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Pollination biology in the dioecious orchid *Catasetum uncatum*: How does floral scent influence the behaviour of pollinators?

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ABSTRACT

Catasetum is a neotropical orchid genus that comprises about 160 dioecious species with a remarkable sexual dimorphism in floral morphology. Flowers of *Catasetum* produce perfumes as rewards, which are collected only by male euglossine bees. Currently, floral scents are known to be involved in the selective attraction of specific euglossine species. However, sexual dimorphism in floral scent and its eventual role in the pollination of *Catasetum* species have never been investigated. Here, we have investigated the pollination of *Catasetum uncatum* and asked: (1) Is floral scent a sexual dimorphic trait? (2) Does pollinarium removal/deposition affect scent emission? (3) Does sexual dimorphism in floral scent and changed scent emission have implications with regard to the behaviour of the pollinators? The frequency and behaviour of floral visitors were observed in non-manipulated flowers (both flower sexes) and in manipulated flowers (pistillate only) in which pollinaria were deposited. Scents of staminate and pistillate flowers (both manipulated and non-manipulated) were collected by using dynamic headspace methods and analysed chemically. Electrophysiological analyses were performed to detect compounds triggering antennal depolarisation in the euglossine species. *C. uncatum* is pollinated mainly by males of *Euglossa nanomelanotricha*. Pollinators were more frequent in pistillate than in staminate inflorescences. Bees approaching staminate flowers frequently flew away without visiting them, a behavioural pattern not observed in pistillate flowers. In the chemical analyses, we recorded 99 compounds, 31 of which triggered antennal depolarisation in pollinators. Multivariate analyses with the electrophysiological-active compounds did not detect differences between the scent composition of staminate and pistillate flowers. Pollinarium removal or deposition resulted in diminished scent emission within 24 h in staminate and pistillate flowers, respectively. Surprisingly, bees discriminated pollinated from non-pollinated pistillate flowers as early as 2 h after pollination. The rapid loss in the attractiveness of flowers following pollinarium removal/deposition can be interpreted as a strategy to direct pollinators to non-pollinated flowers. We have found no evidence that euglossine males discriminate staminate from pistillate flowers by means of floral scent. Instead, we speculate that bees use visual cues, such as sex dimorphic traits, to discriminate flowers of different sexes. Together, our results provide interesting insights into the evolution of floral signals in gender-dimorphic species and into its significance in plant reproductive biology.

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1. Introduction

One of the most fascinating pollination mechanism has evolved among the thousands of neotropical orchids. Some 600 species from at least 55 genera offer only perfumes as floral resources

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(Dressler, 1982; Ramirez et al., 2002), which are collected by male euglossine bees (Apidae: Euglossini), the so-called orchid bees (Vogel, 1966). Males of these bees have unique adaptations to gather and store perfumes. The stored perfumes are then exposed later in perching sites during courtship display, most probably to attract conspecific females (Eltz et al., 2005b; Ramírez, 2009).

Among the perfume-producing orchids, *Catasetum* is particular, because it is one of the few genera (besides *Cycnoches* and *Mormodes* pro parte) in which species are dioecious. Pistillate (female) and staminate (male) flowers of all *Catasetum* species are characterised by a pronounced sexual dimorphism that is involved in a highly specialised pollination mechanism (Dodson, 1962; Dressler, 1981, 1993; Gerlach, 2007; Williams and Whitten, 1983). Staminate flowers of *Catasetum* species bear a catapult-like apparatus that is triggered when a male euglossine bee collects perfume from the osmophores at the labellum, an action that results in the attachment of the pollinarium to its body. Thereby, the bee is forcibly expelled from the flower and flies away with an attached pollinarium. A subsequent visit to a receptive pistillate flower, again for the purpose of perfume collection, may result in pollination (Dodson, 1962).

Catasetum is a species-diverse taxon that embraces about 160 species (Petini-Benelli, 2012). In this genus, reproductive isolation is frequently achieved by pollinator shifts and floral scents are known to play a pivotal role in this sense (Hills et al., 1972). Floral scents of *Catasetum* species have been studied since the late 1960s (Gerlach and Schill, 1991; Hills et al., 1968, 1972; Kaiser, 1993). The pattern emerging from these studies suggests that scent composition is generally species-specific, in spite of the ubiquitous presence of some widespread dominant compounds. This specific scent composition seems to select for specific pollinators, since synthetic compounds offered alone attract more orchid bee species than a mixture of two or more compounds (Williams and Dodson, 1972; Williams and Whitten, 1983). Indeed, *Catasetum* species are generally pollinated by a single or few euglossine species, even in the presence of dozens of syntopic species (Ackerman, 1983; Carvalho and Machado, 2002; Dodson and Frymire, 1961; Gerlach, 2007; Williams and Dodson, 1972; Williams and Whitten, 1983). Recently, Ramírez et al. (2011) have shown that fragrance-producing orchids originated at least three times independently after their fragrance-collecting bee mutualists, suggesting that flower scents evolved under the pressure of the pre-existing sensory biases of their pollinators (see also Schiestl, 2010; Schiestl and Dötterl, 2012). Thus, we can reasonably speculate that each euglossine species exhibits its own olfactory biases (e.g., chemical receptors in the antennae and/or neurological interconnections in the brain) that would explain the preference for the distinct scent-specific *Catasetum* species.

In addition to their pivotal role in the selective attraction of pollinators, floral scents are also of great significance for the reproductive success of dioecious plants, mainly those pollinated by animals. For these plants, it is essential that flowers of both sexes attract the same pollinator species to secure conspecific pollen transfer (Dötterl et al., 2014; Fenster et al., 2004); this might result in selection to reduce sexual divergency. The strength and direction of selection (e.g., convergence or advergence) on floral scents of staminate and pistillate flowers vary greatly and depend on the reward offered by each sex and on the intimacy of the relationship with their pollinators (Ashman, 2009). Selection for intersexual chemical mimicry, for example, is reported for some dioecious species and is particularly strong when one of the sexes (usually female) is non-rewarding, thereby attracting pollinators by mimicking the floral scents of the rewarding sex (Raguso, 2003; Soler et al., 2012). By contrast, selection for divergence can occur if sex-specific scents indicate different rewards that are sought by pollinators for distinct purposes and if this results in more efficient pollen transfer, without disrupting the interaction with the specific pollinator (Okamoto et al., 2013). In the genus *Catasetum* in which flowers of both sexes offer perfume as a reward, we might expect that scents of pistillate and staminate flowers resemble each other to assure the attraction of the same euglossine species (see above).

