Journal of Chemical Ecology (2019) 45:464–473
https://doi.org/10.1007/s10886-019-01072-6

Subtle Chemical Variations with Strong Ecological Significance: Stereoselective Responses of Male Orchid Bees to Stereoisomers of Carvone Epoxide

Katharina Brandt[1] · Stefan Dötterl[2] · Roman Fuchs[2] · Daniela Maria do Amaral Ferraz Navarro[3] · Isabel Cristina Sobreira Machado[4] · Daniel Dobler[5] · Oliver Reiser[5] · Manfred Ayasse[1] · Paulo Milet-Pinheiro[4]

Received: 17 January 2019 / Revised: 27 March 2019 / Accepted: 26 April 2019 / Published online: 21 May 2019

Abstract

Different enantiomers of chiral compounds within floral perfumes usually trigger distinct responses in insects; however, this has frequently been neglected in studies investigating semiochemicals in plant-pollinator interactions. Approximately 1000 neotropical plants produce floral perfumes as the only reward for pollinators, i.e. male euglossine bees. The chiral compound carvone epoxide is a key component of the scent bouquet of many perfume-rewarding plants that are pollinated by males of *Eulaema*. Here, we tested the biological activity of the four carvone epoxide stereoisomers to four *Eulaema* species occurring in the Atlantic Rainforest of NE-Brazil. We determined the stereochemistry of carvone epoxide in the floral scent of several *Catasetum* species, tested whether the antennae of bees respond differentially to these stereoisomers and investigated if there is a behavioural preference for any of the stereoisomers. We found that 1) *Catasetum* species emit only the (−)-*trans*-stereoisomer of carvone epoxide, 2) for *E. atleticana* and *E. niveofasciata* antennal responses to the (−)-*trans*-carvone epoxide were significantly stronger than those to (−)-*cis*-carvone epoxide, 3) the strength and pattern of antennal responses to all 4 stereoisomers (separately tested) did not differ among *Eulaema* species, and 4) there were significant differences in attractiveness of the four stereoisomers to the bees species with the (−)-*trans*-stereoisomer being particularly attractive. We assume (−)-*trans*-carvone epoxide to be the dominant isomer in perfume-rewarding plants pollinated by *Eulaema*. The universal occurrence of carvone epoxide in *Catasetum* species pollinated by *Eulaema*, suggests that this compound has evolved in perfume-rewarding as a specific attractant for *Eulaema* bees as pollinators.

Keywords *Catasetum* · Chirality · Electrophysiology · *Eulaema* · Euglossine bees · Perfume-rewarding plants · Pollinators

Introduction

The biological function of an organic compound is determined by its properties, such as chain length, functional groups, and stereochemistry (König and Hochmuth 2004). Floral scents are often complex blends of volatile organic compounds (VOCs), of which many are chiral (e.g. Williams and Whitten 1983). Chiral compounds have rigid spatial arrangements of atoms that contain no mirror plane, or no centre of inversion (i.e. no rotation-reflection axis), and thus occur in two non-superimposable mirror-image forms (enantiomers; Moss 1996). Stereoisomers are characterized by differences in physicochemical properties and usually trigger distinct biological responses in insects (e.g. Ayasse et al. 2003; Dopman et al. 2010; Erbilgin and Raffka 2000; Linn et al. 2003; Teale et al. 1994). This is especially true for enantiomers, which only differ in physical properties, i.e., their optical rotation.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10886-019-01072-6) contains supplementary material, which is available to authorized users.

✉ Paulo Milet-Pinheiro
miletpinheiro@hotmail.com

[1] Institute of Evolutionary Ecology and Conservation Genomics, University of Ulm, Ulm, Germany

[2] Department of Biosciences, University of Salzburg, Salzburg, Austria

[3] Departamento de Química Fundamental, Universidade Federal de Pernambuco, Recife, Brazil

[4] Departamento de Botânica, Universidade Federal de Pernambuco, Recife, Brazil

[5] Institute of Organic Chemistry, University of Regensburg, Regensburg, Germany

However, most studies that investigate the chemical communication by olfactory cues between plants and their pollinators do neither elucidate the absolute configuration of chiral floral scent compounds nor study their potentially differential role in attracting pollinators (Dötterl et al. 2006; Gerlach and Schill 1991; Hills et al. 1972; Milet-Pinheiro and Gerlach 2017; Schneider et al. 2013; Williams and Whitten 1983). This is also true for perfume-rewarding plants, which are pollinated by male euglossine bees, even if the significance of chirality in these associations has long been outlined (Eltz et al. 1999; Williams and Whitten 1983).

The production of floral perfumes by certain neotropical plants as reward for male euglossine bee pollinators was first recognized by Vogel (1966). His findings led other contemporaneous scientists to investigate the chemistry of floral perfumes in a myriad of euglossine-pollinated plants, which culminated in the identification of many compounds new to science. In 1985, for example, Lindquist et al. (1985) isolated and described the monoterpenoid (2*S*,5*R*)-(−)-2,3-epoxy-5-isopropenyl-2-methyl-cyclohexanone, also known as (−)-*trans*-carvone epoxide, from the floral fragrance of *Catasetum maculatum*. Later, Whitten et al. (1986) stated that this compound is the major component in the floral fragrance of taxonomically diverse angiosperms (several genera of Orchidaceae and one species of *Dalechampia*, Euphorbiaceae) and is a potent attractant for male euglossine pollinators, mainly of the genus *Eulaema*. Since then, *trans*-carvone epoxide, without information about its absolute configuration, has repeatedly been reported in perfume-rewarding plants of many different genera and families (*Dalechampia*, Euphorbiaceae; Armbruster 1989; several genera of Orchidaceae, Kaiser 1993, 2011; *Gloxinia*, Gesneriaceae; Martel et al. 2019; *Anthurium*, Araceae; Schwerdtfeger et al. 2002; *Unonopsis*, Annonaceae; Teichert et al. 2009). Interestingly, it has never been reported as floral scent constituent in non-perfume-rewarding plants (Knudsen et al. 2006). In perfume-rewarding plants, *trans*-carvone epoxide sometimes co-occurs with its diastereoisomer *cis*-carvone epoxide (Kaiser 1993, 2011; Schwerdtfeger et al. 2002). Carvone epoxide is a complex molecule that has three chiral centers. However, because of the epoxide group, only two diastereomers are possible: For each diastereoisomer of carvone epoxide, there are two possible enantiomers, i.e. (1*S*,4*R*,6*S*)-1-methyl-4-(prop-1-en-2-yl)-7-oxabicyclo[4.1.0]heptan-2-one [hereafter (−)-*trans*-carvone epoxide] and (1*R*,4*S*,6*R*)-1-methyl-4-(prop-1-en-2-yl)-7-oxabicyclo[4.1.0]heptan-2-one [(+)-*trans*-carvone epoxide], and (1*S*,4*S*,6*S*)-1-methyl-4-(prop-1-en-2-yl)-7-oxabicyclo[4.1.0]heptan-2-one [(−)-*cis*-carvone epoxide] and (1*R*,4*R*,6*R*)-1-methyl-4-(prop-1-en-2-yl)-7-oxabicyclo[4.1.0]heptan-2-one [(+)-*cis*-carvone epoxide] (Fig. 1).

