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Studies

STUDIES

Sexual dimorphism in floral scents of the neotropical orchid *Catasetum arietinum* and its possible ecological and evolutionary significance

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Abstract

Dioecy in angiosperms is often associated with sexual dimorphism in floral traits other than the sexual organs. Species of the neotropical orchid genus *Catasetum* produce unisexual flowers characterized by a remarkable morphological sexual dimorphism. *Catasetum* species emit strong floral perfumes that act as both signal and reward for male euglossine bee pollinators. Although the role of floral perfumes of *Catasetum* in attracting euglossine pollinators is well investigated, little is known about whether perfumes differ between floral sexes and, if they do, whether this chemical dimorphism influences the pollination ecology of the plants. Taking *Catasetum arietinum* as a model species, our aim was to observe the behaviour of pollinators on male and female flowers and to compare scent properties (i.e. chemical composition, total amount and temporal fluctuation) of male and female flowers. Floral scent samples were collected by using dynamic headspace methods and were analysed via gas chromatography coupled with mass spectroscopy (GC-MS). *Catasetum arietinum* is pollinated by males of two *Euglossa* species (i.e. *E. nanomelanotricha* and *E. securigera*). Bees approached male and female inflorescences of *C. arietinum* in similar proportions but landed significantly more often and spent more time on female flowers, which emitted more scent than male flowers. Furthermore, the amount of scent emitted varied across the different times of sampling, corresponding to the pattern of the diel foraging activity of pollinating bees on male and female flowers. The chemical composition of scents differed significantly between sexes. The two major compounds (*Z*)-methyl-*p*-methoxycinnamate and (*E*)-geranyl geraniol contributed most to this difference. This is the first case of sexual dimorphism reported in orchid floral perfumes. We discuss the influence of sex-specific floral scents on the behaviour of euglossine pollinators and offer new insights into the ecological and evolutionary significance of divergence in floral scents among dioecious plants.

Keywords: Catasetinae; *Catasetum arietinum*; *Euglossa nanomelanotricha*; *Euglossa securigera*; euglossine bees; floral scent; perfume-rewarding orchids; pollination; sexual dimorphism.

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Introduction

Reproductive division into two sexes (i.e. dioecy) is a rare condition in flowering plants (see, e.g., Barrett and Hough 2012). Numerous examples for sexual dimorphism in floral and reproductive traits (e.g. longevity of flowers, Primack 1985; inflorescence architecture, Rourke 1987; total flower number, Barrett 1992; flowering phenology and periodicity, Thomas and LaFrankie 1993; flower size, Delph *et al.* 1996) exist in dioecious plants, including chemical dimorphism in floral perfumes (Ashman 2009; Okamoto *et al.* 2013). The natural selection of dimorphism in floral perfumes (and other floral traits) of animal-pollinated plants is generally constrained by one key condition: male and female flowers have to attract the same pollinator species (Ashman 2009; Dötterl *et al.* 2014); otherwise, pollen transfer will not occur and reproduction will fail. As long as this constraining condition is met, any divergence in floral perfumes might be adaptive, if this results in increased fitness. In an insightful review on dimorphism of floral perfumes in dioecious plants, Ashman (2009) has suggested that both the strength and direction (e.g. convergence/advergence) of selection on the floral scents of male and female flowers depends in particular on the reward (if any) that is offered by each sex and on the biology/behaviour of pollinators. She also mentions that sex-specific compositions of floral perfumes (corresponding to two different rewards) might lead to the directed behaviour of pollinators, if they prefer the scent of one floral sex over the other.

In addition to floral scent composition *per se*, the total amount and timing of scent emission can be a major driver of pollinator behaviour in gender dimorphic plants. Ervik *et al.* (1999) have found that, in phytelephantoid palms (Arecaceae), male inflorescences emit a strong scent that peaks during the first day of anthesis and decreases quickly afterwards, whereas female inflorescences continuously emit a somewhat weaker scent for at least 2 days. Accordingly, the number of visiting beetle pollinators to male inflorescences decreases during the second day of anthesis, whereas female inflorescences are visited constantly for 2 days. Furthermore, Terry *et al.* (2007) have observed, in a Australian *Macrozamia* cycad, a sequential thrips' repellence (push) and attraction (pull) that is caused by daily variations in the emission of specific scent volatiles (i.e. β-myrcene and ocimene) within the cones. These examples indicate that the movement of pollinators can be directed from one floral sex to the other depending on the timing and the total amount of scent emission.

In the neotropical orchid genus *Catasetum* floral perfumes play a crucial role in pollination ecology acting both as an attractant and reward for pollinators (Dressler 1982). Because of specific adaptations, male euglossine bees (Euglossini: Apidae) are able to collect and store these perfumes and expose them later on perching sites, presumably to enable species-specific recognition and/or to signal male genetic fitness to conspecific females (Eltz *et al.* 1999, 2003, 2005b). Unlike most orchids, which have hermaphroditic flowers, representatives of *Catasetum* produce unisexual flowers characterized by a pronounced dimorphism in morphology. This morphological dimorphism is prerequisite for one of the most unusual and fascinating pollination mechanisms reported in angiosperms: when a male euglossine bee collects perfumes at the labellum of a male flower of *Catasetum*, a catapult-like mechanism is triggered. This action results in the attachment of the pollinarium to the bee's body and its prompt expulsion from the flower. When the same bee subsequently enters into the labellum of a female flower, again to collect perfume, the pollinarium is inserted into the stigmatic cleft, resulting in pollination (Dodson 1962).

Following the comprehensive description of the nuances of this interaction between 'perfume flowers' and their perfume-collecting pollinators by Vogel (1966), the chemistry of floral scents of *Catasetum* species has been intensively investigated (e.g. Hills *et al.* 1972; Gerlach and Schill 1991; Kaiser 1993) and the floral bouquets of ~25 *Catasetum* species have been chemically characterized (Milet-Pinheiro *et al.* 2017). The general tendency emerging from these studies suggests that the chemical composition of floral perfumes is species-specific (Hills *et al.* 1968; Gerlach and Schill 1991; Kaiser 1993) and that this specificity is directly involved in the selective attraction of a few euglossine bee species, a feature that therefore acts as an effective pre-mating reproductive barrier (Hills *et al.* 1972). Apart from the floral scent chemistry, also the evolution of *Catasetum*, and other orchids, has been an area of concern discussed in many different studies. For example, it has been suggested that the production of perfumes as a reward in neotropical orchids (including *Catasetum*) evolved subsequent to the origin of perfume-collecting behaviour in euglossine bees (Ramírez *et al.* 2011), and that floral scents evolve under the selective pressures of the sensory/behavioural biases of their euglossine pollinators (Schiestl and Johnson 2013). Furthermore, it has been demonstrated that floral perfumes can act as an isolating mechanism in orchids (Cozzolino and Widmer 2005). Despite all these studies, a lack of knowledge persists over other interesting aspects of floral scent, such as a possible sexual dimorphism in floral perfumes and the ecological and evolutionary implications these aspects might have.

Male euglossine bees are known to change their scent preference after collecting a specific compound intensively (Eltz *et al.* 2005a). Therefore, evolutionary processes might have fostered the selection for divergence in floral perfumes (e.g. chemistry and/or timing) which might be adaptive if this induces euglossine males to visit alternately male and female flowers to collect sex-specific compound(s). In terms of the timing of scent emission, selection for divergence might occur if the movement of pollinators from one flower sex to the other is promoted by this divergence. This is possible when (i) the scent production is initiated earlier in one of the sexes (different timing of scent emission; e.g. Ervik *et al.* 1999) or (ii) the total amount of emitted scent is higher in one of the floral sexes at a specific time of day (different amount of scent emission; e.g. Ashman 2009).

