination System in Bromeliads

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SUMMARY

Perfume flowers (*sensu* Vogel[1]) produce intense scents that function both as attractants and as the sole rewards for pollinators. The scent is collected exclusively by male euglossine bees and used during pre-mating behavior.[2–5] Perfume flowers have evolved independently in 15 angiosperm families, with over 1,000 reported species across the Neotropical region.[6] Members of *Cryptanthus* (Bromeliaceae) represent a puzzling exception among perfume flowers, as flowers produce nectar and do not emit a noticeable scent yet still attract euglossine males.[7] Here, we studied the pollination ecology of *Cryptanthus burle-marxii* and decode the chemical communication between its flowers and euglossine males. Field observations revealed euglossine males and hummingbirds as potential pollinators. The bees always contacted anthers/stigma of *C. burle-marxii* while scraping the petals to obtain chemicals, whereas nectar-seeking hummingbirds normally only contacted the anthers. Based on gas chromatography-mass spectrometry/nuclear magnetic resonance analyses of flower scent samples and bioassays, we identified the diterpene copalol as the only floral scent compound triggering scent-gathering behavior in euglossine males. Unlike euglossine-bee-mediated pollination, hummingbird pollination is ancestral in the *Cryptanthus* clade, suggesting a case of an ongoing pollinator shift[8–10] mediated by the evolution of perfume as a reward. Copalol was previously unknown as a floral scent constituent and represents the heaviest and least-volatile compound known to attract euglossine males. Our study provides the first experimental evidence that semivolatile floral compounds can mediate euglossine bee interactions. Male euglossine pollination in other plant species lacking noticeable floral scents[11–13] suggests that semivolatile-mediated pollinator attraction is more widespread than currently appreciated.

RESULTS AND DISCUSSION

The Mutualists

Cryptanthus burle-marxii Leme is a rare and endangered terrestrial bromeliad, endemic to the Atlantic Rainforest habitat in NE-Brazil.[14,15] It is an andromonoecious and self-incompatible species that depends on pollen vectors for reproduction[16] and presents a pollination system that is highly unusual among bromeliads (see below). Similar to other congeners,[7,17] it has delicate whitish flowers with three reflexed petals that are only basally fused, allowing easy access to the nectar (see STAR Methods for a detailed description on floral morphology), which is produced at the beginning of the anthesis (8.0 ± 3.9 μL and 29.1% ± 2.1% of sugar concentration). Field observations on populations of *C. burle-marxii* in two Atlantic Forest fragments at "Mata Praia do Cupe" and "Mata Nossa Senhora do Oitero" revealed that the flowers are visited by different functional groups of animals, including hummingbirds and insects of different orders (Table S1). Stingless bees collected pollen and floral tissue, hummingbirds and butterflies visited the flowers for nectaring, and male euglossine bees visited the flowers mainly to collect scent and sometimes to additionally drink nectar (Table S1). However, only individuals of the reddish hermit hummingbirds (*Phaethornis ruber*, Trochilidae; Figure 1A) and of male euglossine bees (*Eulaema nigrita*, *E. atleticana*, and *Euglossa* sp.; Figures 1B and 1C) contacted both anthers and stigma and thus probably were pollinators. Based on both the behavior and the frequency of visits to flowers, males of *E. nigrita* were considered the main pollinators of *C. burle-marxii*.

Figure 1. Floral Visitors and Pollinators of *Cryptanthus burle-marxii*

(A and B) Males of *Eulaema nigrita* collecting scent from the flowers of *Cryptanthus burle-marxii*.

(A) A male scraping the petal surface with its anterior tarsal brushes.

(B) Males getting covered with pollen while moving around the flower to scrape all petals. Bees contacted anthers and the stigma while manipulating flowers, promoting pollination (see also Video S1).

(C) A reddish hermit hummingbird (*Phaethornis ruber*; Trochilidae) drinking nectar from flowers. Note the beak contacting one of the anthers.

Photos in (A) and (B) are by Paulo Milet-Pinheiro and (C) is by Tarcila L. Nadia. See also Table S1.

Reddish hermit hummingbirds foraged on flowers of several individuals of *C. burle-marxii* in traplining behavior.[18] As they approach flowers, they insert their beaks into the nectar chamber (Figure 1C), frequently contacting anthers but rarely the laterally bent stigma (Figures 2A–2D; see also[19]). Visits by male euglossine bees, on the other hand, always resulted in contact to both the anthers and stigma. After approaching a flower, they landed on the petals and, to gather scent, scraped them in the middle portion using the specialized hair brushes on their foreleg tarsi (Figure 1A).[1] While moving around the flower to scrape all petals, the male euglossine bees always contacted not only the anthers but also the stigma (Figures 2E and 2F). In some occasions, we observed 2–4 males at once on a single flower (Figure 1B). After the long events of scent gathering (as long as 20 min), the male euglossine bees left and visited the flowers of other individuals, thereby promoting cross-pollination.

Bromeliaceae are pollinated predominantly by vertebrates, especially hummingbirds. In this family, pollination by male euglossine bees is extremely rare.[7,20] Besides *C. burle-marxii*, it has only been reported in *C. dianae*, a species that has a similar pollination system and the same pollinator species as we report here.[7] Although attractive to and pollinated by male euglossine bees, flowers of these two species do not feature typical traits of the perfume flower syndrome,[1] because they also produce nectar as a reward for pollinators and do not emit a strong scent. However, the stereotyped scent-gathering behavior of male euglossine bees[1] observed on flowers of *C. burle-marxii* (and of *C. dianae*)[7] represents unquestionable evidence that these bees collect chemical compounds from the flowers (Video S1).