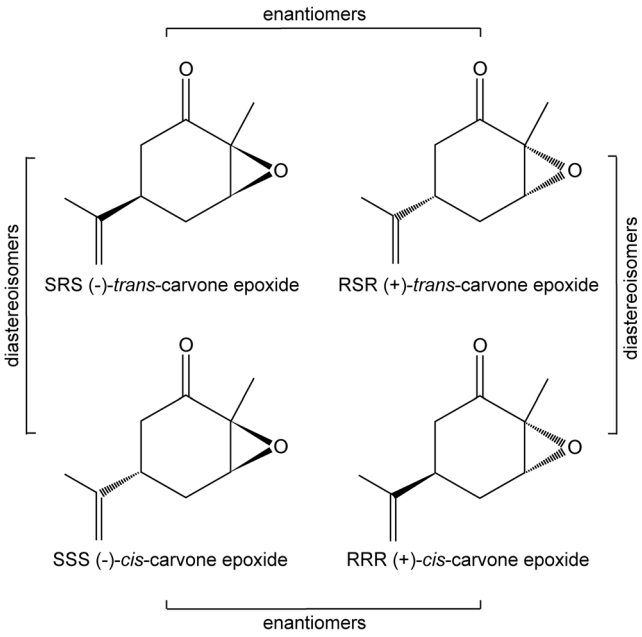


Fig. 1 The four different stereoisomers of carvone epoxide

To our best knowledge, there are so far only two studies testing whether stereoisomers of floral compounds play a distinct role in the attraction of male euglossine bees. In the first study, Williams and Whitten (1983) found that male *Eulaema nigrita* bees are attracted to (−)-α-pinene, while (+)-α–pinene has a repellent effect. In the second study, Schorkopf et al. (2011) showed that the (−)-isomer of ipsdienol triggers stronger antennal responses and attracted more males of *Euglossa cyanura* than its optical isomer or a racemic mixture. Common theories suggest that male euglossine bees use perfumes to attract con-specific females and species-specific perfume blends are assumed to act as pre-mating reproductive barrier among sympatric species (Weber et al. 2016; Zimmermann et al. 2009). Chirality, therefore, may act as an effective reproductive barrier, if different species occurring in the same habitat show distinct preference for one stereoisomer over the other(s).

Catasetum Rich. Ex Kunth. is the most speciose genus of the subtribe Catasetinae (Orchidaceae), embracing about 170 species (Govaerts et al. 2018). In a recent review on floral scent chemistry and pollinators of *Catasetum*, Milet-Pinheiro and Gerlach (2017) confirmed *trans*-carvone epoxide as a main component of the floral scent of many species pollinated by bees of the genus *Eulaema*. However, the absolute configuration of this compound in most species, as well as the possible stereoselective attraction of different *Eulaema* species, remains unknown. In the present study, we synthesized all four carvone epoxide stereoisomers and tested them in electrophysiological and biological assays with *Eulaema* species. In order to put our findings into an ecological context, we

determined the stereochemistry of carvone epoxide in the floral scent of several *Catasetum* species pollinated by *Eulaema*. Our aim was to explore whether males of different *Eulaema* species can distinguish between different stereoisomers of carvone epoxide in electrophysiological analyses and in the field assays. Specifically, we addressed the following questions: 1) Which stereoisomer of carvone epoxide is emitted by five different *Eulaema*-pollinated species of the genus *Catasetum*? 2) Does strength of antennal responses to the four stereoisomers differ within and among species? 3) Do bees prefer a specific stereoisomer in behavioural field assays?

Material and Methods

Plant Species and Collection of Headspace Samples Floral volatiles of five *Catasetum* species [*C. discolor* Lindl. 1844 ($N = 2$), *C. gardneri* Schltr. 1914 ($N = 6$), *C. macrocarpum* Rich. Ex Kunth 1822 ($N = 5$), *C. osculatum* Lacerda & P. Castro 1995 ($N = 3$), and *C. schmidtianum* Miranda & Lacerda 1992 ($N = 1$)] were collected using dynamic headspace methods (Dötterl et al. 2005). Therefore, inflorescences were enclosed in a polyester oven bag (Toppits®) and the floral scent was trapped for 5 h in an adsorbent tube [glass vials; 15 mg Tenax-TA (mesh 60–80) and 15 mg Carbotrap B (mesh 20–40) fixed using glass wool] using a membrane pump (G12/ 01 EB, Rietschle Thomas, Puchheim, Germany). The flow rate was adjusted to 200 mL/min. Volatiles trapped in these adsorbent tubes were eluted with 200 μL *n*-hexane (99.5%, Merck). To detect ambient contaminants, negative controls (empty bags; $N = 1$ for each species) were collected by using the aforementioned methods. All headspace samples were stored in 2 mL screw cap vials at −20 °C until the chemical analyses.

Synthesis of Carvone Epoxide Stereoisomers The carvone epoxide stereoisomers were prepared from (*S*)- or (*R*)-carvone according to procedures reported in the literature (Garver et al. 1976; Takita et al. 2011; Wang et al. 2006; Yasuda et al. 1979). (*R*)-(−)-carvone (98%) and (*S*)-(+)-Carvone (90%) were purchased from Sigma-Aldrich but were not pure. The latter explains the lower enantiopurity of the (+)-*trans*- (92%) and the (−)-*cis*- (85%) epoxides obtained in their synthesis as determined by chiral gas chromatography (Table 1, see also below). Further information on NMR spectra of the synthesized carvone epoxides is included in the supplementary material (see SM1, SM2, SM3, and SM4).

Chiral Gas Chromatography Chiral GC-FID (flame ionization detector) was used to elucidate the isomeric composition of carvone epoxide in headspace samples of the five *Catasetum* species and in the synthesized samples (see above). All five plant species are pollinated by male *Eulaema* bees and have the typical caraway note of species that have carvone epoxide as main constituent in their floral scent bouquet (Kaiser 2011). The headspace samples and the synthetic isomers of carvone epoxide were analyzed on an Agilent 7890A gas chromatograph (Santa Clara, USA) equipped with a fused silica capillary column (30 m × 0.23 mm i.d.), coated with a 0.23 μm film of 0,4% heptakis(2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)-beta-cyclodextrin (DIME-beta-CD) (30%) in SE-52 (70%), the same as described in (Dötterl et al. 2006). Aliquots of 1 μL were injected in splitless mode in a split/splitless injector (Agilent) with temperature set at 250 °C. The temperature of the GC oven was increased from 40 °C to 200 °C at a heating rate of 20 °C per minute. Hydrogen was used as the carrier gas with a constant flow rate of 3 mL/min. The synthetic isomers were injected alone and as a mixture, and the natural samples were injected alone or co-injected with the mixture of the synthetic isomers.