Unlike most *Catasetum* species, which grow in the canopy of tall trees in neotropical rainforests, *Catasetum arietinum* grows predominantly on the stipes of short to medium *Elaeis* palms (Arecaceae). Therefore, this orchid offers a unique opportunity for a detailed investigation of the associations of a *Catasetum* species with its euglossine pollinators and of floral perfumes *in situ*. In this context, we have examined flower morphology (i.e. the size of the flower and the flower opening, plus the location of the osmophores) and pollination biology (i.e. flower development, longevity, sex ratio [SR], pollinators) of *C. arietinum* and have investigated the scent chemistry of this orchid. Thereby, we have addressed the following questions. (i) Which are the effective pollinators of *C. arietinum*? (ii) Does the frequency of visits over the day differ between male and female flowers? (iii) Does the total amount of emitted floral scent differ among floral sexes and/or change over the day? (iv) Does the

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chemical composition of floral scent differ between male and female flowers and/or change over time of day?

Methods

Study site

The investigated population of *C. arietinum* is situated in the surroundings of the farm 'Agua Fria' (600 m a.s.l.; 8°11′19.0″S, 35°28′13.6″W), located in the municipality of Chã-Grande (Pernambuco, NE Brazil), a region of transition between semi-deciduous seasonal Atlantic Forest and the typical Caatinga tropical dry forest of NE Brazil. Additional information on the climate at the study site is given in **Supporting Information—Method M1**.

Study species

Catasetum arietinum is a rare species endemic to the state of Pernambuco (Holst 1999). Plant individuals of this species grow epiphytically predominantly on palm trees with their conical pseudo-bulbs, leaves (linear-lanceolate, wavy margins and three prominent veins) and inflorescences characteristically arching downwards (Holst 1999; Fig. 1A). The flowering period ranges from February to June or July and inflorescences often grow one after another from a newly developing pseudo-bulb (Holst 1999). Normally, plant individuals produce inflorescences that bear either male or female flowers but, in exceptional cases, flowers of both sexes can occur on the same inflorescence (Holst 1999).

The investigated population of *C. arietinum* consisted of 28 different epiphytic plant individuals growing predominantly on palm trees of the genus *Elaeis* (Arecaceae) which were investigated during the two flowering periods: (i) early March to end of August during 2016 and (ii) early March to end of May during 2017. Plant vouchers of the investigated population are stored in the Herbarium Geraldo Mariz (UFPE), Recife, Brazil.

Flower morphology and biology

Flower morphology and size was described from single male (N = 17 from 16 different plant individuals) and female flowers (N = 8 from seven different plant individuals; for details, **see Supporting Information—Method M2**). Furthermore, the location of osmophores was determined in male (N = 2) and female (N = 2) flowers following the method described by Stern *et al.* (1986) and in **Supporting Information—Method M3**.

For monitoring development of flowers from opening to abscission, male (N = 8) and female (N = 4) inflorescences of different plant individuals (one inflorescence per individual) were bagged in pre-anthesis stage using voile bags. We recorded flower longevity and the development of perfume production as

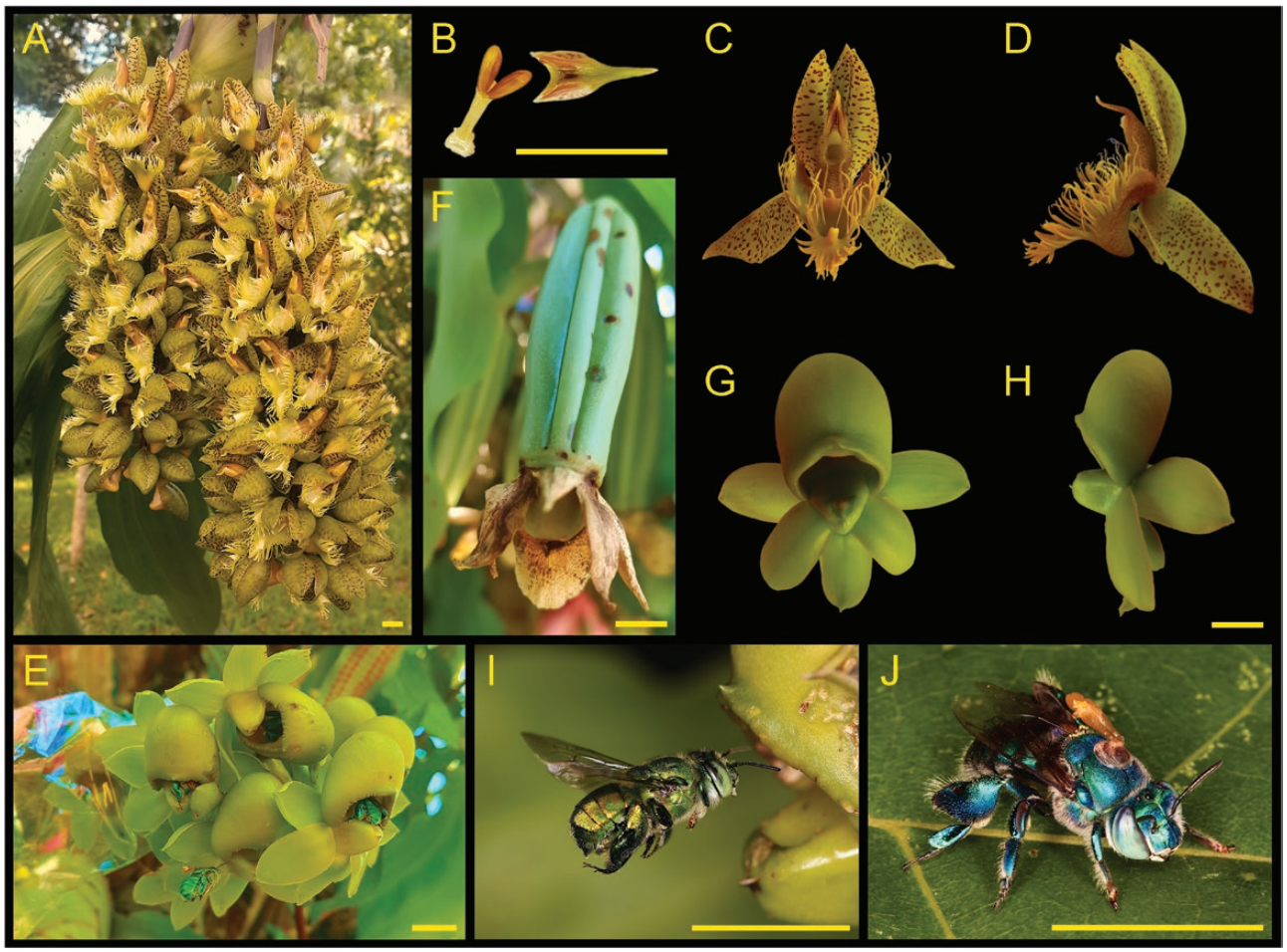


Figure 1. Overview of male and female flowers and pollinators of *Catasetum arietinum*: (A) male inflorescence, (B) pollinarium, (C) male flower front view, (D) male flower side view, (E) female inflorescence with pollinating male bees of *Euglossa securigera*, (F) immature fruit, (G) female flower front view, (H) female flower side view, (I) *E. securigera* and (J) *E. nanomelanotricha* with an attached pollinarium. Scale bar: 1 cm. Photos B, C, D, E, F, G, H by Katharina Brandt; A, I, J by Paulo Milet-Pinheiro.

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perceived by human olfaction. For this, one of us (K.B.) smelled inflorescence daily at 1030 h from a distance of ~2 cm and ranked its strength in a scale (0: no scent, 1: weak scent, 2: medium scent, 3: strong scent). We determined the SR of male and female inflorescences following Opler and Bawa (1978) and Freeman *et al.* (1981) as well as the operational sex ratio (OSR) using the method described by Emlen and Oring (1977). For details on SR and OSR, see **Supporting Information—Method M4**.