Identification of the Semiochemicals Attracting Male Euglossine Bees

Facing the unusual finding that male euglossine bees gathered chemicals from the surface of the petals of a bromeliad, we decided to specifically investigate the semiochemical(s) involved in the communication between *C. burle-marxii* and *E. nigrita*, which was the most frequent euglossine species. We hypothesized that these bees are attracted to the flowers by olfactory cues, in spite of the absence of a notable scent. To test whether the behaviorally active chemical(s) is/are present in flower headspace samples and/or flower solvent extracts, we performed a series of bioassays in an experimental flight cage. We applied flower solvent extract samples (in n-hexane) to artificial flowers and tested them against solvent controls in dual-choice assays. We found that the flower extract triggered more approaches, landings, and, more importantly, the stereotyped scent-gathering behavior displayed by male euglossine bees; solvent headspace samples of the flowers, on the other hand, did not (Figures 3A and 3B). To establish which specific component(s) of the flower extract triggered the scent-gathering behavior displayed by *E. nigrita* males, we performed an additional set of dual-choice assays. Through thin-layer chromatography with silica plates, we isolated four fractions of the original flower extract (hereafter F1, F2, F3′, and F3) and found that only one of them (F3) elicited more behavioral responses by *E. nigrita* males than the control (Figures 1C–1F). In fact, F3 was as effective as a mixture of all fractions (Figure 3G) in eliciting behavioral responses (see Video S2), evidencing that (a) component(s) in F3 act(s) alone as semiochemical(s).

Following these experiments, we analyzed all samples used in the bioassays by gas chromatography coupled to mass spectrometry (GC/MS) to single out the potential semiochemical(s) involved in the attraction of males of *E. nigrita* and in triggering their scent-gathering behavior. The GC/MS analyses revealed that F3 was composed of a single constituent (retention index = 2,262; Figure 4A), which was also detected in the flower extracts, but not in F1, F2, and F3′ (Figure S1) or in solvent headspace samples. The absence of this compound in solvent headspace samples together with the fact these samples did not elicit stereotyped scent-gathering behavior suggests that this compound is released into the air by flowers in minute quantities only, too low to being both detected and behaviorally active. To look for trace amounts in the headspace of flowers, we collected additional headspace samples, now analyzed by thermal desorption-GC/MS.[21] We found low amounts of the same compound detected in F3 and flower solvent extracts (for a detailed list of compounds in headspace samples and solvent extracts, see Table S2), showing that it is indeed volatile and contributes to the olfactory display of flowers of *C. burle-marxii*.

Based on mass spectral comparison alone, we were unable to identify the compound in F3 but were certain its fragmentation bore close similarities (>85%) to that of labdane-related

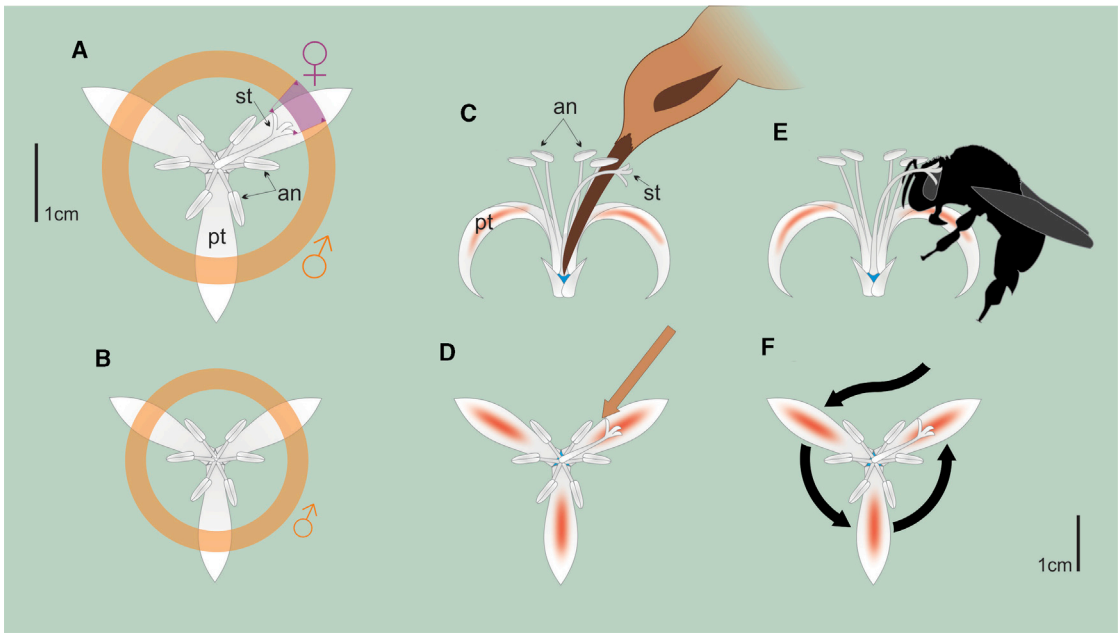

Figure 2. Scheme Illustrating the Morphology of Perfect (Hermaphrodite) and Staminate Flowers of the Andromonoecious Bromeliad *Cryptanthus burle-marxii* (Bromeliaceae) and Their Interactions with Different Pollinators in the Atlantic Forest of Northeastern Brazil

(A) Functional asymmetry of hermaphrodite flowers due to the position of the style.

(B) Symmetric staminate flowers. Although contact with the anthers is possible from approaches in any direction (orange area), contact with the stigma is only possible in a specific direction (purple area).

(C) Side view of a hermaphrodite flower visited by the hummingbird *Phaethornis ruber* (Trochilidae; brown silhouette and arrows) that feeds on nectar (in blue).

(D) Approach direction required from a hummingbird to contact both female and male reproductive structures with its beak. Hummingbirds can approach the flower from multiple directions to get in contact with the anthers, but the contact with the stigma only occurs when they approach a flower from the same side to which the stigma is turned. Hummingbirds appear to empty the floral nectar from a single feeding position only, because we never observed them moving around the flower after approaching it.

(E) Side view of a hermaphrodite flower visited by a male *Eulaema nigrita* bee (Apidae: Euglossini).

(F) Movement sequence performed by an individual of this bee species (black arrows) while collecting scent from all the petals of a flower (red zones), contacting male and female floral reproductive structures with its body.