Bee Species In this study, we tested four different *Eulaema* species known to occur in the Atlantic Rainforest of Pernambuco State, Northeastern Brazil, i.e. *E. atleticana* Nemésio, 2009, *E. marcii* Nemésio, 2009, *E. nigrita* Lepeletier, 1841, and *E. niveofasciata* Friese, 1899. For all species, 12 antennae of different males were tested. Bees were obtained on scent baits [i.e. filter papers impregnated with 30 μL eucalyptol (99%; Merck), benzyl acetate (≥99%; Merck), eugenol (≥98%; Merck), methyl salicylate (≥99%; Merck) or skatole (98%; Merck)], from which they were collected with entomological nets. They were placed in glass vials and kept inside a cooled box until electrophysiological analyses. All bees were collected at the surroundings of the

Table 1 Isomeric composition of synthesized carvone epoxides according to chiral gas chromatography

Percentage	(−)-*trans*- (%)	(+)-*trans*- (%)	(−)-*cis*- (%)	(+)-*cis*- (%)
Sample "(−)-*trans*-"	100	0	0	0
Sample "(+)-*trans*-"	8	92	0	0
Sample "(−)-*cis*-"	0	5	85	10
Sample "(+)-*cis*-"	7	0	0	93

"Mata dos Brennands" (10 m a.s.l; 8°02′30.5"S, 34°57′ 54.1"W), municipality of Recife (Pernambuco State - Northeastern Brazil). Sampling of bees was authorized by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) of the Ministério Brasileiro do Meio Ambiente (permit number 53545–1).

Electroantennographic Measurements To test if the peripheral olfactory circuitry of the four tested *Eulaema* species differently responds to the carvone epoxide stereoisomers, we used electroantennographic analyses (EAG). For each tested bee individual, one of the antennae was excised at the scape using micro-scissors (Castroviejo, Fine Science tools; 69,121 Heidelberg, Germany). Using a stereomicroscope (Stemi 2000-CS, ZEISS, Oberkochen, Germany) and a razor blade, the excised antenna was cut at the tip (last segment of flagellum) and at the base (first segment of flagellum). The excised antenna was mounted between two glass capillaries that were filled with insect Ringer solution (1 L aqua demin containing 5 g of NaCl, 0.42 g of KCl and 0.19 g of CaCl +2H2O) and connected to silver-electrodes. The electrode connected with the base of the antenna was grounded, while the electrode that was connected to the tip transmitted changes of the potential within the antenna to a signal acquisition controller (IDAC-2 Signal acquisition controller; Syntech, Hilversum, the Netherlands). The antennal preparation was placed in front of a glass tube, through which a constant humidified airflow (25 mL/min) was blown, in order to prevent dehydration and to transfer test compounds to the antenna (hereafter scent stimuli).

We applied the scent stimuli to each antennal preparation in constant order [(−)-*trans*-carvone epoxide, (+)-*trans*-carvone epoxide, (−)-*cis*-carvone epoxide and (+)-*cis*-carvone epoxide], each of them in four different ascending concentrations (10^{-8}, 10^{-6}, 10^{-4}, 10^{-2}, *v*/v, in *n*-hexane 99%, Merck), starting and ending with *n*-hexane as negative control.

Because prolonged or repetitive stimulation can lead to decreased antennal responses (Strausfeld and Kaissling 1986), we allowed a resting phase of 90 s between stimuli. For each stimulus, a v-shaped strip of filter paper (0.25 cm^2; VWR International, Darmstadt, Germany) was inserted in a Pasteur pipette (15 cm, VWR International, Darmstadt, Germany). The filter paper inside the Pasteur pipette was impregnated with 10 μL of the respective testing solution. Before connecting the Pasteur pipettes with the stimuli to a stimulus controller (CS-05; Syntech, Hilversum, the Netherlands), *n*-hexane was allowed to evaporate for 1 min. For measurements, air puffs were delivered through the Pasteur pipettes onto the antenna via this stimulus controller (30 ms, with a pulse flow of 25 mL/min). Antennal responses were recorded by Syntech EAG software (EAGPro, v. 2.1; Hilversum, The Netherlands).

We further tested solutions of eugenol and benzyl alcohol in a concentration of 10 μL/mL (10^{-2}) as positive control. Both substances are well known attractants to euglossine bees (Ramírez et al. 2002). Absolute antennal responses to these substances did not differ among the four tested bee species (PERMANOVA: pseudo-$F_{12, 239} = 0.35$, $P = 1$).

Field Assays The attractiveness of the four carvone epoxides (10^{-2} in *n*-hexane, *v*/v) to the different *Eulaema* species (*E. atleticana, E. marcii, E. nigrita, E. niveofasciata*) was tested in field assays at the same localities where we caught bees for electrophysiological measurements. We tested the compounds individually against *n*-hexane from 06:00 to 10:00 h within the time interval in which *Eulaema* bees are most active at our study site (Milet-Pinheiro, pers. obs.). Field assays were performed on 12 different non-consecutive days ($N = 24$ for each test compound). Each of the four carvone epoxides and the solvent control *n*-hexane were tested twice a day for periods of 30 min. To exclude possible influence of temporal variation in foraging activity of bees, we tested the four compounds in different orders. Overall, the four compounds were tested equally among different time periods.

In the field assays, we attached filter papers (size 5 × 5 cm) to a tree using a pin at a height of approximately 180 cm. We used a pipette (LabMate Soft, 10–100 μL; PZ HTL S.A, Warszawa, Poland) to apply 30 μL of each test solution on a filter paper at the beginning of the assay and again 15 min later. Each compound was tested on different trees to avoid a possible learning effect by the bee individuals. The scent emission of 30 μL of the tested isomers corresponded to the mean scent emission of inflorescences of *Eulaema*-pollinated plant species (*Catasetum discolor, C. gardneri*, *C. macrocarpum*, *C. osculatum*, *C. schmidtianum,* Brandt et al., unpublished). If several approaches / landings were obviously from a single bee individual, it was counted as the same response. We did not collect bees during assays to avoid interference in responses by other bees. Thus, it is possible that one single bee individual has responded more than once if it has left the range of vision to come back later during assays. However, considering the frequency of visits and the size of the study field, as well as the large sample size, we believe that eventual repetitive responses by any bee individual might not have biased our results.

Statistical Analyses

Absolute EAG Responses To test for differences in EAG responses among the tested bee species, we first normalized the EAG responses against *n*-hexane using the option provided in the Syntech software. Thereby, we corrected for a change in antennal sensitivity during measurements. These normalized

EAG responses are in the following referred to as "absolute EAG responses". Absolute EAG responses were log-transformed (to achieve normality of residuals and reduce heteroscedasticity). Using these responses, we calculated univariate Euclidean distance matrices (for each compound individually) and performed multifactorial general linear mixed model analyses (LMM) [random factors: individual (nested in bee species), fixed factors: bee species and stereoisomer, covariate: stimulus concentration]. We tested for differences among the four different concentrations and for differences in comparison to *n*-hexane within each species (within-species comparison for a concentration effect). With this analysis strategy on data derived from repeated measurements, any effects on signal amplitudes due to individual antenna length could be corrected statistically. Based on these LMM models, further posterior comparisons were calculated (i.e. pairwise tests).