We also determined the fruit set under natural conditions. For this purpose, we marked all intact female flowers (N = 110 from 17 inflorescences) using numbered labels and recorded the ratio of developing fruits after successful pollination.

Observation of floral visitors

The frequency of flower visitors was recorded on 16 male (N = 465 single flowers) and 13 female (N = 84 single flowers) inflorescences of 23 different plant individuals on 13 non-consecutive days from 0600 to 1630 h. Observation periods of 30 min each were performed in intervals of 30 min (143 h of total observation). We documented the species and the number of flower visitors, thereby distinguishing between approaches without landings (flights towards the inflorescence to a distance closer than 30 cm) and approaches followed by landings on flowers. During observations, we did not collect bees in order to enable the recording of landings and the observation of natural behaviours.

We documented the behaviour of floral bee visitors during the whole period of field work and recorded the duration of hovering and landing for bee individuals on female (N = 25) and on male (N = 20) flowers as well as the attachment of a pollinarium to a bee's body on male flowers and the deposition of attached pollinaria during visits to female flowers. Further, bees were collected with entomological nets, dried and mounted on insect needles for identification. Identification of bees was performed by using taxonomical keys for euglossine bees (Nemesio 2009); other insects were identified with the help of specialists. Voucher specimens were deposited at the entomological collection of the Federal University of Pernambuco (UFPE).

Sampling of floral volatiles and chemical analysis

We sampled scents of male (N = 11) and female (N = 10) flowers of different *C. arietinum* individuals (one flower per individual) by using dynamic headspace methods for the following purposes: (i) to characterize their chemical composition, (ii) to measure their daily fluctuation in total scent emission (i.e. the total amount of scent trapped by the adsorbent filters) and (iii) to determine daily changes in the chemical composition of scent. To obtain flower perfume samples for thermal desorption (TD), we followed the procedure described in Dötterl and Jürgens (2005) and in **Supporting Information—Method M5**. Sampling of floral perfumes was performed using the same flower of an inflorescence four times a day (0600, 0900, 1200 and 1500 h).

For the chemical analysis of TD samples we used a gas chromatograph coupled to a mass spectrometer (GC-MS; Agilent 7890A™ gas chromatograph, Agilent 5975C Series MSD™ mass spectrometer; Agilent Technologies, Palo Alto, CA, USA) (for details on equipment specifications, configurations and on the elucidation/quantification of compounds, see **Supporting Information—Method M6**).

Statistics

We used the Shapiro–Wilk test to test for the normality of distribution within data samples. Further, homogeneity of variances was assessed by using a Levene's test. To compare the amount of male and female inflorescences and single flowers produced by single plant individuals we used the Wilcoxon matched-pairs test for the comparison of two dependent samples. We performed either a Student's t-test (normality) or a non-parametric Mann–Whitney U-test (non-normality), depending on the distribution of data, to test (i) the mean number of flowers, (ii) morphological features of flowers (i.e. height and width of the flower itself and access to the labellum), (iii) scent production during anthesis as perceived by human olfaction (i.e. time until all flowers open, time until strongest scent and time until scent weakening), (iv) longevity of flowers and (v) the hovering and landing times of pollinators on male and female flowers. These analyses were performed in Statistica 7.0.

Using the software PRIMER 6 (version 6.1.15) in combination with the add-on permutational multivariate analysis of variance (PERMANOVA)+ (version 1.0.5), we performed permutational analyses (PERMANOVA) comparing: (i) the frequency of visits to male and female inflorescences throughout the day, (ii) the absolute amount of total scent emitted by a single flower at various times of day, (iii) the relative ratio of compounds (semi-quantitative comparison between floral sexes) and potential changes in this ratio over the day and (iv) the presence/absence of compounds (qualitative comparison between flora sexes) and potential changes over the day (for further details, see **Supporting Information—Method M7**).

To depict variation in floral scent chemistry graphically among samples, we calculated a mean of the emitted scent per day for each individual and used non-metric multidimensional scaling (NMDS; Clarke and Gorley 2006). Analysis of similarities (ANOSIM) was performed to analyse the overlap of sample groups to accompany the simple depiction via NMDS ($-1 < R < 1$: values towards −1 mean that samples within defined groups are less similar than between groups, a value of 0 means groups overlap completely, and the closer the value gets to 1, the larger the differences between groups). We further performed permutational test for homogeneity of multivariate dispersions (PERMDISP) to test for differences in scent variability (dispersion) within floral sexes based on the semi-quantitative and qualitative matrices.

Results

Flower biology and flower development

Within the two blooming seasons, the 28 investigated plant individuals produced 86 inflorescences (49 in 2016 and 35 in 2017), of which 65 were exclusively male and 17 exclusively female. Male and female inflorescences flowered continuously across the blooming season, without a temporal differentiation in flowering onset. Two inflorescences produced both male and female flowers (12 male and one female flowers; 20 male and five female flowers). The SR of the population was 3.82, indicating that almost four times more male than female inflorescences were present; the OSR (5.92; Table 1) indicated the production of almost six times more male flowers than female flowers for the studied population.

Male inflorescences are pendent and bear between 11 and 64 flowers (Fig. 1A). During anthesis, male flowers open steadily from the basis to the tip of the inflorescence. The time until all male flowers of an inflorescence are open generally ranges between 1 and 4 days. The labellum is an open tongue-like structure that is held in downwards-pointing position and has several stout fimbriae at its margins. Flowers bear their lateral sepals on the

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left- and right-hand sides behind the labellum. The pollinarium with two pollinia lies above the labellum (Fig. 1B). Two parallel antennae are extended down towards the inner surface of the labellum in such a way that they pass between three spurs, which are located at this surface. Male flowers of *C. arietinum* are either light green or yellow, spotted with darker dots on sepals and petals and have a pale labellum with fimbriae (Fig. 1C and D).

Female inflorescences of *C. arietinum* are erect and contain up to 13 flowers (Fig. 1E). The flowers of an inflorescence open within 2–4 days. Female flowers differ considerably in their morphology and colour from male flowers. In contrast to male flowers, all female flowers are yellowish-green. The hood-shaped labellum of female flowers has a much deeper cavity than the labellum of male flowers, with a lid containing the stigmatic cleft. If pollinated, female flowers begin to develop an elongated green fruit (Fig. 1F). The lateral sepals and petals of female flowers are located on both sides of the flower beneath the labellum with the dorsal sepal in between the petals (Holst 1999; Fig. 1G and H). Sepals and petals wither during fruit development.

Both male and female flowers of *C. arietinum* are strongly fragrant to the human nose. The osmophores are located on the inner surface of the labellum beneath the antennae and surrounding the three spurs in male flowers and all over the inner surface of the labellum in female flowers. Male flowers start emitting scent on the day after opening and scent emission is strongest between Days 2 and 3, as perceived by human olfaction. If undisturbed by floral visitors, single flowers of male inflorescences usually produce scent until they wither after 5–7 days. After pollinarium removal, scent emission ceases within 1–2 days and male flowers start to wither on the subsequent day. Perfume production in female flowers normally begins 2 days after flower opening and is already strong. Female flowers last 2–3 weeks if visitors are excluded, showing a significantly longer lifespan than male flowers. In female flowers, perfume is emitted as long as the flowers are open but emission weakens considerably after 1–2 weeks, as perceived by human olfaction. If pollinated, they stop producing perfume and start developing a fruit on the subsequent day. In total, 25 % of flowers that were exposed to pollinators (N = 116 flowers) developed a fruit.