Flowers in (C) and (E) were represented without a petal so that the interior of the floral tube is visible. an, anthers; pt, petal; st, stigma. Left scale bar refers to (A), whereas right scale bar refers to (B)–(F). See also Table S1.

diterpenoids, such as biformene and sclarene (Figures 4A and 4B).[22] An analysis of F3 by 2D nuclear magnetic resonance (NMR) spectroscopy identified the structure of the active compound as labda-8(17),13(*E*)-dien-15-ol (synonyms: 9,10-anti-copalol or simply copalol; Figure 4). All ^{1}H and ^{13}C chemical shifts (Table S3) are identical to reported values of copalol[23–25] and to those of synthetic (+)-copalol[26] measured under identical conditions (see Supplemental Information for 2D NMR spectra of the isolated natural product and synthetic (+)-copalol with complete assignments). Although the NMR assignment can clearly distinguish copalol from the diastereomer syn-copalol, which shows different chemical shifts (Table S3), it cannot distinguish the enantiomers (+)-copalol and (−)-copalol.

After unambiguous identification of the labdane-related diterpene copalol, we performed another series of bioassays where we tested synthetic (+)-copalol[26] against a solvent control. Similar to the findings with the crude flower solvent extracts, as well as F3, we found that, compared to solvent controls, copalol triggered more approaches, landings, and scent-gathering behavior in *E. nigrita* males (Figure 3H), confirming that it is the only compound in flowers of *C. burle-marxii* that functions as a semiochemical for male euglossine bees.

Labdane-related diterpenes are common in plants and well known for their function as phytohormones (i.e., gibberellins).[27–29] They also mediate interactions between plants and other organisms, especially to fight against micro-organisms and herbivores.[27,28,30] Our findings with copalol add two mutualistic functions to labdanes, i.e., signaling to and rewarding of pollinators. Copalol was first isolated as a natural product in 1969 from mycelia of the fungal plant pathogen *Gibberella fujikuroi*.[31] Since then, it was also reported in other natural sources, such as plant essential oils,[24,32] marine bacteria,[33] and propolis[25] but never as a floral scent compound (for a review on floral scent compounds, see Knudsen et al.[34]).

With a molecular weight of 290.5 g/mol, predicted boiling point of 378°C ± 11°C, and predicted vapor pressure of 3.8×10^{-5} Pa,[35] copalol replaces the also behaviorally active hexahydrofarnesyl acetone,[36] with a molecular weight of 268.5 g/mol, predicted boiling point of 317°C ± 10°C, and predicted vapor pressure of 5.3×10^{-2} Pa, as the heaviest and least volatile compound known to attract male euglossine bees as a single compound. Hexahydrofarnesyl acetone was identified from the hindleg pockets of male euglossine bees,[36] and it is known as a major component of the scent bouquet of the perfume-

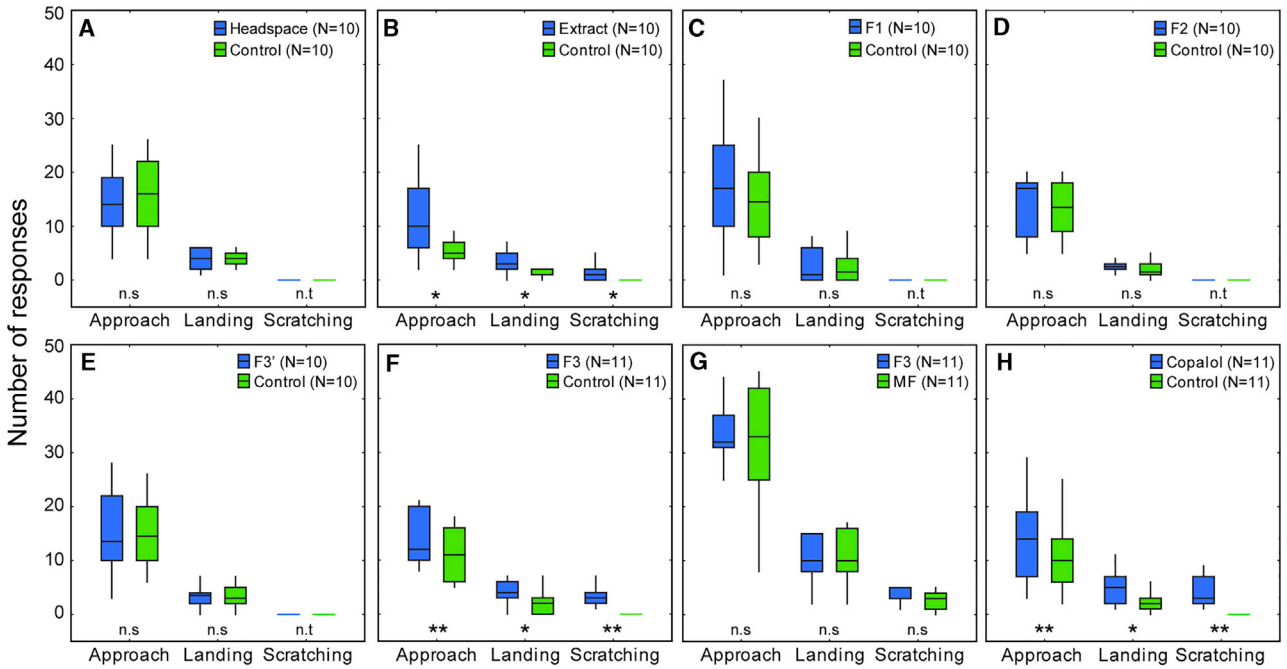

Figure 3. Bioassays Testing the Behavioral Activity of Floral Samples, Fractions, and Synthetic Copalol to Male Bees of *Eulaema nigrita*

Number of behavioral responses (approach, landing, and scratching) displayed by bees in dual-choice assays testing flower headspace samples (A), flower solvent extracts (B), and individual (F1–F3; C–F) or mixed (MF; G) fractions thereof and (+)-copalol (H). Boxplot (median; box: percentile 25%–75%; whiskers: non-outlier range). Symbols and abbreviations: asterisks indicate significant difference (*$p < 0.01$; **$p < 0.001$); n.s., non-significant difference ($p > 0.05$) and n.t., not tested (when bees did not display one of the three behaviors to either treatment or control artificial flowers).