In a recent work, euglossine males were shown to change their scent preference after collecting a given compound intensively (Eltz et al., 2005a). Consequently, selection for sexual divergence might occur if subtle dimorphism in flower scent induces a greater flow of pollinators from staminate to pistillate flowers (or vice versa) as males become "satiated" with a compound (or bouquet) that is typical for a given sex and if this results in increased plant fitness. Moreover, in *Catasetum* species, the pollinarium attachment and the catapult mechanism are assumed to cause aversive behaviour in euglossine pollinators, which avoid visiting further staminate flowers, but not pistillate flowers (Romero and Nelson, 1986). Obviously, this implies that pollinators are able to differentiate between staminate and pistillate flowers possibly by dimorphism in floral scent.

In perfume-producing orchids, pollinarium removal or deposition commonly leads to diminished scent emission and flower longevity, which ultimately result in flowers being no longer attractive to pollinators (Carvalho and Machado, 2002; Dodson, 1962; Hills et al., 1999; Martini et al., 2003; van der Pijl and Dodson, 1969). This phenomenon has an important implication for the reproductive success of the plants, since euglossine males then forage preferably for non-pollinated flowers. Curiously, the consensus that scent emission is reduced after pollinarium removal or deposition in *Catasetum* (and other perfume-producing orchids) is based mainly on human olfaction (see, for example, Carvalho and Machado, 2002; Dodson, 1962; Janzen, 1981; Martini et al., 2003; van der Pijl and Dodson, 1969) and not on quantitative analysis. Similarly, studies that experimentally associate a reduction of scent emission to a diminished attractiveness of flowers to pollinators are scarce. Thus, the exact chemical changes in floral scent production and composition after pollinarium removal/deposition and their implication with regard to the floral attractiveness to pollinators await further experimental investigations.

In this study, we have investigated the pollination ecology of *C. uncatum* Rolfe and its interaction with euglossine flower visitors. This epiphytic species is abundant in the Catimbau National Park in Pernambuco (Northeast Brazil), where it grows exclusively on the stipes of short palms (1–3 m high) of *Syagrus coronata* (Mart.) Becc. (Arecaceae) (Fig. 1a). These circumstances allow the detailed investigation of individuals in a natural population, something that is otherwise difficult for most *Catasetum* species, which are frequently rare and associated with the canopy environment in neotropical rainforests. Furthermore, in the Catimbau National Park, only three euglossine species occur (Schlindwein et al. unpubl. res.), which facilitates a comparative approach to the way that co-occurring euglossine species perceive the flower scents of this orchid by means of antennal receptors. In this scenario, we have performed a comprehensive multi-faceted study combining chemical and electrophysiological analyses with behavioural assays to investigate the role of floral scents in several aspects of the pollination ecology of the dioecious *C. uncatum*. We addressed the following questions: (1) Which flower visitors are the effective pollinators? (2) Do pollinators differentiate staminate from pistillate flowers? (3) Is the floral scent composition a sexually dimorphic character? (4) Do electrophysiological antennal responses to *C. uncatum* floral scents differ between visiting and co-occurring non-visiting euglossine bees? (5) Does pollinarium removal and/or deposition in staminate and pistillate flowers, respectively, interfere in flower longevity, scent emission and attractiveness to pollinators?