In summary, male inflorescences produce a higher number of single flowers that differ in their morphological features (including the height of flower opening), but not in their size, from female flowers. In comparison to male flowers, female ones bloom longer, thereby emitting floral scent for a longer time (Table 1).

Floral visitors

We recorded male bees of two species of *Euglossa*, namely *Euglossa securigera* (Fig. 1I) and *E. nanomelanotricha* (Fig. 1J), on the flowers of *C. arietinum*. *Euglossa securigera* was 10.4-fold more abundant than *E. nanomelanotricha*. For information on observed flower visitors other than bees, see **Supporting Information—Results R1**.

Bees approached the inflorescences from downwind and hovered in front of the flowers for a few seconds. On male flowers, male bees landed on the labellum facing upwards. After landing, the bees showed a typical perfume-collecting behaviour (Eltz *et al.* 2005b; Eltz 2010) during which they may have contacted one or both antennae of the flower, triggering the ejection of the pollinarium. The pollinarium was then glued to the dorsal part of the thorax (mesoscutum) by means of the viscidium. In cases of pollinarium attachment, volatile collection on the flower was interrupted promptly and the bees left the male inflorescence.

The morphology of female flowers forces visiting bees to crawl inside the cavity of the labellum after landing. The labellum of female flowers was entered facing either up- or downwards depending on where male euglossine bees had previously landed. The time during which male bees hovered in front of flowers and the period of collecting volatiles once landed on the flowers was significantly longer on female than on male flowers of *C. arietinum* (Table 1). When backing out of the labellum (upside down), an attached pollinarium was sometimes inserted into the stigmatic cleft, resulting in pollination of the flower.

Bees approached male and female inflorescences of *C. arietinum* equally frequent (PERMANOVA: pseudo-$F_{1, 297}$ = 0.94, P = 0.34; Fig. 2A) and the number of approaching bees differed significantly, depending on the distinct times of observation during the day (PERMANOVA: pseudo-$F_{10, 297}$ = 13.31, P < 0.001). Thereby, the temporal course of initiation, peak and cessation of approaches was similar among floral sexes (interaction between sexes and time of day; PERMANOVA: pseudo-$F_{10, 297}$ = 0.58, P = 0.83). Pairwise analyses revealed that the number of

Table 1. Comparison of sex ratio, morphological features, flower development and behaviour of pollinators among male and female flowers of *C. arietinum*. [a]Wilcoxon matched-pairs test; [b]Student's *t*-test; [c]non-parametric Mann–Whitney *U*-test; *Observation of bagged inflorescences without visitors; significant difference at α = 0.05 indicated in bold.

	Male (mean ± SD)	N	Female (mean ± SD)	N	Statistics
Number of inflorescences/flowers					
Number of inflorescences per plant individual	2.4 ± 1.75	28	0.68 ± 1.09	28	Z = 3.24, **P < 0.01**[a]
Number of flowers per inflorescence per plant individual	23.42 ± 13.65	28	2.13 ± 3.48	28	Z = 4.44, **P < 0.001**[a]
Flower morphology					
Flower height (mm)	56.86 ± 1.90	17	52.93 ± 2.47	8	Z = 1.02, P = 0.31[c]
Flower width (mm)	56.29 ± 3.35	17	49.33 ± 2.86	8	t = 1.31, P = 0.2[b]
Height of flower opening (mm)	25.80 ± 0.96	17	14.55 ± 0.90	8	t = 7.32, **P < 0.001**[b]
Width of flower opening (mm)	16.42 ± 0.80	17	18.32 ± 0.81	8	t = –1.46, P = 0.16[b]
Flower development*					
Days until all flowers open	3.38 ± 1.06	8	2.75 ± 0.96	4	Z = 1.02, P = 0.31[c]
Days until strongest scent	2.75 ± 0.46	8	2.50 ± 1.73	4	Z = 0.93, P = 0.35[c]
Days until scent weakens	5.25 ± 1.16	8	10.25 ± 0.96	4	t = –7.38, **P < 0.001**[b]
Longevity	6.75 ± 1.04	8	20.25 ± 6.02	4	Z = –2.63, **P < 0.01**[c]
Behaviour of pollinators					
Hovering time (s)	4.06 ± 0.76	20	3.08 ± 0.67	25	t = –4.57, **P < 0.001**[b]
Handling time (s)	13.67 ± 2.3	20	21.8 ± 1.5	25	t = 4.11, **P < 0.001**[b]

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approaching *Euglossa* bees to male and female inflorescences of *C. arietinum* was highest between 0800 and 1330 h. We did not observe any euglossine visitors before 0700 h and after 1430 h.

When considering only the visits to male inflorescences, 17.5 % of the approaching *Euglossa* bees landed on flowers. Some bees (3.9 % of approaching/6.6 % of landing bees to male inflorescences) had a pollinarium attached from previous visits on male flowers. Among the bees landing on male flowers, 10.2 % triggered the catapult-like mechanism resulting in the attachment of the pollinarium (Fig. 2B). Furthermore, 2.4 % of the bee individuals that landed on male flowers had a pollinarium attached and obtained a second one (data not shown). On female inflorescences, 49.3 % of the approaching bees landed and some (4.5 % of approaching bees/6.9 % of landing bees on female inflorescences) had at least one attached pollinarium. Among the bee individuals that landed to collect perfumes on female flowers, 2.1 % deposited the pollinarium into the stigmatic cleft (Fig. 2B). For a comparison of data (total numbers of bees per species observed approaching with or without landing on flowers, removing/bearing pollinaria, and the deposition of pollinaria to female flowers) between *E. nanomelanotricha* and *E. securigera*, see **Supporting Information—Results R2**.