Relative EAG Responses We standardized the absolute EAG responses in relation to responses to the reference substances (i.e. eugenol and benzyl alcohol). The relative response (%) to a carvone epoxide stereoisomer in four different concentrations was calculated as the absolute EAG response of an individual divided by the mean response of the same individual to the two reference substances. This standardization was necessary since absolute EAG responses are influenced by the length of the antenna (Nagai 1981; Roelofs 1984), which differed between the four tested bee species.

To test whether (1) a single bee species responds differently to the four stereoisomers and (2) different bee species respond differently to the four stereoisomers, we followed the procedure of Brandt et al. (2017). After standardization of responses, we used the multiple measurements of an individual (antenna) to the different concentrations of a specific compound and calculated a linear regression line using R (v 3.5.1). The intercept of this line was extracted and represents the position where the regression line crosses the Y-axis. This value is a measure for the overall strength of responses. Using the intercept instead of absolute responses, allows us to avoid repetitive statistical analyses for each of the four different concentrations. These individual intercepts were analyzed with a linear mixed model using species, stereoisomers and their interaction as fixed factors and individuals as random factor.

All procedures concerning linear mixed models (LMM) were performed with R (v3.5.1; R Core Team 2018) and some of its additional packages. Here 'lme4' (v. 1.1.18; Bates et al. 2014) and 'lmerTest' (v. 3.0.1; Kuznetsova et al. 2017) were used for designing, calculating and testing overall effects of linear mixed effect models, 'emmeans' (v. 1.2.4; Lenth 2018), and 'multcomp' (v. 1.4.8; Hothorn et al. 2008) for further post-hoc tests and pairwise comparisons.

Field Assays To test for differences in the number of visits by a specific bee species to the four stereoisomers, we calculated a pairwise Euclidean distance matrix and performed a two-factorial PERMANOVA analysis (factors: stereoisomer and bee species) followed by post-hoc analyses.

PERMANOVA is a technique for testing the simultaneous response of one or more variables to one or more factors in an

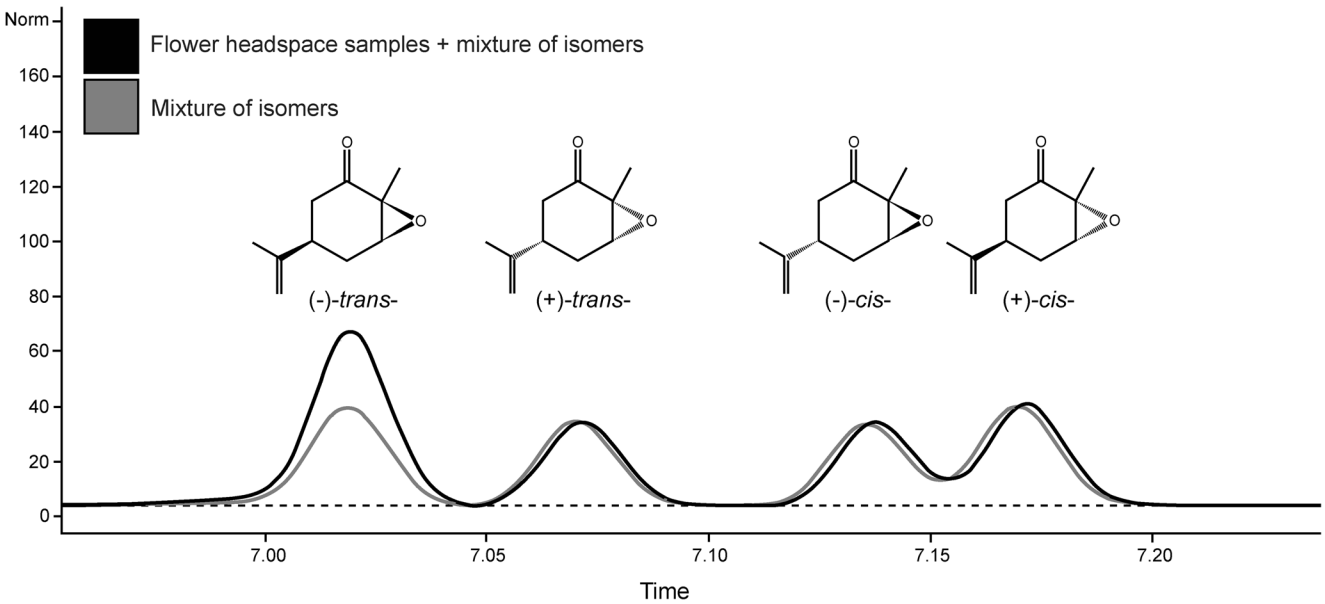
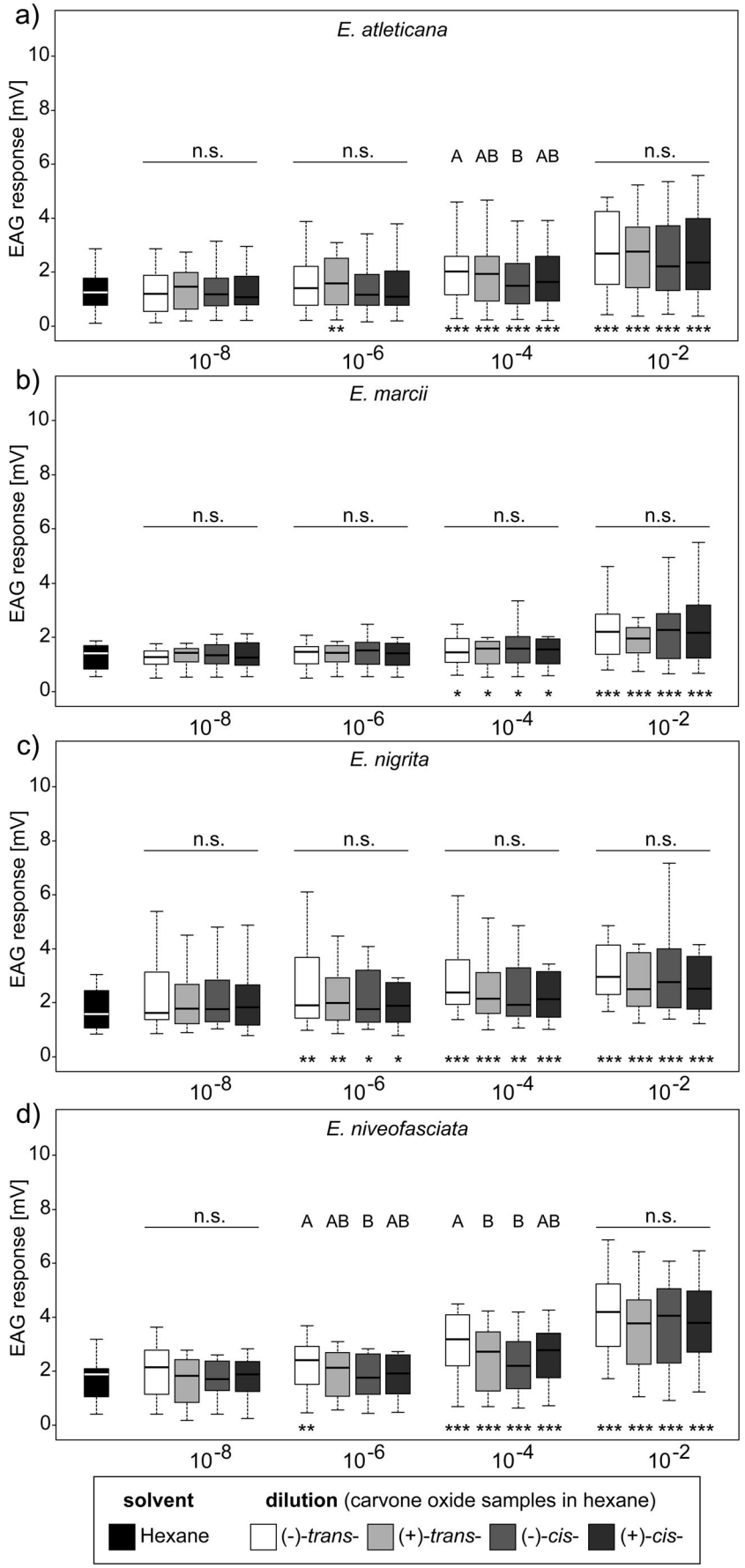