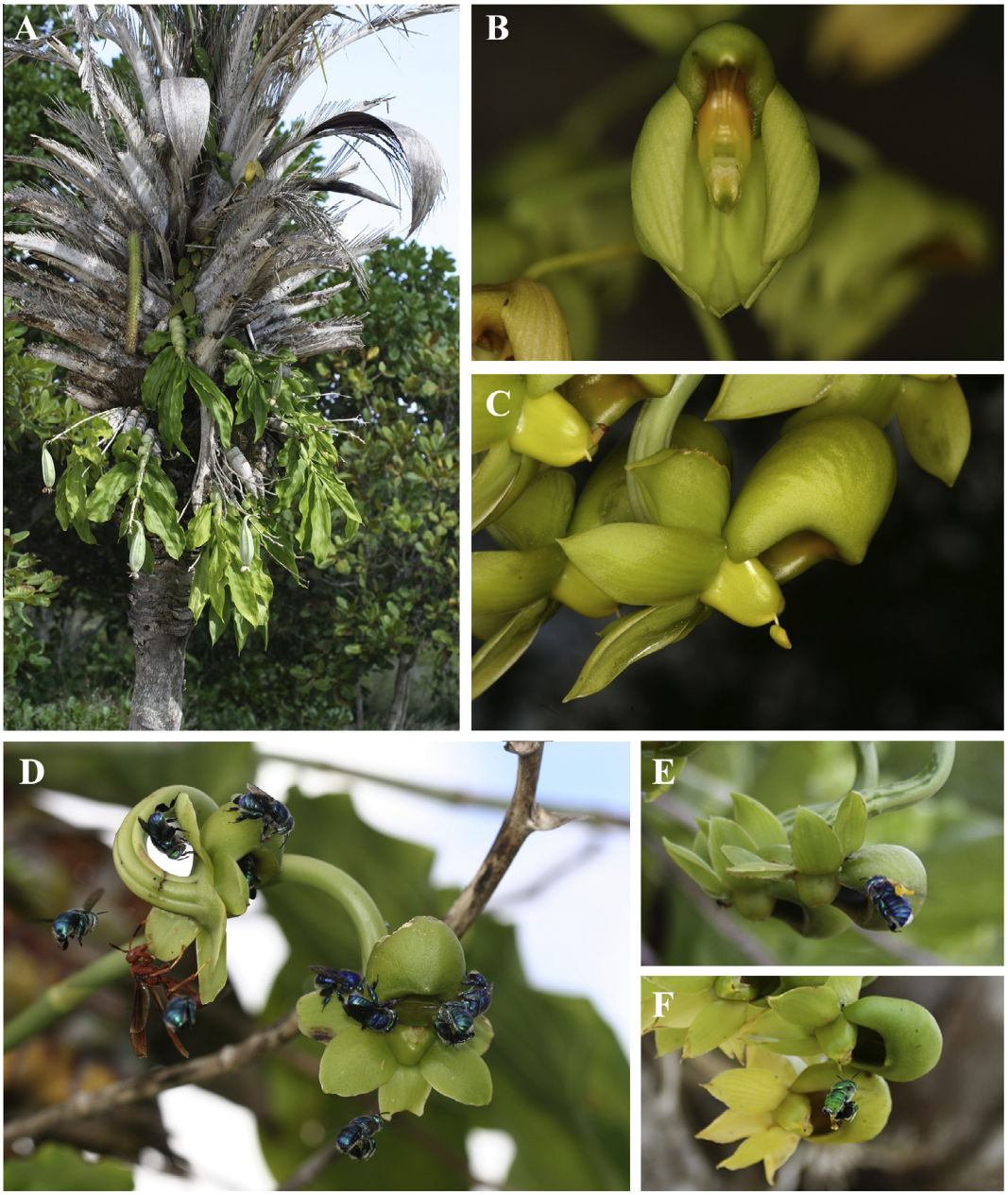

Fig. 1. *Catasetum uncatum* growing on palm stipes of *Syagrus coronata* (A). Staminate (B) and pistillate (C) flowers of *C. uncatum*, showing a remarkable sexual dimorphism. Euglossine pollinators of *C. uncatum*: *Euglossa nanomelanotricha* (D and E) and *Eg. carolina* (F). A pollinarium attached to the thorax of *Eg. nanomelanotricha* (E).

2. Results

2.1. Flower biology

Staminate and pistillate flowers of *C. uncatum* are green, becoming yellowish throughout their lifespan, and show a morphological sexual dimorphism (Fig. 1b and c). The osmophores are situated on the inner side of the labellum in both staminate and pistillate flowers. Blooming begins early in March and lasts until late May. The flowers open from the base to the top of the inflorescences continuously through the day and all flowers of a given inflorescence are open only after 2–3 days. Staminate flowers start scent emission (as detectable by the human nose) a few hours after flower opening, whereas pistillate flowers are scented after 3 d. The average longevity of bagged staminate (10 ± 1.6 days) and pistillate (30 ± 2.5 days) flowers differs significantly (*t*-test: $T = -42.4$, d.f. = 109, $P < 0.001$). The senescence event in staminate and pistillate flowers is triggered by the removal and deposition of the pollinarium, respectively, also resulting in reduced life times. In staminate flowers, the first signals of senescence (i.e., wilting and colour change from greenish to yellowish) are perceivable as soon as 2 h after pollinarium removal. In pistillate flowers, the same senescence signals are observed only by one day after pollination. Staminate and pistillate flowers wither completely 3 and 5 days after pollinarium removal or deposition, respectively. To the human nose, a reduction in scent emission following pollinarium removal or deposition is only perceived on the following day (for chemical details see below).

Among the 43 inflorescences used to determine the sexual ratio, 24 were staminate and 19 pistillate. Staminate inflorescences had on average more flowers than pistillate ones (17 vs. 5; $T = 10.03$, d.f. = 41, $P < 0.001$), reflecting a sexual ratio of flowers of 3.4:1.

2.2. Pollination ecology

Only males of two euglossine species were observed collecting scents from flowers of *C. uncatum*: *Euglossa nanomelanotricha* and *Eg. carolina* (Fig. 1d–f). In spite of the different flower morphology of staminate and pistillate flowers, the euglossine bees displayed a highly similar perfume-collecting behaviour at flowers of both sexes. The bees approached and hovered in front of the flowers for a few seconds, alighted at the column or directly at the labellum, entered the labellum in an upside-down position and then scratched its interior surface with the long hairs of their anterior tarsi. At this moment, either removal or deposition of pollinarium could occur, depending on whether the bee was visiting a staminate or pistillate flower, respectively. While scratching the inner surface of the labellum of staminate flowers (where the osmophores are situated), the bees could contact one or both floral antennae (lateral extensions of the rostellum that hold the anther cap and stipe of the pollinarium under tension), triggering the ejection of the pollinarium. The pollinarium was attached to the mesoscutum of the thorax of the bee by the non-removable glue of the viscidium. Carrying a pollinarium, the bees became potential pollinators of pistillate flowers. After gathering scents in a pistillate flower, a process that takes on average 21 s ± 3.3 s.d. (N = 74 visits), the bees backed out of the flower and, during this movement, the pollinarium could be inserted into the stigmatic slit in the apical portion of the column (for a scheme, see Dodson, 1962). After leaving a flower, bees hovered for a few seconds (mean 3.7 s ± 1.8 s.d., N = 74 visits) in front of it, while transferring the volatiles from the fore- to the mid- and, finally, to the hind legs.

Bee scent-collecting behaviour was shown to be essentially the same in staminate or pistillate flowers; however, after leaving a flower, the bees behaved differently. In the case of the abrupt event of pollinarium attachment, bees were forcibly expelled from the staminate flowers (about 30 cm) and promptly flew away. Contrastingly, during visits to pistillate flowers, the bees were never catapulted out of the flowers and frequently visited consecutively the same flower (on average 7.5 visits ± 3.2 s.d., N = 20). Furthermore, bees remained for a long time at a given pistillate inflorescence (on average 11.5 min ± 9.9 s.d., N = 20), collecting scents from all flowers. Bees approaching staminate flowers frequently flew away without visiting them, a behaviour pattern that we did not observe in pistillate flowers.

The frequency of visits also differed between staminate and pistillate inflorescences. Males of *Eg. nanomelanotricha* and *Eg. carolina* visited significantly more pistillate than staminate inflorescences (75 vs. 11 visits; PERMANOVA: Pseudo-F_1 = 19.5, P < 0.01). Frequency of visits varied significantly through the day (Pseudo-F = 17.7, P < 0.001) and pairwise comparisons indicated that visits to flowers were more abundant between 0800 h and 1200 h (Fig. 2). The effect of daytime on the frequency of visits during the day was different for staminate and pistillate flowers (Pseudo-$F_{1,5}$ = 10.3, P < 0.001). Whereas visits to pistillate flowers varied significantly throughout the day, visits to staminate flowers were constant. We observed, however, a marginal difference in the frequency of visits to staminate flowers between the time interval 1000–1200 h and the intervals 0600–0800 h, 1400–1600 h and 1600–1800 h (Fig. 2).