rewarding aroid *Anthurium thrinax*.[37] Differently from volatile organic compounds (VOCs) (boiling point 50°C/100°C–240°C/260°C),[38] which are long known to be involved in the attraction of male euglossine bees by perfume flowers,[39] the importance of semivolatile organic compounds (SVOCs) (boiling point 240°C/260°C–380°C/400°C)[38] in these interactions has remained largely overlooked.[40] In fact, this is the first experimental evidence that male euglossine bees acquire SVOCs from perfume flowers. In other associations, such as those involving sexually deceptive orchids, SVOCs (e.g., long-chain alkanes and alkenes) are well known to attract male insects and to be more important than VOCs in eliciting short-range attraction and pseudo-copulatory behavior.[41–43] Our findings, together with the fact that SVOCs of exogenous origins are quite common (and frequently dominant) in extracts of hindleg pockets of many euglossine bee species,[3,44] suggest that such compounds might mediate many more associations between perfume flowers and male euglossine bees (and probably other pollination systems) than is currently appreciated. Indeed, within the otherwise aroma-rich universe of perfume flowers, besides *Cryptanthus*, there are representatives of the orchid genera *Paphinia*, *Sievekingia*, and *Catasetum* that are scentless to the human nose[11,12] but nevertheless are attractive to perfume-seeking male euglossine bees.

Perfume Flowers: Ecological and Evolutionary Perspectives

Pollination by male euglossine bees is a derived condition within the Bromeliodeae clade, among which hummingbirds are the ancestral pollinators.[8,45] This scenario points to an early or incipient phase of pollinator shift from vertebrate to male euglossine bee pollination in *Cryptanthus* and suggests that perfume flowers in this genus have evolved from nectar flowers. The low amount of nectar in flowers of *Cryptanthus* spp. (no more than 10 μL; our data and Siqueira and Machado[7]) when compared to hummingbird-pollinated taxa across the Bromeliodeae clade,[46–52] together with the fact that the most effective pollinators (i.e., male euglossine bees) primarily forage for scent, indicate that nectar is a plesiomorphic trait that might gradually be suppressed across the evolutionary history of the recently evolved *Cryptanthus* lineage.[8]

Perfume flowers have appeared independently in several Neotropical families under distinct evolutionary/ecological contexts. Similar to *C. burle-marxii*, it is also likely that the only case of pollination by male euglossine bees among Gesneriaceae, i.e., *Gloxinia perennis*,[53] has evolved in a clade having hummingbirds as ancestral pollinators.[54] More frequently, however, perfume flowers are derived from food-deceptive flowers pollinated by male and female euglossine and/or non-euglossine bees (Orchidaceae: Catasetinae, Zygopetalinae, and Stanhopeinae)[55,56] from resin- (Euphorbiaceae: *Dalechampia*)[57,58] and pollen-rewarding flowers (Solanaceae: *Cyphomandra*)[59,60] pollinated by female bees (usually from euglossine) or from beetle-pollinated species offering food and/or mating sites (Araceae: *Anthurium* and *Spathiphyllum*[61] and Annonaceae: *Unonopsis*[62,63]). It is noteworthy mentioning that, independent of the origin, pollination by male euglossine bees is always a derived condition and there is no evidence so far that other