Fig. 2 Chromatograms of a synthetic mixture of the four carvone epoxide isomers ((−)-*trans*-, (+)-*trans*-, (−)-*cis*-, and (+)-*cis*-; grey curve) and the co-injection of this mixture with a flower headspace sample of *Catasetum macrocarpum* (black curve)


a)
E. atleticana
b)
E. marcii
c)
E. nigrita
d)
E. niveofasciata
EAG response [mV]
n.s.
A AB B AB
A B B AB
10^-8
10^-6
10^-4
10^-2
solvent
Hexane
dilution (carvone oxide samples in hexane)
(-)-trans-
(+)-trans-
(-)-cis-
(+)-cis-

◀ **Fig. 3** Absolute EAG responses of male bees of a) *E. atleticana*, b) *E. marcii*, c) *E. nigrita* and d) *E. niveofasciata* ($N = 12$ each) to different concentrations of the four carvone epoxides and to the control *n*-hexane (%; Median, 25% - 75%, confidence interval of 95%). Asterisks indicate significant differences when compared to *n*-hexane (linear mixed model; * / ** / *** = $P \leq 0.05$ / 0.01 / 0.001). Different letters indicate significant differences between tested stereoisomers within a concentration

ANOVA experimental design based on a similarity/distance matrix with permutation methods (Anderson et al. 2008). The PERMANOVA and the Euclidean resemblance matrices were calculated in PRIMER 6 (version 6.1.15; PRIMER-E Ltd. 2012) in combination with the add-on PERMANOVA + (version 1.0.5; PRIMER-E Ltd. 2012).

Results

Chemical Configuration of Carvone Epoxide in Catasetum Spp. Co-injections of the synthetic stereoisomers of carvone epoxide with flower headspace samples revealed that all five *Catasetum* species, i.e., *C. discolor*, *C. gardneri*, *C. macrocarpum*, *C. osculatum*, and *C. schmidtianum* emit only the (−)-*trans*- stereoisomer of carvone epoxide (Fig. 2).

Electroantennographic Measurements

Absolute EAG Responses All four stereoisomers of carvone epoxide elicited positive antennal responses in the four species (i.e. *E. nigrita*, *E. niveofasciata*, *E. atleticana,* and *E. marcii*) with the highest responses found towards (−)-*trans*-carvone epoxide (LMM substance differences: $F_{3,657} = 8.98$, $P < 0.001$). Depending on species and stereoisomer, positive responses were detected either at the highest concentrations of the stereoisomers tested (10^{-4} and 10^{-2}) or additionally at a lower concentration of 10^{-6}. There were no positive responses to concentrations lower than 10^{-6} in any tested species (Fig. 3). Within *E. atleticana* and *E. niveofasciata*, but not within the other species, there were differences among the four stereoisomers at a given concentration (LMM overall differences species x substance: $F_{9/657} = 4.47$, $P < 0.001$); see also Fig. 3). In particular, for these two species antennal responses to the (−)-*trans*-carvone epoxide were significantly stronger than those to (−)-*cis*-carvone epoxide.

Relative EAG Responses The overall strength (intercept of regression lines) of antennal responses to the different stereoisomers was similar among all bee species (main test LMM species x substance: $F_{9,132} = 1.86$, $P = 0.057$). However, antennal sensitivity of *Eulaema* species to each stereoisomer of carvone epoxide differed significantly (main test LMM substance: $F_{3,132} = 7.38$, $P < 0.001$; Fig. 4). In *E. nigrita* only, the responses to (−)-*trans*-carvone epoxide were stronger than to the other tested stereoisomers.

Field Assays In total 235 euglossine males were attracted by carvone epoxides and no bee responded to the solvent control *n*-hexane. Beside euglossine bees, no other insect responded to the compounds. Among the four *Eulaema* species occurring in the study area, only *E. marcii* was not attracted by any of the stereoisomers (Fig. 5). For the other three tested bee species, the attractiveness differed significantly among the stereoisomers (*PERMANOVA:* pseudo-$F_{12,479}$: 11.68, $P < 0.001$). Both *trans*-carvone epoxide enantiomers always attracted more bees than the solvent control, with the (−)-*trans*-stereoisomer being more attractive than the (+)-*trans*-stereoisomer in two of the species (*E. atleticana*, *E. nigrita*). With the exception of *E. nigrita,* none of the *cis*-carvone epoxide enantiomers attracted more bees than the solvent control (Fig. 5).

Discussion

In our study, we found that 1) all tested *Catasetum* species emit only the (−)-*trans*-stereoisomer of carvone epoxide, 2) within *E. atleticana* and *E. niveofasciata* antennal responses to the (−)-*trans*-carvone epoxide were significantly stronger than those to (−)-*cis*-carvone epoxide, 3) the strength and pattern of antennal responses to all 4 stereoisomers (separately tested) did not differ among *Eulaema* species, and 4) there were significant differences in attractiveness of the four stereoisomers of carvone epoxide to the bee species with the (−)-*trans*-stereoisomer being particularly attractive.

Our enantioselective analyses confirm previous studies (Lindquist et al. 1985, Whitten et al. (1986) by showing that the (−)-*trans*-stereoisomer of carvone epoxide is a key component in the scent profile of perfume-rewarding plants. The absence of relevant variation in the isomeric pattern of carvone epoxide among such plants suggests that reproductive isolation by selective attraction of euglossine bees is not mediated by different isomeric patterns of this compound. Instead, plants with carvone epoxide in their profile also seem to selectively attract their specific euglossine pollinators by complex blends of volatiles consisting of a few major compounds and a myriad of minor constituents, as suggested for other plant species (Dodson et al. 1969; Whitten et al. 1986). Indeed, plants producing carvone epoxide also release a high number of other components, including chiral ones (see e.g. Kaiser 1993; Milet-Pinheiro et al. 2015b; Whitten et al. 1988; Williams and Whitten 1983).