Eg. nanomelanotricha was by far the most frequent pollinator. Out of the 86 recorded flower visits, 89% were from males of this species (88% thereof to pistillate and 12% to staminate flowers). Males of *Eg. carolina* accounted for nine visits, from which seven were to pistillate and two to staminate flowers. Although abundant at the study site (Schlindwein et al. unpubl. res.), we did not observe males of *Eulaema nigrita* visiting flowers of *C. uncatum*. The fruit set of marked flowers exposed to pollinators was 20% (N = 135).

2.3. Field bioassays

The choice bioassays performed in the field to evaluate the effect of pollination in flower attractiveness showed that non-pollinated pistillate flowers attracted more males of *Eg. nanomelanotricha* than pollinated pistillate flowers, during the 2 h of observation after pollinarium deposition (47 vs. 25 flower visits;

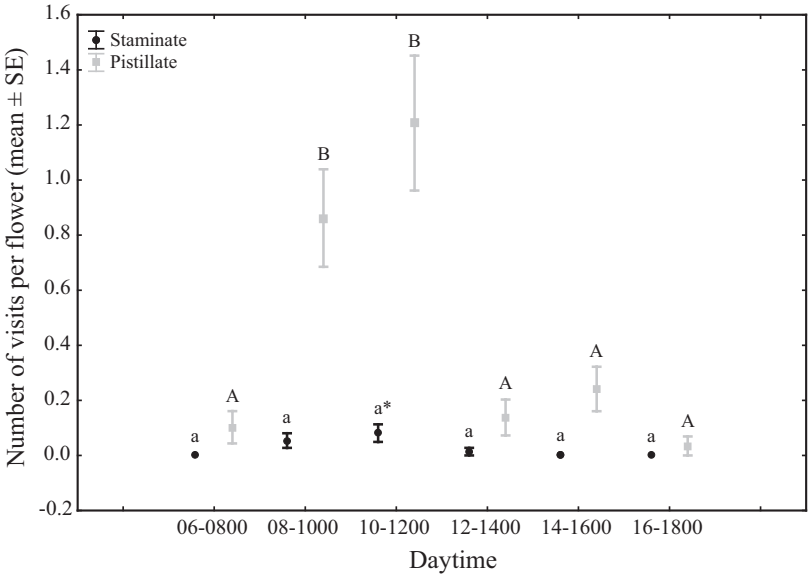

Fig. 2. Frequency of visits by male euglossine pollinators (*Euglossa nanomelanotricha* and *Eg. carolina* pooled) to staminate (N = 6; 74 flowers) and pistillate (N = 6; 29 flowers) inflorescences of *Catasetum uncatum* recorded throughout the day. Upper and low cases indicate pairwise comparisons during the day within pistillate and staminate flowers, respectively (PERMANOVA, P < 0.05). Asterisk indicates marginally significance differences between the time interval 1000–1200 h and the intervals 0600–0800 h (P = 0.058), 14–1600 h (P = 0.056) and 1600–1800 h (P = 0.052) as tested by PERMANOVA analyses.

Chi-square = 6.72, d.f. = 1, $P < 0.01$). Given the low number of visits by *Eg. nanomelanotricha* to staminate flowers, we performed this bioassay only with pistillate flowers.

2.4. Composition of the floral scent bouquet of C. uncatum

We identified 99 floral compounds overall in *C. uncatum*, including monoterpenes (37), sesquiterpenes (30), aromatics (22), aliphatics (1) and to nitrogen-containing compounds (1) (Table 1). Eight compounds were not assigned to specific classes. Thirteen compounds were found exclusively in pistillate flowers and eight exclusively in staminate flowers (Table 1). However, these compounds were not necessarily found in all samples of a given sex and were frequently recorded only in trace amounts.

Monoterpenes were the dominant (relative amount) compound class in the scent bouquet of both staminate and pistillate flowers, followed by aromatics and sesquiterpenes. They contributed, respectively, to 44.2%, 32.3% and 2.8% in median to the total amount of scent emitted by staminate flowers, and to 20.5%, 4.1% and 0.8% to the total amount of scent emitted by pistillate flowers. Aliphatics and N-compounds were found only in trace amounts in flowers of both sexes. Eucalyptol and veratrole were by far the most abundant compounds of the scent bouquet in both flower sexes (Table 1).

2.5. Electrophysiology

The GC–EAD analyses revealed that the antennae of male bees of *Eg. carolina*, *Eg. nanomelanotricha* and *El. nigrita* responded similarly to the compounds found in the scent of staminate flowers of *C. uncatum*. We recorded 25 antennal responses in individuals of the three species, with most of them occurring in all three species (Fig. 3). Since some of the compounds co-eluted in these analyses, the antennal responses were not necessarily related to a single compound. The compounds triggering antennal responses were aromatics (15 compounds), monoterpenes (10), sesquiterpenes (5) and one N-compound. We found three EAD-active compounds that occurred only in some of the staminate but not in pistillate flowers, namely benzyl acetate, methyl o-anisate and 3,4-dimethoxybenzaldehyde.

2.6. Scent emission and composition in staminate and pistillate flowers

The total amount of scent emitted by staminate (median: 147 ng/flower/min) and pistillate flowers (44.9 ng/flower/min) did not differ significantly ($F_{1,18} = 0.003$, $P = 0.96$). Scent emission varied greatly among individuals, ranging from 4 to 285 ng/flower/min in staminate flowers and from 2 to 779 ng/flower/min in pistillate flowers.

Similarly, the composition of scent bouquets of staminate and pistillate flowers did not differ significantly either qualitatively (PERMANOVA: Pseudo-$F_1 = 2.03$, $P = 0.08$) or semi-quantitatively (PERMANOVA: Pseudo-$F_1 = 1.42$, $P = 0.2$). Furthermore, the variability (dispersion) in the semi-ntitative (PERMDISP: $F_{1,16} = 4.3$, $P = 0.1$) and qualitative (PERMDISP: $F_{1,16} = 4.3$, $P = 0.23$) scent pattern of staminate and pistillate flowers did not differ significantly (Fig. 4).