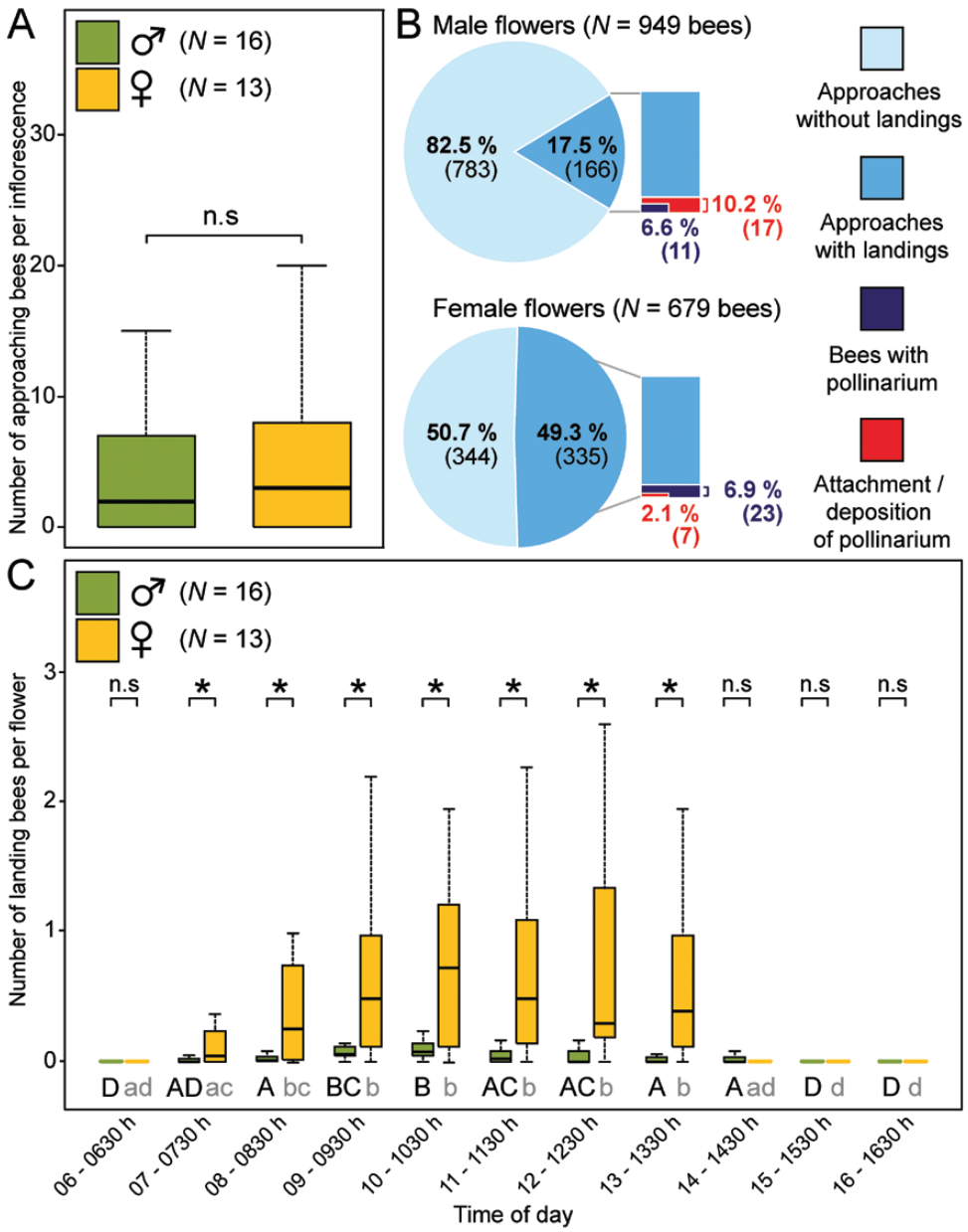


Figure 2. (A) Number of approaching male *Euglossa* bees (*E. nanomelanotricha* and *E. securigera* pooled) to male (*N* = 16) and female (*N* = 13) inflorescences of *Catasetum arietinum*. (B) Pollinarium transfer by *Euglossa* bees on male and female flowers of *C. arietinum*. For each type of observed behaviour (approaches without and with subsequent landings) or characteristic (with attached pollinarium, attachment/deposition of pollinarium) relative percentages and total numbers (in brackets) are given. (C) Frequency of male euglossine bees (*E. nanomelanotricha* and *E. securigera* pooled) landing on male (*N* = 16 inflorescences) and female flowers (*N* = 13 inflorescences) of *C. arietinum* throughout the day. Different letters indicate significant differences for the number of visitors on single male flowers (capital letters: A, B, C, D) and single female flowers (regular letters: a, b, c, d) between the different time intervals of observation. Asterisks indicate significant differences ($P \leq 0.05$; PERMANOVA) between visits to male and female flowers at a given time of day. n.s. indicates no significant differences ($P > 0.05$; PERMANOVA).

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Approaching bees landed more frequently on female than on male flowers (PERMANOVA: pseudo-$F_{1, 297}$ = 31, P < 0.001; Fig. 2C) and the number of bees landing on flowers differed significantly between different times of observation (PERMANOVA: pseudo-$F_{10, 297}$ = 3.26, P < 0.01). The temporal course of initiation, peak and cessation of landings on flowers differed significantly between floral sexes (interaction between sexes and time of day; PERMANOVA: pseudo-$F_{10, 297}$ = 2.66, P < 0.01): on both male and female inflorescences, *Euglossa* bees initiated foraging activity in the morning (~0700 h) and foraging ended in the afternoon (~1430 h). However, pairwise comparisons revealed a peak of landing bees between 0900 and 1030 h on male flowers and between 0800 and 1330 h on female flowers (Fig. 2C).

Total perfume emission and daily fluctuation of scent

The total amount of scent emitted by single flowers varied among individuals ranging from 7 to 1042 ng min^{-1} in male flowers and from 27 to 3395 ng min^{-1} in female flowers, with female flowers emitting significantly more scent (PERMANOVA: pseudo-$F_{1, 76}$ = 15.22, P < 0.001; Fig. 3A). Flowers emitted more scent between 0900 and 1200 h (PERMANOVA: pseudo-$F_{3, 76}$ = 6.26, P < 0.001; Fig. 3B) than at the rest of the day, irrespective of floral sex (interaction between sexes and time of day; PERMANOVA: pseudo-$F_{3, 76}$ = 1.94, P = 0.13).

Chemical composition of floral scent bouquet

In the headspace samples of *C. arietinum*, we (tentatively) identified 52 compounds belonging to the following compound classes: sesquiterpenes (21 compounds), monoterpenes (15), aromatics (11), isoprenoid hydrocarbons (2), aliphatics (1), irregular terpenes (1) and nitrogen-containing compounds (1). Of these 52 identified compounds, eight were found exclusively in female flowers and three exclusively in male flowers. However, compounds were not necessarily found in all samples of a given sex and some were recorded only in trace amounts (Table 2). Sesquiterpenes represented the dominant compound class in male and female flowers. (*E*,*E*)-α-farnesene was the major compound within the scent bouquet of flowers of both sexes (24.3 ± 25.9 SD in male and 23.2 ± 23.1 SD in female flowers), followed by (*Z*)-methyl-*p*-methoxycinnamate (18.6 ± 20.9 SD) in male flowers and by (*E*)-geranyl geraniol (21.2 ± 24.8 SD) in female flowers. In addition, 38 unidentified compounds were present in the floral scent of *C. arietinum*. Of these unidentified compounds, one was found exclusively in male flowers and seven occurred exclusively in female flowers (Table 2).

Multivariate statistical analyses revealed that the scent bouquet of male and female flowers differed semi-quantitatively (PERMANOVA: pseudo-$F_{1, 57}$ = 3.04, P < 0.05; Fig. 4) and qualitatively (PERMANOVA: pseudo-$F_{1, 57}$ = 3.41, P < 0.05; **see Supporting Information—Results R3**) and the chemical composition changed over the day (semi-quantitative PERMANOVA: pseudo-$F_{3, 57}$ = 5.20, P < 0.001; qualitative PERMANOVA: pseudo-$F_{3, 57}$ = 3.59, P < 0.001). While semi-quantitative changes over the day were independent of sexes (interaction between sexes and time of day; PERMANOVA: pseudo-$F_{3, 57}$ = 1.60, P = 0.07), the floral sex dictated which chemical compounds were affected by qualitative changes (interaction between sexes and time of day; PERMANOVA: pseudo-$F_{3, 57}$ = 1.77, P < 0.05). Dispersion/variability of scent bouquet of male and female flowers was similar both semi-quantitatively (PERMDISP: $F_{1, 19}$ = 2.09, P = 0.23) and qualitatively (PERMDISP: $F_{1, 19}$ = 3.24, P = 0.14).