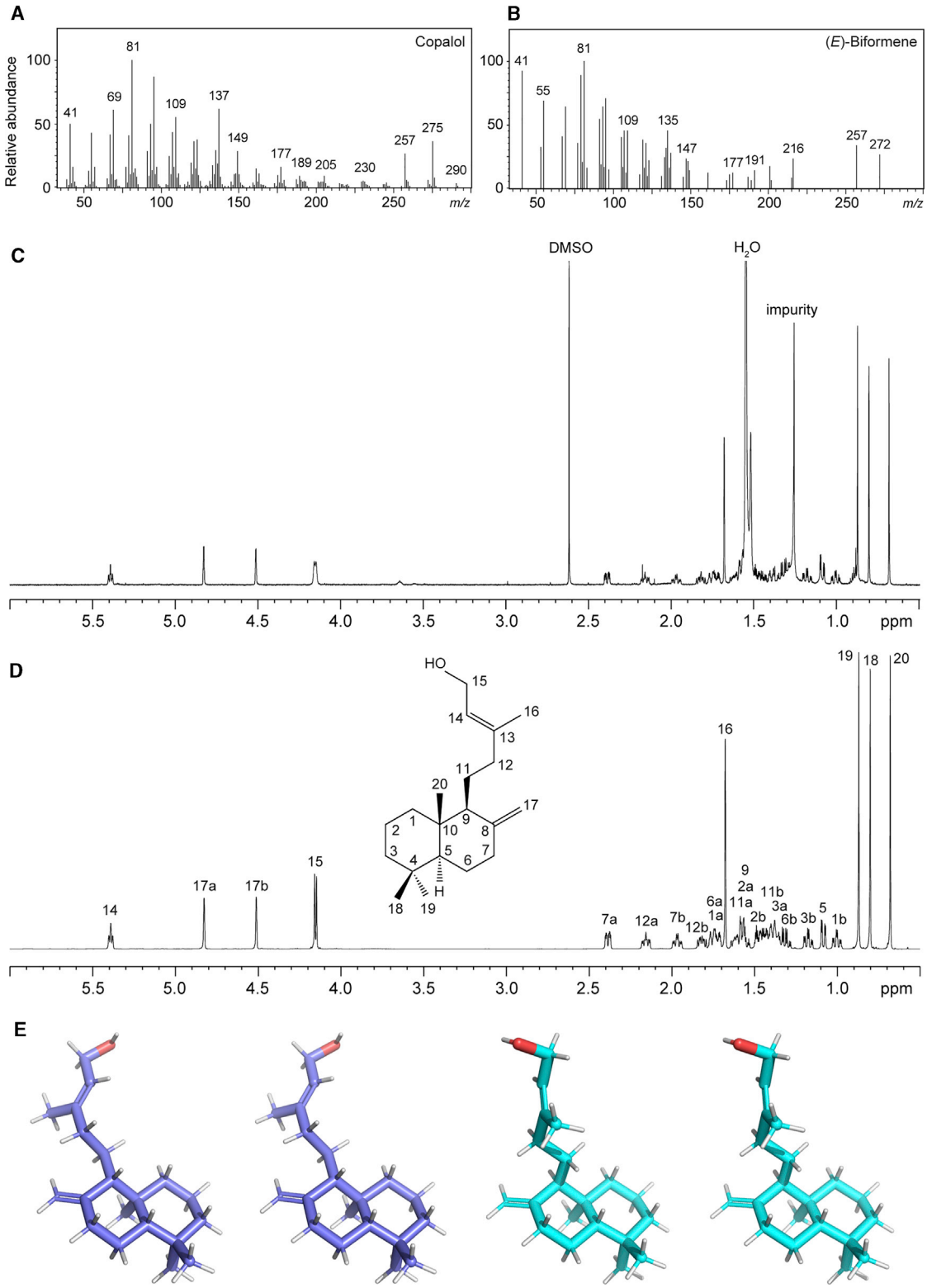

Figure 4. Analyses and Identification of the Floral Semiochemical of *Cryptanthus burle-marxii* by GC/MS and NMR Spectroscopy

(A and B) Mass spectrum of copalol (A) and of the labdane-related diterpene biformene (B).

(C and D) ^{1}H 1D spectrum of fraction 3 (C) and synthetic (+)-copalol (D). The structure of copalol with atom labeling is shown as inset.

(E) Stereo figure of (+)-copalol (blue structure at the left side) and syn-copalol (cyan structure at the right side).

Further information on the TLC separation and GC/MS analyses are found in Figure S1 and Table S2 and additional NMR data in Figures S2 and S3 and Table S3.

pollination syndromes have derived from it. This indicates that perfume flowers represent a very successful pollination strategy in Neotropical ecosystems, possibly because of the intrinsic advantages of these bees as pollinators, such as high abundance, predictability, efficiency, and potential for long-distance gene flow and outcrossing.[64–67]

Conclusions

Our study describes an unusual case of pollination by male euglossine bees in bromeliads (a family predominantly associated with pollination by vertebrates), in a system that challenges the general view of perfume flowers in many typical aspects. The flowers of *Cryptanthus burle-marxii* do not emit a noticeable scent and produce nectar, thereby attracting hummingbirds in addition to male euglossine bees as potential pollinators. Reconstruction of the ancestral pollination system in the Bromelioideae clade[8] suggests that *C. burle-marxii* (and possibly other congeners) is undergoing a shift either from hummingbird to male euglossine bee pollination or binary pollination by both functional groups, mediated by the emergence of scent and (yet) retention of nectar as floral rewards. Although unscented to the human nose, the flowers of *C. burle-marxii* emit a complex bouquet of compounds that includes the semivolatile diterpene copalol, which is the key attractant of the main pollinators, i.e., male *Eulaema nigrita* bees. The occurrence of semivolatile semiochemicals mediating these interactions, together with our inability to easily detect them by scent alone and the apparent low attractiveness of "scentless" flowers to euglossine males,[11] raises the possibility that botanists and pollination ecologists have overlooked an entire phenotypic class in angiosperms, parallel to the way perfume and oil flowers were overlooked before the pioneering work of Vogel[1,68] or the ultraviolet patterns of floral pigmentation, invisible to the human eye, were overlooked before the use of UV-sensitive photography and spectrometry.[69–71]

We have observed a low frequency of male euglossine bees on flowers of *C. burle-marxii*, a trend shared by other "scentless" species of perfume flowers.[11] This suggests that semivolatile-driven systems might indeed be less conspicuous, probably because these compounds are released into the air in small amounts and have a reduced spatial reach, which might culminate in a lower overall attractiveness to bees. Additionally, semivolatiles are assumed to make male leg-pocket contents (perfumes) species specific,[72,73] meaning that only one or a small subset of the species in any given community of euglossine bees will be attracted. Therefore, semivolatiles might mediate the most specific (and inconspicuous) interactions between euglossine bees and perfume flowers, and we believe that this could well be true for other chemistry-based pollination systems. Our findings open a new and promising universe into which future studies dealing with plant-pollinator interactions might be headed. Technical hindrances in working with semivolatiles (e.g., detection, synthesis, and experimentation), however, might still preclude the unveiling of their role in pollinator attraction in many systems.

STAR★METHODS

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

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2020.11.012.

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AUTHOR CONTRIBUTIONS

Conceptualization, P.M.-P., J.B.O., and I.C.M.; Behavioral Designs, P.M.-P.; NMR Experiments and Analysis, M.S. and L.-L.G.; Project Administration, P.M.-P. and I.C.M.; Investigation, P.M.-P., A.D.-M., J.B.O., N.S.L.A., A.C.G.C., S.A.-L., M.F.R.S., A.C.D.M., M.S., and S.D.; Formal Analysis, P.M.-P. and A.D.-M.; Writing – Original Draft, P.M.-P. and A.D.-M.; Writing – Review & Editing, all authors; Funding Acquisition, J.B.O. and I.C.M.; Resources, P.M.-P., D.M.A.F.N., L.-L.G., M.S., S.D., and I.C.M.; Supervision, P.M.-P. and I.C.M.

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		
Cryptanthus burle-marxii plants	This work	Voucher (UFP 46.561) at the Herbarium Geraldo Mariz (UFP), Federal University of Pernambuco.
Eulaema and *Euglossa* bees	This work	Vouchers at the insect collection of the Floral and Reproductive Biology Lab, Federal University of Pernambuco
Chemicals, Peptides, and Recombinant Proteins		
(+)-Copalol	Gundersen Laboratory, University of Oslo	[26]
n-Dodecane (99%)	Sigma-Aldrich	D221104; CAS: 112-40-3
Tenax TA (60-80 mesh)	Sigma-Aldrich	11982
Carbotrap (20-40 mesh)	Sigma-Aldrich	20287
$CDCl_3$ (99.8 atom% D) with 0.03% tetramethylsilane (TMS)	Armar Europe, Leipzig, Germany	013400,2050
n-Hexane (99.5%)	Sigma-Aldrich	32293; CAS: 110-54-3
Software and Algorithms		
Agilent MSD Productivity ChemStation	Agilent Tech.	https://www.agilent.com
Topspin 3.2	Bruker Biospin	https://www.bruker.com/de/service/support-upgrades/software-downloads/nmr.html
SPARKY 3	University of California, San Francisco	https://www.cgl.ucsf.edu/home/sparky/
Statistica v. 7.0	StatSoft, Inc	https://www.statsoft.de
Other		
Membrane pump	Rietschle Thomas	G12/01 EB
Insect tent	Bugdorm	BugDorm-2120
5 mm NMR-Tubes, Type 5TA	Armar Europe, Leipzig, Germany	032100,5045

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Paulo Milet-Pinheiro (paulo.milet@upe.br).