The emission of (−)-*trans*-carvone epoxide is commonly found within several genera (including *Catasetum*) of different plant families and is considered to be a result of evolutionary convergence in floral reward of perfume-rewarding plants

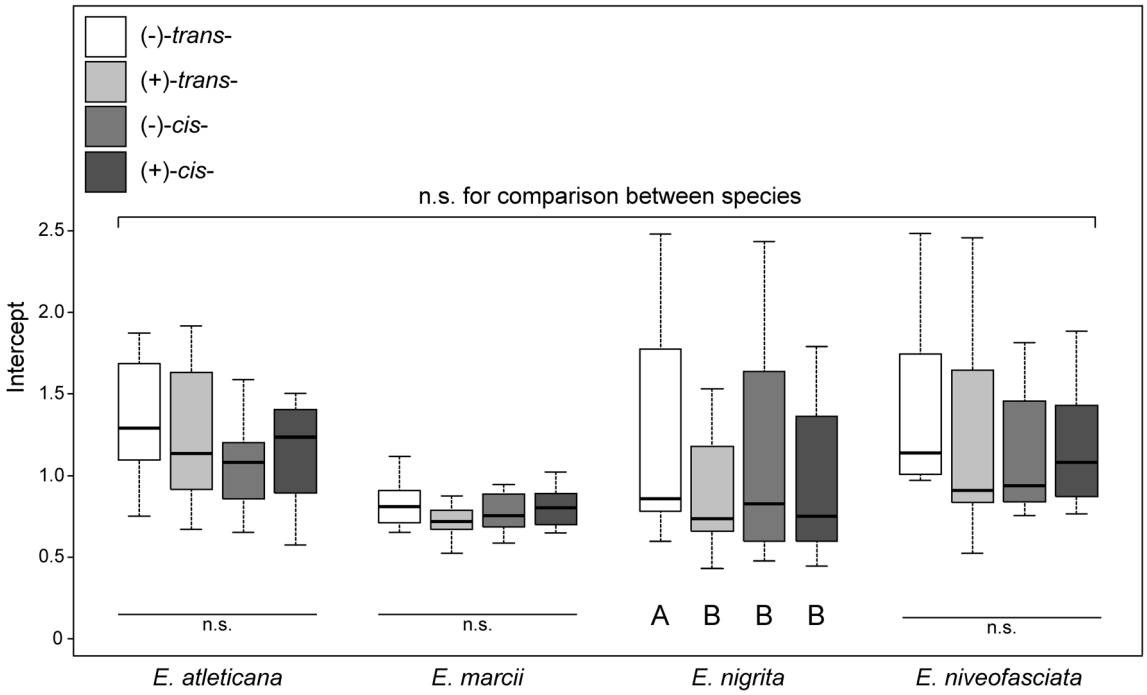


Fig. 4 Intercept of regression lines (strengths of responses) of standardized EAG responses for tested bee species (Median, 25% - 75%, confidence interval of 95%). Different letters indicate significant differences among tested stereoisomers within a species

(Whitten et al. 1986). This underlines the function of (−)-*trans*-carvone epoxide as main attractive signal for a selective subset of euglossine visitors (Whitten et al. 1986) and its possible role as a private communication channel for euglossine bees (Raguso 2008; Whitten et al. 1986). This is supported by our finding that only euglossine males are attracted by carvone epoxides. Although we observed a few *Euglossa* individuals on scent baits with carvone epoxide [mainly on the (−)-*trans*] in the field assays, the universal presence of this compound in floral perfumes of *Catasetum* species pollinated by *Eulaema*, as well as its absence in floral perfumes of *Euglossa*-pollinated species (Milet-Pinheiro and Gerlach

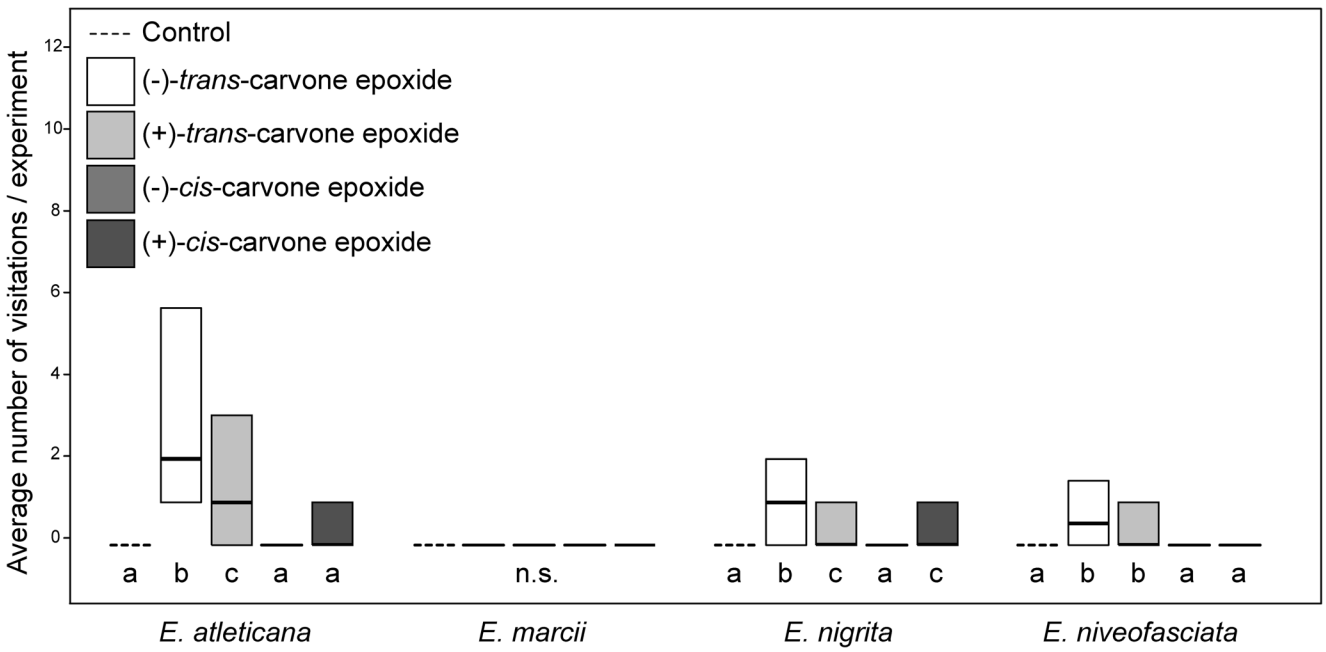


Fig. 5 Number of visits to the four carvone epoxides and the solvent control (Median, 25% - 75%). Different letters indicate significant differences in visits within bee species to the four different stereoisomers and to the solvent control. "n.s" indicates that *E. marcii* responded similarly to the different stereoisomer of carvone epoxide and the solvent control

2017), suggest that this compound evolved as a specific attractant of *Eulaema* bees as pollinators.