2.7. Influence of daytime and removal/deposition of pollinarium on scent emission

The total amount of scent emitted by flowers varied significantly throughout the day [$F_{3,54} = 18.8$, $P < 0.001$; Fig. 5]; flowers emitted on average a larger amount of scents in the morning than in the afternoon. No differences were found between the sexes [$F_{1,18} = 0.002$, $P = 0.96$] and treatments [i.e., removal/deposition of pollinarium; $F_{1,18} = 1.7$, $P = 0.2$]. Effects of daytime [interaction sex × daytime; $F_{3,54} = 0.69$, $P = 0.56$] and treatment [interaction sex × treatment; $F_{1,18} = 0.0003$, $P = 0.98$] were the same in both sexes. Non-manipulated and manipulated flowers showed similar changes in scent emission during the course of the day, in which flowers were treated. In the morning following pollinarium removal/deposition, however, manipulated flowers produced a lower amount of scents than in the previous morning, whereas non-manipulated flowers produced a similar high amount of volatiles [interaction daytime × treatment; $F_{3,54} = 11$, $P < 0.001$; Fig. 5].

Qualitatively, scent pattern changed in response to daytime (PERMANOVA: Pseudo-$F_{3,94} = 6.7$, $P < 0.001$) and treatment (Pseudo-$F_{1,94} = 10.8$, $P < 0.001$) and to the interaction of these two factors (Pseudo-$F_{3,94} = 10.9$, $P < 0.001$). Effects of daytime (interaction sex × daytime; Pseudo-$F_{3,94} = 1$, $P = 0.46$) and treatment (interaction sex × treatment; Pseudo-$F_{1,94} = 1.2$, $P = 0.36$) were the same in both sexes.

Semi-quantitatively, scent pattern changed in response to daytime (Pseudo-$F_{3,94} = 2.7$, $P < 0.01$), treatment (Pseudo-$F_{1,94} = 2.9$, $P < 0.05$) and to the interaction of these two factors (Pseudo-$F_{3,94} = 2.9$, $P < 0.01$). Effects of daytime (interaction sex × daytime; Pseudo-$F_{3,94} = 1.1$, $P = 0.36$) and treatment (interaction sex × treatment; Pseudo-$F_{1,94} = 1.4$, $P = 0.23$) were the same in both sexes.

3. Discussion

Our results showed that flowers of *C. uncatum* attracted exclusively males of two *Euglossa* species, whereas they did not stimulate visitations of the locally abundant third euglossine species, *El. nigrita*. We also found that visits were much more frequent to pistillate than to staminate flowers, in spite of the much higher number of the latter flowers in the population. This indicates that male bees discriminate flowers of the two sexes and prefer pistillate over staminate flowers. However, we have found no evidence that males are chemically guided to visit pistillate flowers after visiting staminate flowers, since statistical comparisons considering EAD-active compounds did not reveal clear sexual dimorphism (either in total amount or in scent composition and temporal scent pattern) in floral scents of *C. uncatum*. Finally, we show that scent emission fluctuates during the course of a day and that pollinarium removal/deposition results in diminished scent emission. Reduced scent emission in the afternoon and after pollination results in flowers being less attractive to pollinators and reflects also the natural behaviour of the euglossine bees being less active in collecting fragrances in the afternoon and from pollinated flowers.

3.1. Pollination mechanism, effective pollinators and natural fruit set

C. uncatum depends exclusively on male bees of *Eg. nanomelanotricha* and *Eg. carolina* as pollinators. These two species carried pollinaria and pollinated pistillate flowers and together accounted for a fruit set of 20%. However, males of *Eg. nanomelanotricha* were the main pollinators, since they were almost 10 times more frequent than those of *Eg. carolina*. Interestingly, males of the locally abundant *El. nigrita* neither visited nor approached flowers of *C. uncatum*, even if other plant species of the perfume-flower syndrome were scarce in the Catimbau National Park (Schlindwein et al. unpubl. res.). Clearly, some particular features in floral scents prevent visits by *El. nigrita*; possibly, attractive compounds are absent or deterrent compounds are present in the scent bouquet of *C. uncatum*. The second scenario is much more likely, since scent baits with the two major compounds of *C. uncatum* (i.e., eucalyptol and veratrole) attract *El. nigrita* in the field either alone or in mixture (Milet-Pinheiro, unpubl. res.). Furthermore, the experimental addition of skatole (the most potent known attractant of *El. nigrita*)

Table 1
Relative amount (median, minimum and maximum) of volatile compounds in staminate ($N = 9$) and pistillate ($N = 9$) non-manipulated flowers of *Catasetum uncatum*, 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 samples in which compounds were recorded.