Material availability

This study did not generate any new or unique reagents.

Data and Code Availability

This study did not generate any unique datasets or code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Study sites

The field study was conducted in two Atlantic Forest fragments, situated in the municipality of Ipojuca, NE-Brazil: (1) Reserva Particular do Patrimônio Natural (RPPN) Nossa Senhora do Oiteiro de Maracaípe (08°31'48''S, 35°01'05''W, 12 m a.s.l.) with ca. 75 ha and (2) Mata da Praia do Cupe (8°27'44''S, 34°59'25''W, 6 m a.s.l.) with ca. 12 ha. The climate is Tropical Rainy As'[74] with annual means for temperature and rainfall of 26°C and 1,600 mm, respectively. The rainy season extends from April to September, with most rainfall between May and July. The dry season lasts from October to March, with the driest period between October and December.[75]

Study species

Cryptanthus Otto & A. Dietr. is a genus of terrestrial bromeliads endemic to Brazil. It encompasses ca. 80 species, which occur predominantly in the Atlantic Forest domain.[14] *Cryptanthus burle-marxii* Leme is endemic to northeastern Brazil, listed as vulnerable in the Red List of the Brazilian Flora.[15] It is found exclusively in areas of Restinga, an ecoregion of the Atlantic Rainforest characterized by heterogeneous vegetation composed of herbaceous, shrubby, and arboreous layers growing on sandy plains that typically occur along the coastal zone of northeastern Brazil. Individuals normally form dense populations that grow on the leaf-litter layer under shaded to semi-shaded lighting, at moist and nutrient-rich conditions.

Cryptanthus burle-marxii blooms continuously from April to December, with a flowering peak between early August and late October.[16] Its flowers have three reflexed petals, not more than 3 cm long, that are only basally connate, allowing easy access to nectar in the small chamber, no matter from which direction floral visitors approach. In a classic botanical perspective, this configuration fits the less complex and generalist flower model.[76] *Cryptanthus burle-marxii* and other congenerics are andromonoecious, a derived character among Bromeliaceae that is exclusive to this genus.[10,77] The six stamens of both staminate and hermaphrodite flowers extend from the center of the floral axis, raising the anthers symmetrically toward all directions above the nectariferous chamber. This builds a clear actinomorphic symmetry in male flowers. In hermaphrodite flowers, however, the actinomorphic symmetry observed for male flowers is compromised due to a limp style, which moves awkwardly the conduplicate-patent stigma toward the petals, promoting a directional reverse herkogamy (Figures 2A and 2B; see also[78]).

A voucher specimen was deposited at the Herbarium Geraldo Mariz (UFP), Federal University of Pernambuco (UFP 46.561).

METHOD DETAILS

Nectar measurements

Volume and sugar concentration of nectar were measured in 15 plants (N = 1 flower per individual) using a graduated microsyringe (25 μL Hamilton, NY, USA) and a pocket refractometer (0%–32%; Atago, Tokyo, Japan), respectively. In order to prevent floral visitors to consume nectar, we covered whole plants of *C. burle-marxii* with voile bags before anthesis, at about 0500h. For each flower, we collected nectar twice, at 0830h and 1200h. Data on volume and concentration of nectar at 1200h is not shown, since flowers were empty at this time.

Flower visitors and pollinators

Floral visitors of *C. burle-marxii* were monitored *in situ* by focal observations from flower opening to abscision, i.e., between 0500h and 1400h. For the focal observations, we selected five to ten flowering individuals and recorded all flower visits. In total, we monitored flowers for 240 hours on 27 non-consecutive days at different months of 2014. During observations, we recorded the behavior of visitors on flowers, as well as the resource sought. To determine effective pollinators, the frequency of visiting species, and whether they contacted stigmas and anthers, as well as the flights performed among conspecific plant individuals were considered. For documentation and better description of the behavior of floral visitors, we took photographs using digital cameras. During the field work, in days in which focal observations were not performed, flower-visiting insects were captured with entomological nets, identified in the lab, and deposited in the insect collection of the Floral and Reproductive Biology Lab, at the Federal University of Pernambuco. Hummingbirds were identified by comparing our photographic records with those in the specalized literature.[79]

Collection of flower headspace samples and flower solvent extracts

Flower headspace samples and flower solvent extracts of *C. burle-marxii* were collected for two main purposes: 1) to perform bioassays in the experimental flight cages and 2) to chemically characterize its floral scent bouquet.

Flower headspace samples of *C. burle marxii* (n = 10) were collected using standard dynamic headspace methods.[80,81] Flowers were excised from 10 different plants (one flower per individual) and enclosed within a polyester oven bag (8 × 5 cm; Toppits). The air inside the bag was then trapped for 2 hours in an adsorbent tube, through which air was drawn at a rate of 200 mL min^{-1} using a membrane pump (G12/01 EB, Rietschle Thomas, Puchheim, Germany). The adsorbent tubes consisted of glass tubes (length: 10cm; inner diameter: 4 mm) filled with 50 mg of a 1:1 mixture of Tenax-TA (mesh 60–80; Supelco, Bellefonte, Pennsylvania, USA) and Carbotrap B (mesh 20–40, Supelco, Bellefonte, Pennsylvania, USA), which was held in the tubes using glass wool. Volatiles trapped in the adsorbent tubes were then eluted with 200 μL of hexane (99.5%, Sigma-Aldrich). To control for non-floral (vegetative) volatiles and for volatiles released by tissue damage (since flowers were excised), headspace samples of plant individuals from which flowers had been removed were collected following the same aforementioned methods.