In comparison with other specialist bees, e.g. oligoleges, that are able to perceive components involved in host finding at concentrations as low as 10^{-8} (see e.g. Brandt et al. 2017), the antennae of *Eulaema* bees to carvone epoxides do not seem to be that sensitive. Though comparisons of sensitivity among bees to different compounds in EAG measurements have to be considered with caution, it is interesting that the volatile host recognition cues of oligolectic bees are only produced in trace amounts (Milet-Pinheiro et al. 2015a), whereas carvone epoxide is typically produced in high amounts by *Eulaema*-pollinated perfume-producing plants (Armbruster 1989; Milet-Pinheiro and Gerlach 2017; Schwerdtfeger et al. 2002; Whitten et al. 1986). Therefore, a high sensitivity to carvone epoxide might not be required for effective communication between pollinators of the genus *Eulaema* and their perfume sources.

One interesting aspect in our study was the fact that male bees of the species *E. marcii* were not attracted by any of the tested carvone epoxide stereoisomers in the field assays, although the electroantennographic measurements revealed that they are able to perceive these compounds starting at a concentration of 10^{-4}. These findings illustrate that antennal sensitivity is not always correlated with the attractiveness of a compound (see e.g. Gemeno et al. 2003; Jhumur et al. 2007). In contrast to what we observed for *E. marcii*, the results for the other three tested bee species (*E. atleticana*, *E. nigrita* and *E. niveofasciata*) demonstrate a relationship between antennal detection of the different carvone epoxide stereoisomers and their attractiveness to bees. Both the antennal responses and the results of the behavioral field assays point to a preference for (−)-*trans*-carvone epoxide over the other tested stereoisomers. This preference might be even more pronounced, because the purity of the different carvone epoxides we tested was different. If bees were indeed more sensitive to (−)-*trans*-carvone epoxide, than responses to (+)-*trans* and (+)-*cis* isomers, which contained about 7–8% of the (−)-*trans* isomer (see Table 1), might generally have been amplified. In the case of the (−)-*cis*- isomer, which did not contain (−)-*trans*-carvone epoxide, significant antennal and behavioral differences within a tested species were always found in relation to the (−)-*trans*-carvone epoxide. Irrespective of this potential bias, our findings of the antennal responses and the field assays are in agreement with the study of Schorkopf et al. (2011), who demonstrated that antennal sensitivity to ipsdienol enantiomers correlate with its attractiveness. More precisely, male *Euglossa cyanura* did not only exhibit a behavioral preference for (−)-ipsdienol, but the antennae also showed stronger electroantennographic responses to the (−)- than to the (+)-isomer. Furthermore, the findings of our electroantennographic measurements and the results of our field assays suggest that *Eulaema atleticana*, *E. nigrita*, and *E. niveofasciata* possess different olfactory receptors for detecting distinct stereoisomers. If bees would have just one receptor for detecting the various carvone epoxides, we would have expected that their attractiveness is the same. In other plant-insect interactions where chiral compounds are involved, there are examples for both cases, i.e. different stereoisomers are detected by the same or different receptor(s) (e.g. Schneider et al. 2013).

Altogether our findings suggest that (−)-*trans*-carvone epoxide is the key stereoisomer in *Eulaema*-pollinated plant species releasing carvone epoxide. It is very likely that this stereoisomer is responsible for the attraction of various, but not all *Eulaema* species. Stereoisomeric patterns of this compound are very similar within *Catasetum* meaning that plants do not split niches by emitting different enantiomers of carvone epoxide. Therefore, reproductive isolation among species of this genus (and other perfume-rewarding plants) might rather be acquired through other isolating mechanism, such as different blooming times, geographical distribution and specific placement of pollinarium at pollinators' body (Hills et al. 1972). Future investigations elucidating the absolute configuration of other chiral compounds and testing the selective attractiveness of different stereoisomers/stereoisomeric patterns to euglossine bees might reveal whether stereochemistry is involved in reproductive isolation of perfume-rewarding plants.

Acknowledgements We thank Tiago César and Juan Fernández Gómez for providing *Catasetum* plants for sampling of floral perfumes and Hans Saegesser Santos for performing preliminary EAG analyses. This work was supported by the Deutsche Forschungsgemeinschaft (AY 12/12-1), the Deutscher Akademischer Austauschdienst (DAAD grant number 57210526), Coordenação de Aperfeiçoamento de Nível Superior (CAPES, Brazil - Finance Code 001 - grant to P.M.P.), Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (grant number FACEPE/BCT - 0288-2.05/17 to P.M.P.), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (grant number CNPq/PDJ - 152077/2016-2 to P.M.P).

References

Anderson MJ, Gorley RN, Clarke KR (2008) PERMANOVA+ for PRIMER: Guide to software and statistical methods, PRIMER-E, Plymouth

Armbruster WS (1989) Three new species of Dalechampia (Euphorbiaceae) from Venezuelan Guayana. Brittonia 41:44–52

Ayasse M, Schiestl FP, Paulus HF, Ibarra F, Francke W (2003) Pollinator attraction in a sexually deceptive orchid by means of unconventional chemicals. Proc R Soc Lond B Biol Sci 270:517–522

Bates D, Mächler M, Bolker B, Walker S (2015) Fitting Linear Mixed-Effects Models Usinglme4. J Stat Softw 67:1–48

Brandt K, Dötterl S, Francke W, Ayasse M, Milet-Pinheiro P (2017) Flower visitors of Campanula: are Oligoleges more sensitive to host-specific floral scents than Polyleges? J Chem Ecol 43:4–12

Dodson CH, Dressler RL, Hills HG, Adams RM, Williams NH (1969) Biologically active compounds in orchid fragrances. Science 164: 1243–1249

Dopman EB, Robbins PS, Seaman A (2010) Components of reproductive isolation between north-american pheromone strains of the european corn borer. Evolution 64:881–902. https://doi.org/10.1111/j.1558-5646.2009.00883.x

Dötterl S, Wolfe LM, Jürgens A (2005) Qualitative and quantitative analyses of flower scent in Silene latifolia. Phytochemistry 66:203–213

Dötterl S, Burkhardt D, Weißbecker B, Jürgens A, Schütz S, Mosandl A (2006) Linalool and lilac aldehyde/alcohol in flower scents: Electrophysiological detection of lilac aldehyde stereoisomers by a moth. J Chromatogr 1113:231–238

Eltz T, Whitten WM, Roubik DW, Linsenmair KE (1999) Fragrance Collection, Storage, and Accumulation by Individual Male Orchid Bees. J Chem Ecol 25:157–176. https://doi.org/10.1023/a:1020897302355

Erbilgin N, Raffa KF (2000) Opposing effects of host monoterpenes on responses by two sympatric species of bark beetles to their aggregation pheromones. J Chem Ecol 26:2527–2548

Garver L, Van Eikeren P, Byrd JE (1976) A facile synthesis of (+)-pinol from (−)-carvone. J Organomet Chem 41:2773–2774

Gemeno C, Leal WS, Mori K, Schal C (2003) Behavioral and electrophysiological responses of the brownbanded cockroach, Supella longipalpa, to stereoisomers of its sex pheromone, supellapyrone. J Chem Ecol 29:1797–1811

Gerlach G, Schill R (1991) Composition of orchid scents attracting Euglossine bees. Bot Acta 104:379–384. https://doi.org/10.1111/j.1438-8677.1991.tb00245.x