Compounds	KRI	Staminate		Pistillate	
		Median (min/max)	*N*	Median (min/max)	*N*
Aliphatics					
(*E*)-2-Nonenal	1164	0 (0/0.2)	1	0 (0/0.4)	1
Aromatics					
Anisole	918	0.1 (0/1.6)	5	tr (0/0.4)	5
Benzaldehyde*	966	0 (0/0.1)	1	–	0
Guaiacol*	1088	0.5 (0/5.4)	8	0.1 (0/34.5)	6
Methyl benzoate*	1098	0.3 (0/6.6)	8	0 (0/1.5)	3
Veratrole*	1150	18.7 (0/57.1)	8	3.14 (0.1/92)	9
Benzyl acetate*	1169	0 (0/0.2)	3	–	0
Ethyl benzoate*	1173	0 (0/0.1)	2	0 (0/3.2)	1
Methyl salicylate*	1197	0.3 (0.1/0.8)	9	tr (0/6.8)	6
Estragole	1200	–	0	0 (0/18.4)	3
3,4-Dimethoxytoluene	1234	–	0	0 (0/tr)	1
Chavicol	1253	–	0	0 (0/7)	3
p-Anisaldehyde	1261	tr (tr/0.1)	5	tr (tr/tr)	2
1,2,3-Trimethoxybenzene	1307	tr (tr/0.1)	3	tr (tr/0.1)	3
Methyl o-anisate	1336	0 (0/tr)	2	–	0
Eugenol*	1356	–	0	0 (0/0.1)	1
1,2,4-Trimethoxybenzene	1370	0 (0/tr)	1	0 (0/tr)	2
p-Methoxyphenyl ethyl alcohol	1370	0 (0/0.1)	1	0 (0/tr)	1
Methyl p-anisate	1378	0.1 (0/0.3)	8	tr (0/3.7)	6
(*E*)-Methyl cinnamate	1390	tr (0/0.1)	6	tr (0/3.3)	7
p-Anisyl acetate	1420	0 (0/0.1)	3	0 (0/tr)	1
Veratraldehyde	1481	0 (0/tr)	2	-	0
(*Z*)-Methyl-p-methoxycinnamate	1591	0.1 (0/2)	6	0.4 (0/9.1)	7
(*E*)-Methyl-p-methoxycinnamate	1678	0.4 (0/1)	7	0.6 (0.1/13.2)	9
Benzyl benzoate	1775	tr (0/0.2)	5	tr (0/0.8)	5
Monoterpenes					
α-Thujene	929	0.2 (0/0.8)	7	tr (0/0.4)	4
α-Pinene*	937	1.7 (0/4.8)	8	0.8 (0/4.4)	7
Sabinene*	976	1.7 (0/3)	8	0.3 (0/3.8)	6
ß-Pinene*	982	0 (0/1.5)	4	0 (0/0.7)	2
ß-Myrcene*	990	3.2 (0/39.9)	7	2.7 (0/78.4)	7
δ-3-Carene*	1011	0.1 (0/0.2)	6	0 (0/2.1)	3
α-Terpinene	1022	0 (0/0.3)	3	0 (0/0.1)	2
p-Cymene	1029	0 (0/0.3)	4	0 (0/tr)	2
Limonene*	1034	3.7 (0.6/27.9)	9	2.2 (0/39.3)	8
ß-Phellandrene*	1038	0 (0/tr)	1	0 (0/0.4)	1
Eucalyptol*	1039	30.9 (0/66.2)	8	14 (0/65.4)	8
(*E*)-ß-Ocimene*	1051	tr (0/0.2)	5	0 (0/0.2)	2
γ-Terpinene	1064	0.3 (0/0.9)	7	0 (0/0.6)	4
Terpinolene	1092	tr (0/0.3)	5	0 (0/0.1)	2
p-Mentha-2,4(8)-diene	1092	tr (0/0.3)	5	0 (0/tr)	1
6,7-Epoxymyrcene	1093	0 (0/0.7)	2	0 (0/0.2)	2
p-Cymenene	1095	tr (0/0.1)	5	0 (0/tr)	2
Linalool*	1103	0.5 (0/0.8)	7	0.4 (0/0.8)	5
(*Z*)-Limonene oxide	1139	0 (0/tr)	0	0 (0/tr)	2
(*E*)-Limonene oxide	1143	0 (0/0.1)	3	0 (0/0.1)	2
Ipsdienol	1145	0 (0/8.7)	2	0 (0/0.6)	2
δ-Terpineol	1178	tr (0/0.2)	6	0 (0/0.2)	4
α-Terpineol	1200	0.9 (0/2.8)	8	tr (0/2.5)	5
(*Z*)-Dihydrocarvone	1204	0 (0/tr)	4	tr (tr/0.1)	1
(*E*)-Dihydrocarvone	1211	0 (0/0.1)	4	0 (0/tr)	1
Verbenone	1215	0 (0/tr)	1	0 (0/0.1)	3
(*E*)-Carveol	1225	–	0	0 (0/tr)	1
Exo-2-Hydroxycineole	1234	0 (0/0.4)	2	0 (0/0.2)	2
(*Z*)-Ocimenone	1234	–	0	0 (0/tr)	1
(*Z*)-p-Mentha-1(7),8-dien-2-ol	1236	–	0	0 (0/tr)	1
(*Z*)-Carveol	1238	–	0	0 (0/tr)	2
Carvone	1250	0 (0/tr)	2	0 (0/tr)	1
Isopiperitenone	1275	0 (0/tr)	2	0 (0/tr)	3
Methyl geranate	1322	–	0	0 (0/0.3)	3
Piperitenone	1345	0 (0/tr)	1	0 (0/tr)	3
N-compounds					
Indole*	1296	0 (0/0.1)	4	tr (0/13.7)	6
Sesquiterpenes					
7-epi-Sesquithujene	1391	0.1 (tr/1)	9	0 (0/0.2)	4
ß-Elemene*	1394	0 (0/0.3)	3	0 (0/0.7)	3
Sesquithujene	1417	0 (0/0.6)	4	0 (0/0.1)	3

Table 1 (*continued*)

Compounds	KRI	Staminate		Pistillate	
		Median (min/max)	*N*	Median (min/max)	*N*
α-Santalen	1424	0 (0/tr)	1	0 (0/0.1)	2
ß-Caryophyllene*	1429	tr (0/21.6)	5	0.3 (0/64.1)	5
(*E*)-α-Bergamotene	1438	1.3 (0.2/24.6)	9	0.3 (0/8.7)	7
(*Z*)-ß-Farnesene*	1445	0.1 (0/2.2)	8	0 (0/0.1)	4
(*E*)-ß-Farnesene*	1454	tr (0/0.7)	7	0 (0/0.1)	4
α-Humulene*	1461	0 (0/0.5)	4	tr (0/1)	5
ß-Santelene	1465	0 (0/tr)	2	0 (0/tr)	1
γ-Curcumene	1478	tr (0/0.1)	5	0 (0/tr)	3
Ar-Curcumene	1485	tr (0/0.9)	7	0 (0/0.1)	4
(*Z*,*E*)-α-Farnesene	1488	0.1 (0/1.2)	8	0 (0/0.5)	4
α-Zingiberene	1498	tr (0/0.1)	5	0 (0/tr)	2
(*E*,*E*)-α-Farnesene	1504	0 (0/tr)	1	0 (0/4)	1
ß-Bisabolene	1511	1.1 (0.1/21.3)	9	0.2 (0/4.4)	6
ß-Sesquiphellandrene	1529	0.1 (0/2.1)	8	0 (0/0.2)	3
13 unidentified sesquiterpenes		0.3 (0/0.9)		0 (0/8.4)	
Unknown					
8 unidentified compounds		0.4 (0/1.7)		0 (0/1.2)	

* Identification based on authentic standards.