Discussion

Catasetum arietinum is pollinated exclusively by male bees of the genus *Euglossa*, which are highly abundant at the study site (Milet-Pinheiro and Schlindwein 2008). We detected scent

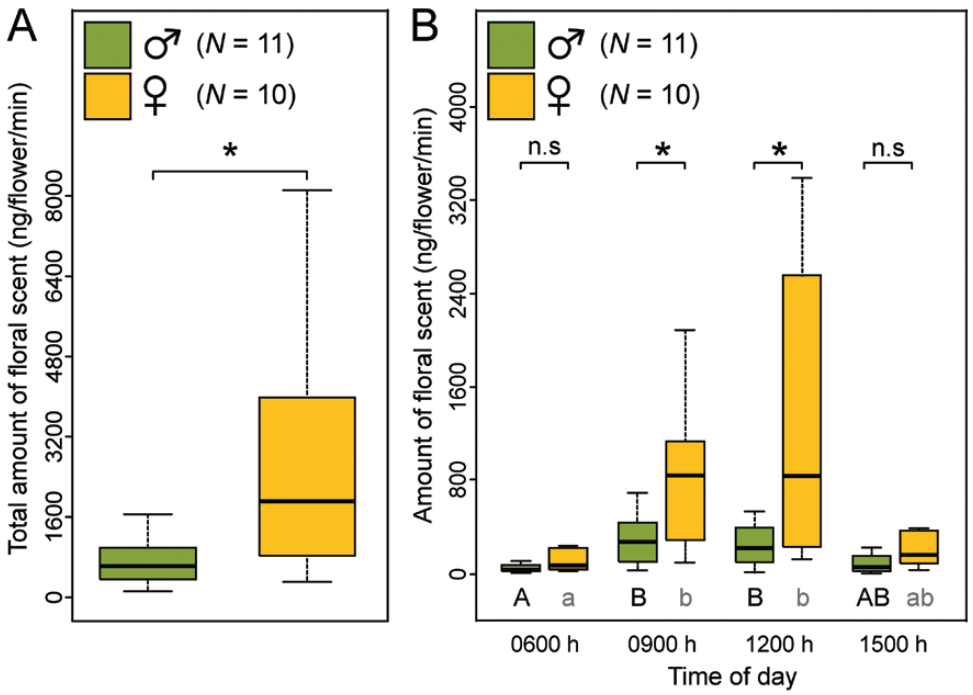


Figure 3. (A) Comparison of the total amount of floral scent emitted by male and female single flowers over the day (all four times of sampling pooled). (B) Amount of floral scent emitted by male and female single flowers of *Catasetum arietinum* at the four different times of sampling. Different letters indicate significant differences among time of day for male (capital letters: A, B) and female (regular letters: a, b) flowers. Asterisks indicate significant differences ($P \leq 0.05$; PERMANOVA) for the total emission of floral scent between male and female flowers at a given time of day. n.s. indicates no significant differences ($P > 0.05$; PERMANOVA).

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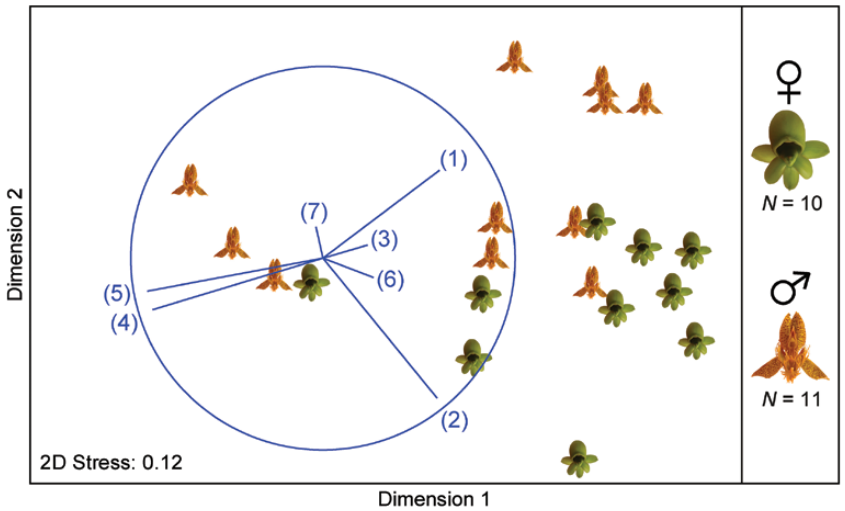


Figure 4. 'Scent space' representation of male and female flowers of *Catasetum arietinum* visualized in two dimensions by non-metric multidimensional scaling (NMDS) based on semi-quantitative Bray–Curtis similarities (ANOSIM: $R = 0.2$, $P < 0.01$; fourth root transformation, stress value = 0.12). Symbols represent the average scent pattern for each individual. Vectors represent the Pearson correlation for compounds most responsible for the dissimilarity in scent composition between floral sexes as indicated in a SIMPER analysis: (1) (*E*,*E*)-α-farnesene, (2) (*E*)-geranyl geraniol, (3) (*Z*)-methyl-*p*-methoxycinnamate, (4) (*E*)-β-bergamotene, (5) β-bisabolene, (6) (*E*)-methyl-*p*-methoxycinnamate and (7) eucalyptol. Vector length and direction indicate the strength of the correlation of the compound on the ordination plot; the large circle indicates the maximum vector length.

foraging on flowers starting at around 0700 h and ending at 1430 h with a peak of landings from 0900 to 1130 h and from 0800 to 1330 h, on male and female flowers, respectively. Although almost four times more male than female inflorescences were recorded in the population (and almost six times more male than female single flowers), bees approached male and female inflorescences of *C. arietinum* equally frequently. However, after approaching inflorescences, bees landed significantly more often on female than on male flowers. Concomitantly, we found that the amount of scent emitted was much higher in female than in male single flowers. Scent emission fluctuated across the day and this fluctuation matched the activity of pollinators on flowers. Chemical analyses showed that the perfume bouquet of *C. arietinum* was a complex blend consisting of three dominant (i.e. (*E*,*E*)-α-farnesene in flowers of both sexes, (*Z*)-methyl-*p*-methoxycinnamate in male and (*E*)-geranyl geraniol in female flowers) and several minor compounds and revealed a sexual dimorphism in the chemical composition of floral scents. Furthermore, the chemical composition of scent changed over the day regarding (i) the relative amounts and (ii) the presence/absence of distinct compounds.

Two species of *Euglossa* (i.e. *E. nanomelanotricha* and *E. securigera*) were observed to carry pollinaria from male to female flowers of *C. arietinum*. *Euglossa securigera* was more frequent in the study area and turned out to be the more effective pollinator, whereas *E. nanomelanotricha* participated in the pollination of *C. arietinum* to a significantly lower extent. Apart from *E. nanomelanotricha* and *E. securigera*, eight other euglossine species are known to occur in this area: *E. carolina* and two other unidentified *Euglossa* species, four different species of the genus *Eulaema* (i.e. *El. niveofasciata*, *El. atleticana*, *El. marcii* and *El. nigrita*) and one *Eufriesea* species (*Eu. nordestina*). However, bees of these species never approached nor visited flowers of *C. arietinum*. These findings represent another example of highly specific pollinator attraction via distinct floral blends, a feature that is a major criterion for pre-mating reproductive isolation in *Catasetum* and other perfume-rewarding plants (Williams and Dodson 1972; Ramírez *et al.* 2011; Milet-Pinheiro and Gerlach 2017).

Euglossine bees are known to be attracted by floral perfumes over long distances, indicating that scent-seeking behaviour might be initially driven by the amount of scent emitted by a whole inflorescence, rather than the scent of individual flowers. This is supported by our observations that male euglossine bees approach male and female inflorescences of *C. arietinum* in similar proportions. Curiously, after approaching inflorescences, bees land significantly more on female flowers, suggesting that bees prefer female flowers and discriminate them from male flowers. These behavioural observations are consistent with our results showing that female single flowers of *C. arietinum* emit a considerably higher total amount of scent than male flowers. Since male and female flowers of this *Catasetum* species are similar in size, the discrepancy in scent emission cannot be a simple allometric consequence of larger flower size (see, e.g., Ashman 2009). Moreover, in perfume-rewarding plants, floral perfumes are exceptionally important because they act as both attractant and reward (Dressler 1982). Consequently, the higher total amount of floral perfumes in female single flowers of *C. arietinum* might lead to a higher foraging efficiency for bees, since the same amount of perfumes can be collected by visiting a few female flowers as opposed to many male flowers. Thus, female flowers represent a more profitable floral reward compared with male flowers. This might affect the behaviour of euglossine pollinators (i.e. choice between landing/not landing on male or female flowers) at the short-distance level, explaining the observed higher frequency of landings on female flowers.