Govaerts R, Bernet P, Kratochvil K, Gerlach G, Carr G, Alrich P, Pridgeon AM, Pfahl J, Campacci MA, Holland Baptista D, Tigges H, Shaw J, Cribb P, George A, Kreuz K, Wood J. (2018) World checklist of Orchidaceae. Facilitated by the Royal Botanic Gardens, Kew Published on the Internet; http://wcsp.science.kew.org/. Accessed 17 Aug 2018

Hills HG, Williams NH, Dodson CH (1972) Floral fragrances and isolating mechanisms in the genus Catasetum (Orchidaceae). Biotropica 4:61–76. https://doi.org/10.2307/2989728

Hothorn T, Bretz F, Westfall P (2008) Simultaneous inference in general parametric models. Biom J 50:346–363

Jhumur US, Dötterl S, Jürgens A (2007) Electrophysiological and behavioural responses of mosquitoes to volatiles of Silene otites (Caryophyllaceae). Arthropod-Plant Interact 1:245–254. https://doi.org/10.1007/s11829-007-9022-3

Kaiser R (1993) The scent of orchids: olfactory and chemical investigations. Elsevier Science Publishers, Amsterdam

Kaiser R (2011) Scent of the vanishing flora, vol 1. Wiley-VCH, Weinheim

Knudsen JT, Eriksson R, Gershenzon J, Ståhl B (2006) Diversity and distribution of floral scent. Bot Rev 72:1–120

König WA, Hochmuth DH (2004) Enantioselective gas chromatography in flavor and fragrance analysis: strategies for the identification of known and unknown plant volatiles. J Chromatogr Sci 42:423–439

Kuznetsova A, Brockhoff PB, Christensen RHB (2017) lmerTest Package: Tests in Linear Mixed Effects Models. J Stat Softw 82:1–26

Lenth R (2018) Estimated marginal means, aka least-squares means. R package version 1.1. The R project

Lindquist N, Battiste MA, Whitten WM, Williams NH, Strekowski L (1985) Trans-carvone oxide, a monoterpene epoxide from the fragrance of Catasetum. Phytochemistry 24:863–865

Linn C, O'Connor M, Roelofs W (2003) Silent genes and rare males: a fresh look at pheromone blend response specificity in the European corn borer moth, Ostrinia nubilalis. J Insect Sci 3(15):1–6

Martel C, Gerlach G, Ayasse M, Milet-Pinheiro P (2019) Pollination ecology of the Neotropical gesneriad *Gloxinia perennis*: chemical composition and temporal fluctuation of floral perfume. Plant Biol. https://doi.org/10.1111/plb.12974

Milet-Pinheiro P, Gerlach G (2017) Biology of the Neotropical orchid genus Catasetum : a historical review on floral scent chemistry and pollinators. Perspect Plant Ecol Evol Syst 27:23–34. https://doi.org/10.1016/j.ppees.2017.05.004

Milet-Pinheiro P, Ayasse M, Dötterl S (2015a) Visual and olfactory floral cues of Campanula (Campanulaceae) and their significance for host recognition by an oligolectic bee pollinator. PLoS One 10:e0128577

Milet-Pinheiro P, Navarro D, Dotterl S, Carvalho AT, Pinto CE, Ayasse M, Schlindwein C (2015b) Pollination biology in the dioecious orchid Catasetum uncatum: how does floral scent influence the behaviour of pollinators? Phytochemistry 116:149–161. https://doi.org/10.1016/j.phytochem.2015.02.027

Moss G (1996) Basic terminology of stereochemistry (IUPAC recommendations 1996). Pure Appl Chem 68:2193–2222

Nagai T (1981) Electroantennogram response gradient on the antenna of the European corn borer, Ostrinia nubilalis. J Insect Physiol 27:889–894

R Core Team (2018) R: A Language and Environment for Statistical Computing. The R Project, Vienna https://www.R-project.org

Raguso RA (2008) Wake up and smell the roses: the ecology and evolution of floral scent. Annu Rev Ecol Evol Syst 39:549–569

Ramirez S, Dressler RL, Ospina M (2002) Abejas euglosinas (Hymenoptera: Apidae) de la région neotropical: listado de especies con notas sobre su biología. Biota Colomb 3:7–118

Roelofs WL (1984) Electroantennogram assays: rapid and convenient screening procedures for pheromones. In: Techniques in pheromone research. Springer, Berlin, pp 131–159

Schneider MA, Dötterl S, Seifert K (2013) Diastereoselective synthesis of a lilac aldehyde isomer and its electrophysiological detection by a moth. Chem Biodivers 10:1252–1259

Schorkopf DLP, Mitko L, Eltz T (2011) Enantioselective preference and high antennal sensitivity for (−)-Ipsdienol in scent-collecting male orchid bees, Euglossa cyanura. J Chem Ecol 37:953–960

Schwerdtfeger M, Gerlach G, Kaiser R (2002) Anthecology in the neotropical genus Anthurium (Araceae): a preliminary report. Selbyana 23:258–267

Strausfeld CZ, Kaissling K-E (1986) Localized adaptation processes in olfactory sensilla of Saturniid moths. Chem Senses 11:499–512. https://doi.org/10.1093/chemse/11.4.499

Takita S, Yokoshima S, Fukuyama T (2011) A practical synthesis of (−)-kainic acid. Org Lett 13:2068–2070

Teale SA, Hager BJ, Webster FX (1994) Pheromone-based assortative mating in a bark beetle. Anim Behav 48:569–578

Teichert H, Dötterl S, Zimma B, Ayasse M, Gottsberger G (2009) Perfume-collecting male euglossine bees as pollinators of a basal angiosperm: the case of Unonopsis stipitata (Annonaceae). Plant Biol 11:29–37

Vogel S (1966) Parfümsammelnde Bienen als Bestäuber von Orchidaceen undGloxinia. Oesterr Bot Z 113:302–361

Wang Q, Huang Q, Chen B, Lu J, Wang H, She X, Pan X (2006) Total synthesis of (+)-Machaeriol D with a key Regio-and Stereoselective SN2′ reaction. Angew Chem 118:3733–3735

Weber MG, Mitko L, Eltz T, Ramírez SR (2016) Macroevolution of perfume signalling in orchid bees. Ecol Lett 19:1314–1323

Whitten WM, Williams NH, Armbruster WS, Battiste MA, Strekowski L, Lindquist N (1986) Carvone oxide: an example of convergent evolution in euglossine pollinated plants. Syst Bot 11:222–228

Whitten WM, Hills HG, Williams NH (1988) Occurrence of ipsdienol in floral fragrances. Phytochemistry 27:2759–2760

Williams NH, Whitten WM (1983) Orchid floral fragrances and male euglossine bees: methods and advances in the last sesquidecade. Biol Bull 164:355–395

Yasuda A, Yamamoto H, Nozaki H (1979) A Stereoselective 1, 3-transposition reaction of Allylic alcohols. Bull Chem Soc Jpn 52: 1757–1759

Zimmermann Y, Ramírez SR, Eltz T (2009) Chemical niche differentiation among sympatric species of orchid bees. Ecology 90:2994–3008