In addition to the solvent flower headspace samples, we collected headspace samples for thermal desorption (n = 10), which is a more sensitive technique for analyzing floral volatile compounds.[21] For this, we followed the same protocol as described above, but used adsorbent tubes for thermal desorption. The adsorbent tubes consisted of ChromatoProbe quartz microvials from Agilent Inc. (length: 20 mm; inner diameter: 2 mm), cut at the closed end and filled with 3 mg of a 1:1 mixture of Tenax-TA (mesh 60–80, Supelco) and Carbotrap (mesh 20–40, Supelco). The mixture was fixed in the tubes by using glass wool.

Besides the headspace samples, we collected flower solvent extracts (n = 19). For this purpose, the petals of five flowers from different individuals (1 or 2 flowers per individual) were inserted for 2 min in a screw cap vial (2 mL; Uniglas) containing 1 mL hexane (99.5%, Sigma-Aldrich). Flower extracts were then concentrated to a volume of 200 μL using a gentle stream of N_2, of which 100 μL

were used for biossays and 100 μL were kept for chemical analyses. For quantification, we mixed 10 ng of n-dodecane (99%, Sigma-Aldrich) as internal standard in each sample.

Flower headspace samples, as well as flower solvent extracts, were stored in screw cap vials that were kept at −20°C until bioassays and/or chemical analyses.

Fractioning of flower solvent extracts

In order to establish the specific component(s) that are responsible for the chemical-gathering behavior displayed by *E. nigrita* males, we collected a more concentrated flower solvent extract, which was first fractioned and then used for both further bioassays and semiochemical elucidaction.

In detail, we immersed ca. 600 flowers of *C. burle-marxii* for 2 min in 20 mL hexane, which was reduced to 2 mL using a gentle stream of N_2. The resulting volume was subjected to thin layer chromatography (20 × 10 cm on silica gel with fluorescent indicator F_{254}) using distilled dichloromethane as eluent, resulting in four fractions (F1 = 0.92, F2 = 0.68, F3′ = 0.35, F3 = 0.30; stained by vanillin/H_2SO_4/ethanol). These fractions were carefully recovered, extracted with distilled hexane (5 mL), and the solvent reduced again to 2 mL under N_2. The resulting samples of each fraction were used in bioassays, analyzed by Gas Chromatography coupled to Mass Spectrometry (GC/MS; see below) and compared against the crude floral extract. F3, which was shown to be behaviorally active, was additionally analyzed by Nuclear Magnetic Resonance (NMR; see below).

Sampling and rearing of bees

The bees used in bioassays were lured in the field with filter paper disks impregnated with skatole, captured with entomological nets, placed in a cool box and transported to the lab, where they were individually marked with paint markers (Edding®, Germany), and finally released in an insect tent (size 60 cm x 60 cm x 60 cm; Bugdorm-2120 Insect Tent; BD2120). The tent was kept within a room at the Department of Botany (Federal University of Pernambuco) at 28 ± 1°C under 70 ± 5% relative humidity and a 14 h photoperiod. Bees were allowed an adaptation phase of at least two days prior to the beginning of bioassays and were fed using artificial flowers filled with sugar water (distilled water with sucrose 25%). The artificial flowers were made of a blue and yellow ethylene-vinyl acetate foam (EVA; ca. 3cm width x 5cm long) and a 2 mL Eppendorf tube.

Flight cage bioassays

The semiochemicals involved in interactions between *E. nigrita* and *C. burle-marxii* were assessed in bioassays, which were performed between September and December 2017 and 2018. In 2017, we tested the behavioral activity of (*i*) flower headspace samples and flower solvent extracts, whereas in 2018 we tested (*iii*) fractions of the extracts and (*iv*) synthetic standard of (+)-copalol that was synthesized according to the procedure reported earlier.[26] In the bioassays, we offered the bees two artificial flowers simultaneously, which were treated as follows: (1) flower headspace sample versus solvent control (hexane) (N = 10); (2) flower solvent extracts versus solvent control (N = 10); (3) fraction 1 (hereafter F1) versus solvent control (N = 10); (4) fraction 2 (F2) versus solvent control (N = 10); (5) fraction 3 (F3′) versus solvent control (N = 10); (6) fraction 4 (F3) versus solvent control (N = 11); (7) F3 versus mixtures of fractions F1 + F2 + F3′ + F3 (MF) (N = 11) and (8) synthetic copalol versus solvent control (N = 11). For biossays (1), (2), (3), (4) and (5) we had 10 replicates, whereas for bioassays (6), (7) and (8) we had 11 replicates. The number of tested bees (i.e., present in the flight cage) varied slightly among experiments (10-20 bees). Due to mortality, we constantly replaced bees to keep the number of test subjects similar across bioassays, so that we tested about 50 different individuals for each dual-choice bioassay.

Each bioassay lasted 20 min. The positions of the paired artificial flowers, which were placed 20 cm apart, were exchanged after 10 min. For bioassays (1) and (2), we applied 100 μL of the samples and 100 μL of solvent (50 μL at the first and 50 at the second part of bioassay). For bioassays 3-6, we applied 20 μL (10 μL in each part) of fractions and of the solvent control, whereas for the bioassay (7) we applied 5 μL of F3 (2.5 μL in each part) and 20 μL of MF (10 μl in each part). For the bioassay (8), we applied 20 μL (10 μL in each part) of synthetic copalol (dissolved in hexane) and of the solvent control. 100 μL of the flower headspace samples were equivalent to the amount of volatile compounds trapped in our headspace samplings, whereas 100 μL of the solvent extracts, as well as 20 μL of the fractions, were equivalent to the chemicals extracted from about 2 flowers. We prepared a solution of copalol in hexane so that aliquots of 20 μL matched the total amount of copalol extracted from about 2 flowers.