Interestingly, former studies on the scent emission and visitation rates of sexually dimorphic plants that offer rewards other than scent (i.e. nectar and pollen) provide results that contrast with those reported here. In *Fragaria virginiana* (Rosaceae), for example, hermaphroditic flowers that possess functional male organs usually produce a higher amount of scent and/or a larger number of compounds than female flowers (Ashman *et al.* 2005). Further, female flowers have been suggested to suffer inadequate visitation or are even avoided by pollinators (Williams *et al.* 2000; Ashman and Diefenderfer 2001; Ashman 2009). The various findings for the ratios of scent emission and visitation frequency of male and female flowers

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Table 2. Relative amount (mean ± SD) of volatile compounds in headspace samples of male (*N* = 11) and female (*N* = 10) flowers of *C. arietinum*. Volatiles are grouped in compound classes and listed according to elution on an HP-5 column. KRI: Kovats retention index. Trace amounts: tr < 0.1 %. *n* = number of sampled flowers in which compounds were recorded. *Identification based on authentic standards.

Compounds	KRI	Male (mean ± SD)	*n*	Female (mean ± SD)	*n*
Aliphatics					
Methyl hexanoate	927	0.2 ± 0.9	(4/11)	tr ± 0	(2/10)
Aromatics					
Styrene*	894	1 ± 3	(10/11)	0.1 ± 0.2	(7/10)
Anisole*	917	0.5 ± 2.2	(5/11)	tr ± 0	(1/10)
Methyl benzoate*	1089	2.8 ± 4.8	(5/11)	0	(0/10)
p-Vinylanisole*	1158	0	(0/11)	0.5 ± 1.6	(3/10)
1,4-Dimethoxybenzene*	1167	0	(0/11)	0.1 ± 0.3	(3/10)
Benzyl acetate*	1169	0.5 ± 2.5	(2/11)	tr ± 0	(1/10)
Methyl salicylate*	1191	0.2 ± 0.6	(4/11)	0.1 ± 0.4	(7/10)
Methyl *p*-anisate*	1373	0.8 ± 1.3	(5/11)	tr ± 0.1	(3/10)
(*E*)-Methyl cinnamate*	1378	tr ± 0.3	(2/11)	0	(0/10)
(*Z*)-Methyl-*p*-methoxycinnamate*	1593	18.6 ± 20.9	(8/11)	7.2 ± 12.2	(7/10)
(*E*)-Methyl-*p*-methoxycinnamate*	1684	3.9 ± 4.5	(8/11)	6 ± 11	(6/10)
Irregular terpenes					
6-Methylhept-5-en-2-one*	985	4.5 ± 4.3	(11/11)	5.2 ± 7	(10/10)
Isoprenoid hydrocarbons					
β-Springene	1920	0.5 ± 1.1	(6/11)	2.2 ± 2.5	(9/10)
α-Springene	1969	tr ± 0.1	(4/11)	0.2 ± 0.2	(8/10)
Monoterpenes					
α-Pinene*	939	0.1 ± 0.2	(6/11)	0.2 ± 0.5	(3/10)
Camphene*	954	0.7 ± 2.4	(8/11)	tr ± 0.1	(4/10)
Sabinene*	975	tr ± 0	(2/11)	0.2 ± 0.6	(2/10)
β-Myrcene*	990	tr ± 0.1	(1/11)	0.4 ± 1.1	(2/10)
α-Terpinene*	1017	0.1 ± 0.3	(5/11)	tr ± 0	(1/10)
p-Cymene*	1024	0.7 ± 1.8	(8/11)	0.2 ± 0.5	(5/10)
Limonene*	1029	2.2 ± 4.5	(10/11)	0.5 ± 1.1	(8/10)
Eucalyptol*	1031	3.2 ± 6.5	(11/11)	4.9 ± 12.2	(9/10)
(*E*)-β-Ocimene*	1050	0	(0/11)	0.1 ± 0.2	(4/10)
Bergamal	1056	0.1 ± 0.2	(7/11)	0.3 ± 0.4	(9/10)
Linalool*	1096	0	(0/11)	tr ± 0	(5/10)
(*E*)-4,8-Dimethyl-1,3,7-nonatriene*	1118	0	(0/11)	0.2 ± 0.4	(8/10)
Geranylacetone*	1455	1.8 ± 2.2	(10/ 11)	2 ± 1.7	(10/10)
(*E*,*E*)-Geranyl linalool	2027	tr ± 0	(1/ 11)	0.1 ± 0.1	(8/10)
(*E*)-Geranyl geraniol*	2192	3.3 ± 12.2	(5/ 11)	21.2 ± 24.8	(10/10)
N-containing compounds					
Indole*	1291	tr ± 0.1	(2/ 11)	0.4 ± 1.7	(3/10)
Sesquiterpenes					
Lavender lactone	1039	0	(0/ 11)	tr ± 0	(5/10)
α-Copaene*	1376	tr ± 0.1	(2/ 11)	tr ± 0.1	(3/10)
7-epi-Sesquitujene*	1391	0.1 ± 0.2	(5/ 11)	0.2 ± 0.4	(3/10)
Sesquitujene	1405	0	(0/ 11)	tr ± 0	(2/10)
(*Z*)-α-Bergamotene	1412	0.1 ± 0.2	(4/ 11)	0.1 ± 0.2	(3/10)
α-Dihydro ionone	1412	tr ± 0.1	(1/ 11)	tr ± 0	(1/10)
(*E*)-β-Caryophyllene*	1429	0.1 ± 0.3	(7/ 11)	tr ± 0.1	(3/10)
(*E*)-α-Bergamotene	1434	10.4 ± 15.8	(6/ 11)	8.3 ± 15.7	(6/10)
7-epi-β-Santalene	1447	tr ± 0	(1/ 11)	tr ± 0	(3/10)
(*E*)-β-Farnesene*	1456	0.5 ± 0.5	(11/ 11)	0.7 ± 0.5	(10/10)
ar-Curcumene	1480	tr ± 0.1	(3/ 11)	0.1 ± 0.2	(3/10)
γ-Curcumene	1482	tr ± 0	(1/ 11)	tr ± 0.1	(2/10)
(*E*)-β-Bergamotene	1487	0.2 ± 0.4	(4/ 11)	0.3 ± 0.6	(3/10)
(*Z*,*Z*)-α-Farnesene*	1493	0.4 ± 0.7	(8/ 11)	0.8 ± 0.9	(9/10)
α-Zingiberene	1493	tr ± 0.1	(2/ 11)	tr ± 0.2	(1/10)
(*E*,*E*)-α-Farnesene*	1505	24.3 ± 25.9	(9/ 11)	23.2 ± 23.1	(9/10)
β-Bisabolene*	1505	10.9 ± 18	(5/ 11)	4.2 ± 7.9	(3/10)
β-Curcumene	1515	tr ± 0	(2/11)	0.2 ± 0.4	(3/10)
Sesquicineole	1516	0.1 ± 0.3	(5/11)	tr ± 0.1	(3/10)
β-Sesquiphellandrene	1522	0.6 ± 1	(6/11)	0.4 ± 0.6	(6/10)
(*E*)-Nerolidol*	1563	0	(0/11)	tr ± 0	(3/10)
Unknown compounds					
38 unknown compounds		6.2 ± 13.8		9 ± 14.1	

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might be the result of the respective male gamete-dispersing strategy. For plants with male flowers that continuously offer a small amount of pollen to pollinators, these flowers require frequent visits. Thus, male flowers of non-perfume-rewarding dimorphic plants have to keep up their attraction to pollinators, possibly by the emission of a higher amount of floral scent. In *Catasetum* (and other perfume-rewarding plants), however, a single visit by pollinators to a male flower may be enough to remove the pollinarium and, consequently, all male gametes at once (Dodson 1962; Holst 1999). Usually, euglossine pollinators land directly on the labellum of male flowers of *Catasetum* which is almost the correct position to trigger the attachment of the pollinarium (see, e.g., Johnson and Edwards 2000). Contrary, the deposition of pollinaria on female flowers might be more difficult, because euglossine bees can access the labellum of female flowers from different positions and it requires the visiting bee to crawl backwards outside the cavity of the labellum, exactly at the position at which the stigmatic cleft is located on a lid. This idea is also supported by our results, showing a higher proportion of attached pollinaria on male flowers in comparison to the deposition of pollinaria on female flowers of *C. arietinum*. These findings indicate that the deposition of a pollinarium might rely on multiple visits by a pollinator and/or on an extended handling time on the flowers which might be achieved by a higher emission of floral scent in female flowers.