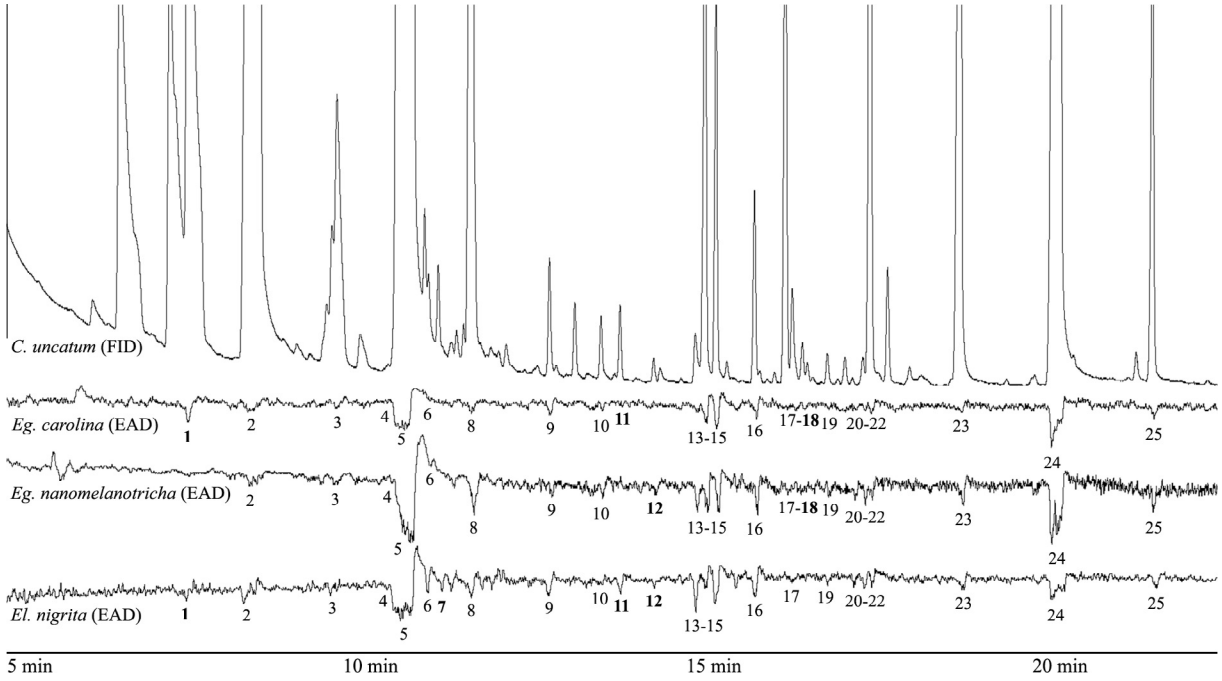

Fig. 3. Examples of coupled gas chromatographic and electro-antennographic detection (GC–EAD) of a staminate scent sample of *Catasetum uncatum* by using antennae of males of the three euglossine species (*Euglossa nanomelanotricha*, *Eg. carolina* and *Eulaema nigrita*). Numbers indicate the compounds triggering antennal responses: (1) ß-myrcene; (2) limonene, ß-phellandrene, eucalyptol; (3) linalool; (4) (*Z*)-limonene oxide + (*E*)-limonene oxide; (5) ipsdienol + veratrole; (6) benzyl acetate; (7) δ-terpineol; (8) methyl salicylate + α-terpineol; (9) p-anisaldehyde; (10) indole; (11) 1,2,3-trimethoxybenzene; (12) methyl-o-anisate; (13) 1,2,4-trimethoxybenzene + p-methoxyphenylethyl alcohol; (14) methyl p-anisate; (15) (*E*)-methyl cinnamate; (16) p-anisyl acetate; (17) (*Z*)-ß-farnesene; (18) (*E*)-ß-farnesene; (19) veratraldehyde; (20) (*Z*,*E*)-α-farnesene; (21) (*E*,*E*)-α-farnesene; (22) ß-bisabolene; (23) (*Z*)-methyl-p-methoxycinnamate; (24) (*E*)-methyl-p-methoxycinnamate; (25) benzyl benzoate. Numbers in bold represent responses that were found either in only one or two bee species.

to flowers of *C. uncatum* does not change its attractiveness to this bee species (Milet-Pinheiro, unpubl. res.). Taken together, these findings are in agreement with other studies that show that the attractiveness of one or a few scent compounds can be suppressed by other compound(s) (Dodson et al., 1969; Williams and Dodson, 1972).

To shed some light on which compounds from the complex floral bouquet of *C. uncatum* could be involved in attracting or deterring floral visitors, we performed GC–EAD analyses with all three euglossine species occurring in the Catimbau National Park. Surprisingly, with a few exceptions, the antennae of all species responded to the same compounds, suggesting that peripheral receptors are highly conservative in orchid bees, not only on an intra- (as also pointed out by Eltz et al., 2006), but also on an inter-generic level. In this sense, EAG analyses, which allow the measurement of the strength of antennal reactions to compounds (either individually or in mixtures), might provide more elucidating results. Schiestl and Roubik (2003), for example, have shown that the strength of antennal reactions of two euglossine species to individual compounds (or binary mixtures) can be increased or reduced by the presence of a further compound. This suggests that, in complex mixtures, the inhibitory effect of some compounds can counterbalance the excitatory effect of others at the peripheral (i.e., receptor) level (see also Hallem et al., 2004).