The bioassays were recorded using a digital camera (Canon EOS 80D) and analyzed later by a treatment-blind observer. The behavioral responses of the bees were recorded as: 1) approach - flights toward the artificial flowers to a distance $\leq$ 2 cm followed by a short hovering; 2) landing - approaches followed by landings at the artificial flowers; 3) scent gathering - scraping of the scent source with the tarsal brushes. Responses could not be attributed to specific individuals because bees removed the color marking within no more than 2 days of captivity.

The bioassays were conducted on sunny days between 0700 and 1100 h, when the bees were most active. We allowed a resting phase of 10-15 min between bioassays to avoid habituation of bees.

Gas chromatography coupled to mass spectrometry

Flower headspace samples, flower solvent extracts, as well as the fractions obtained from extracts were analyzed on a mass spectrometer (Quadrupole 5975C, Agilent, Palo Alto, CA, USA) coupled to an Agilent gas chromatograph (HP 7890A), equipped with an Agilent J&W non-polar HP-5ms column (30 m x 0.25 mm id.; 0.25 μm film thickness) and a thermal separation probe (TSD; Agilent technologies). Aliquots of the solvent samples (1 μL) were placed in a quartz microvial, which was loaded into the probe and inserted

into the modified GC injector. Samples for thermal desorption were directly loaded into the probe and inserted into the modified GC injector. The GC injector worked at a temperature of 250°C in splitless mode. For flower headspace samples, GC oven temperature was set at 40°C for 2 min, then increased at a rate of 4°C min^{-1} to 230°C, then held steady for 5 min. For flower solvent extracts, GC oven temperature was set at 60°C for 2 min, then increased at a rate of 10°C min^{-1} to 300°C, then held steady for 5 min. Electronic flow control was employed to maintain a constant helium carrier gas flow of 1.0 mL min^{-1}. Helium (He) carrier gas flow was maintained at a constant pressure of 7.0 psi. MS Source and quadrupole temperatures were set at 230°C and 150°C, respectively. Mass spectra were taken at 70 eV (in EI mode) with a scanning speed of 1.0 $scan^{-s}$ from *m/z* 35–350.

Compounds were identified by comparing to those mass spectra and retention indices from commercially available mass spectral libraries (MassFinder 4, NIST11, Adams, and Wiley Registry 9th Edition), integrated to the software Agilent MSD Productivity ChemStation (Agilent Technologies, Palo Alto, USA). Confirmation of the identity of some of the compounds was obtained by comparison of both mass spectrum and GC retention index with those of authentic standards available in our compound collection.

Nuclear magnetic resonance spectroscopy (NMR)

The fraction that elicited chemical-gathering behavior from male *Eulaema nigrita* was analyzed by NMR spectroscopy after evaporating solvent (hexane) using a gentle stream of N_2. NMR spectra of the natural sample were measured on a 600 MHz AVANCE III HD spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a $^{1}H/^{13}C/^{15}N/^{31}P$ QXI probe at 298 K. $CDCl_3$ with 0.03% TMS (Armar, Leipzig, Germany) was used as solvent. The synthetic (+)-copalol was analyzed on Bruker AVII 600 MHz. The 1D ^{13}C spectrum of fraction 3 was measured with 65536 transients, 65536 points and a recycle delay of 2 s. The 2D ^{1}H-^{1}H TOCSY spectra were acquired with a mixing time of 120 ms, or 12 ms (resulting in a COSY-type spectrum) using 4 transients, 2048 × 512 points and a recycle delay of 1 s . Spectra were processed with Topspin 3.2 (Bruker Biospin) and analyzed by Sparky (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco). Spectra were referenced to internal TMS.

Identification of copalol in *Cryptanthus burle-marxii* extracts by 2D NMR spectroscopy

Initial NMR analysis in C_6D_6 of the crude extract revealed a strong component with a spin-system similar to (*Z*)-biformene[22] and copalol[26,82], but since the applied solvent was different to the previously reported NMR data and the aliphatic region displayed an enormous amount of signals of different molecules, a fractionation was inevitable.

Fraction 3 (F3) dissolved in $CDCl_3$ was analyzed in detail by NMR spectroscopy. Both the ^{1}H and the ^{13}C chemical shifts matched the ones reported for copalol, which were measured in the same solvent.[26,82] The one-dimensional spectra were identical to the synthetic compound (Figure 4) as well as the two-dimensional ^{1}H-^{13}C HSQC spectrum (Figures S2A–S2D).

We used standard 2D NMR spectra, COSY, TOCSY, HSQC, and HMBC, to completely assign all ^{1}H and ^{13}C chemical shifts.

The most characteristic signals are the two signals of the olefinic methylene group at C9 that lead to many useful correlations in the TOCSY (Figure S3) and the HMBC (Figure S2F), providing a starting point to assign the ring systems but also the methylpentenol side chain. The other characteristic signals originate from H15 and H14 that are neighbors as indicated by a COSY correlation (Figure S3B) and their ^{13}C correlations (Figure S2) show characteristic chemical shifts, C15 of 59.5 ppm, which is typical for an attached oxygen and C14 of 123.0 ppm that is typical for an olefinic carbon. Via correlations to H16 (Figure S3) and weak correlations to the H12 methylene protons, the terminal part of the molecule can be linked to C12 and C11, which in turn is connected to C9. Linking C9 further with C5, C6, and C7 is straight-forward. Connecting the remaining part of the molecule has to rely on long-range couplings to cross the quaternary carbons C4 and C10. Prominent $^{n}J_{CH}$ long-range couplings are visible from the methyl groups H18, H19, and H20 (Figure S2F), connecting to the already assigned C5 and C9, but also to C1 and C3 so that the second ring can be completely assigned using COSY correlations. Out of the three methyl groups, H18 and H19 show common correlations to C3 and C4 and to each other's carbon, which identifies the $C(CH_3)_2$.

QUANTIFICATION AND STATISTICAL ANALYSIS

The behavioral responses of bees to the paired treatments in the dual-choice bioassays were compared using Wilcoxon Matched Pairs t tests (one test for each behavior category). We did not perform statistical analyses when bees did not display one of the three behaviors to either treatment or control artificial flowers. All statistical analyses were performed in Statistica *v*. 7.0.[83]