Additional to the sexual dimorphism in total scent emision between male and female flowers, the chemical quantification revealed a clear fluctuation in scent emission by flowers of *C. arietinum*, a phenomenon that is commonly reported in angiosperms (e.g. Altenburger and Matile 1988, 1990; Jakobsen and Olsen 1994). The total amount of scent emitted by flowers of *C. arietinum* over the day matches the time of foraging activity of *Euglossa* bees as reported in our observations on male and female flowers. Moreover, our results are in accordance with those of Milet-Pinheiro *et al.* (2015) for *C. uncatum* and also with those of earlier studies on daily foraging patterns of other *Euglossa* species on artificial scent baits (Armbruster and McCormick 1990; Armbruster and Berg 1994). The coordination between the peak of scent emission and the activity of pollinators is assumed to be a result of the interplay between thermal ecology of euglossine bees (Armbruster and Berg 1994) and temperature effects of the plant's circadian rhythm (Altenburger and Matile 1988). Considering that neurological biases of male orchid bees shape the evolution of floral scent chemistry of perfume-rewarding plants (Ramírez *et al.* 2011; Schiestl and Johnson 2013), it is reasonable to assume that timing of floral scents in *Catasetum* is an adaptive response to behavioural biases of pollinators (i.e. time of foraging activity), in order to achieve maximum resource efficiency (Fenske and Imaizumi 2016). Furthermore, by producing a lower amount of scent when male orchid bees are less active, flowers of *C. arietinum* might be less apparent to herbivores without compromising pollinator attraction. Indeed, herbivores are known to be able to locate perfume-rewarding flowers by using the same compounds sought for by male orchid bees (Nunes *et al.* 2016).

The attraction and behaviour of insects are influenced not only by the total emission of scents, but also by its chemical composition (Ashman *et al.* 2005). Male euglossine bees are known to change their scent preference after collecting a specific compound intensively (Eltz *et al.* 2005a). Therefore, we find it particularly interesting that our chemical analyses have revealed not only a discrepancy in perfume chemistry between male and female flowers of *C. arietinum*, but also changes in the semi-quantitative and qualitative composition during the day. The production of distinct sex-specific floral scents at different times of the day enables different floral resources to be offered to euglossine pollinators in the form of variable chemical repertoires (see also Ashman 2009). Different relative amounts and/or the presence or absence of constituents within the floral scent might be important preconditions leading the bees to distinguish between male and female flowers and could explain the observed discrepancy in behaviour of euglossine bees on flowers of distinct sexes. In particular, their preference for landing on female flowers might be elicited by certain constituents (possibly above a specific threshold value) that occur within the floral scent and that are either produced exclusively in female flowers or are offered in higher amounts than in male flowers. However, such a divergence in perfumes of the distinct floral sexes and the time of day can only be selected if the floral scent continues to draw the same species of pollinators. If these conditions are met, sexual dimorphism in floral scents can stimulate bees to visit alternately flowers of different sexes, promoting constant cross-pollination (Dötterl *et al.* 2014). Our results suggest that this is indeed the case in *C. arietinum*, since we have been able to observe successful pollination in the investigated population, despite the sexual dimorphism in floral scent.

In accordance with our results for *C. arietinum*, a higher percentage of landings on female single flowers has been documented in the species *C. uncatum* (Milet-Pinheiro *et al.* (2015). However, in the case of *C. uncatum* no sexual dimorphism in floral scent composition was reported. Therefore, it is possible that the presence of further chemical compounds, not found in the headspace samples of *C. uncatum*, could influence the quality of floral reward and the behaviour of euglossine bees. Moreover, the occurrence of sexually mono- and dimorphic scent patterns within the genus *Catasetum* might reflect the outcome of distinct selective pressures (possibly in the form of different main pollinators). Similar results for the co-occurrence of sexual mono- and dimorphism within a given genus have previously been reported by Huang *et al.* (2015) for various *Glochidion* species, showing that host specificity can lead to distinct evolutionary outcomes in closely related species. An investigation of the floral scent bouquets of additional *Catasetum* species, especially with regard to the potential sexual dimorphism in floral scents, might lead to deeper insights into its evolutionary significance for the genus.

The finding that both the total emission and composition of the scents of male and female flowers differ in *C. arietinum*, but not in *C. uncatum* (Milet-Pinheiro *et al.* 2015), further leads to the assumption that the phenomenon described by Romero and Nelson (1986) influences the frequency and behaviour of visiting euglossine pollinators; the authors assume that the attachment of a pollinarium to a bee on male flowers of *Catasetum* causes aversive behaviour, resulting in fewer visits to other male flowers. Therefore, if male bees learn to associate male-specific perfumes with the traumatic event of pollinarium attachment, they will avoid male flowers, whereas female flowers can be visited with no such adverse effects. Such behaviour would increase both the direction of visits from male to female flowers and the chance of pollination of female flowers by a pollinarium-carrying bee, which finally might impact positively the plant fitness (Romero and Nelson 1986; Milet-Pinheiro and Gerlach 2017). Future experimental bioassays testing this potential aversive behaviour of male euglossine bees might shed some further light onto this question.

Because attraction of the same visitor to both male and female flowers is essential for conspecific pollen transfer,

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olfactory cues are usually similar between the floral sexes in dioecious plants (Fenster *et al.* 2004). However, examples also exist showing that, under the precondition that the same pollinating species is attracted to flowers or floral structures of distinct sexes, sexually dimorphic floral scent could have evolved to signal an alternative reward (not only distinct floral perfumes, but also nectar, pollen, etc.) provided by each sex, driving pollinators to visit both male and female flowers (Ashman 2009; Okamoto *et al.* 2013). Our study offers a further remarkable example of a distinct total emission and composition of scent between male and female flowers of a dioecious plant and demonstrates, for the first time, sexual dimorphism in orchid floral perfumes.

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Contributions by the Authors

Conceived and designed experiments: P.M.P., M.A., S.D., K.B. Performed the experiments: K.B., P.M.P. Analysed the data: K.B., P.M.P. Wrote the paper: K.B. Revised the paper: P.M.P., S.D., M.A., I.C.M., D.M.A.F.N.

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Supporting Information

The following additional information is available in the online version of this article—

Method M1. Climate at the study site.

Method M2. Flower morphology.

Method M3. Staining of osmophores.

Method M4. Determination of sex ratio (SR) and operational sex ratio (OSR).

Method M5. Sampling of floral volatiles.

Method M6. Chemical analysis of thermal desorption (TD) samples.

Method M7. Permutational analyses (PERMANOVA).

Results R1. Other flower visitors of Catasetum arietinum.

Results R2. Pollinarium attachment and deposition per pollinator species.

Results R3. Qualitative analysis of floral scent bouquet.

Data D1. Data needed to reproduce all analyses.

Data

All data necessary to reproduce any analyses in this study has been provided as **Supporting Information Data D1**.

Conflict of Interest

The authors declare that there is no conflict of interest.

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