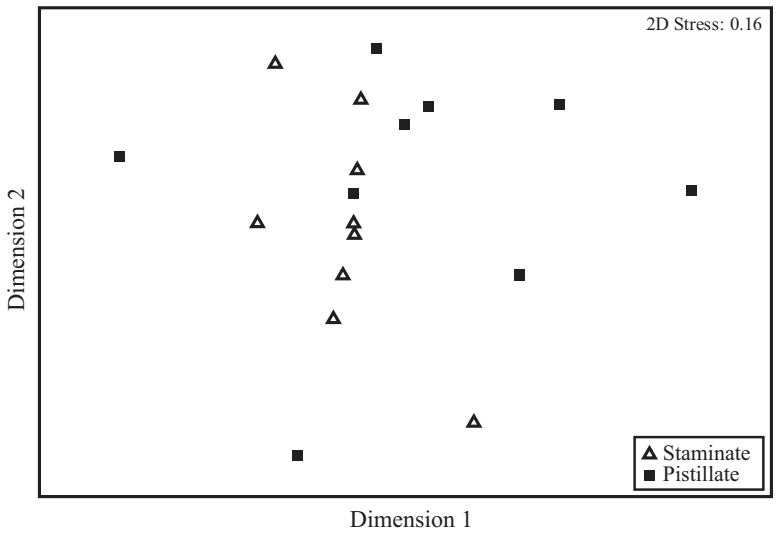

Fig. 4. Comparison of floral scent bouquets of staminate and pistillate flowers of *Catasetum uncatum* based on semi-quantitative Bray–Curtis similarities plotted in non-metric multidimensional scaling (NMDS). Dots represent the average scent pattern for each individual.

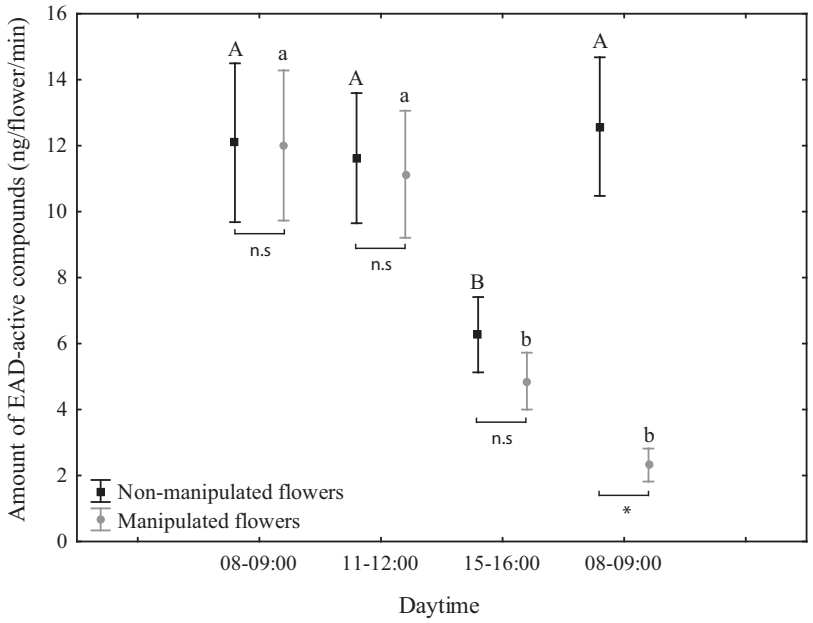

Fig. 5. Amount of EAD-active compounds (mean of the square root ± standard error; $N = 11$ flowers per daytime and treatment) emitted by non-manipulated and manipulated flowers (sexes were pooled, since they behaved in a similar way; see text) of *Catasetum uncatum*. Different letters indicate significant differences among means within a treatment at different daytimes (ANOVA repeated measures, Tukey post hoc tests, $P < 0.05$). Asterisk indicates significant difference ($P < 0.05$) between non-manipulated and manipulated flowers at a given daytime, n.s. not significantly different at $\alpha = 0.05$.

Studies involving electrophysiological analyses (at the receptor, neuron and brain level) of pollinating and non-pollinating bees with regard to their potential scent source and investigations of the significance of electrophysiologically-active compounds (tested singly and in mixtures) on bee behaviour will help to explain the way that floral scents shape the ecology and evolution of this and other interactions involving orchid bees and perfume-producing plants.

3.2. Floral scent composition and differential visitation in staminate and pistillate flowers

C. uncatum produces a complex floral scent bouquet composed of a few major compounds (mainly eucalyptol and veratrole) and a myriad of other minor compounds. We have recorded about 100 volatiles, which represent by far the most complex perfume bouquet recorded for a *Catasetum* species. The number of volatiles released by flowers of other *Catasetum* species has been shown to range from 3 to 30 (Cancino and Damon, 2007; Gerlach and Schill, 1991; Hills et al., 1972; Whitten et al., 1986). Whereas this remarkably difference might represent an actual higher complexity of the scent bouquet of *C. uncatum*, it might also reflect (at least partially) methodological issues. In the present study, headspace samples were collected with small adsorbent filters that were analysed by thermal desorption. This technique might be more sensitive than the usual analyses of solvent headspace samples (Dötterl et al., 2005). Furthermore, most of the work characterising the flower scent bouquet of *Catasetum*

species was performed between the 1970s and 1990s with GC–MS systems that were certainly less sensitive than those on the market nowadays and used here. Taken together, all these methodological issues probably contributed to the discrepancy in the complexity of the scent bouquet reported for *C. uncatum* as compared with those of other *Catasetum* species.

The biological activity of compounds occurring in perfume-producing plants has been extensively tested in the field (see among others, Dodson, 1970; Dodson et al., 1969; Hills et al., 1972; Williams and Dodson, 1972) but most studies have focused on major compounds, whereas compounds occurring in minor amounts have been frequently neglected. In the GC–EAD analyses, we have found 31 biologically active compounds, among them compounds that occur in extremely low amounts but that might also play an important role in attracting or deterring floral visitors. Possibly, important information is held in these minor compounds (see also Eltz et al., 1999). For example, highly subtle differences in chemical composition might be involved in the discrimination of staminate and pistillate flowers by euglossine pollinators.

In the field, we found that male *Euglossa* bees visited pistillate flowers more frequently than staminate flowers, indicating that they indeed discriminate flowers of different sexes. Given the great ability of euglossine males to learn scents that they have previously collected (Eltz et al., 2005a), we can reasonably speculate that floral scent is involved in this discrimination. Euglossine males are assumed to become aversive to staminate flowers because: (1) they are shocked by the unexpected ejection and attachment of the pollinarium and (2) they must carry the extra weight of the pollinarium (Romero and Nelson, 1986). Obviously, this aversive behaviour could have a positive effect on the reproductive success of the plants, i.e., if pollinarium-loaded bees avoid staminate flowers, the chance of visiting and pollinating a pistillate flower increases. With this perspective, selection could act to promote divergence in floral scents of staminate and pistillate flowers (see also Okamoto et al., 2013). However, the scent analyses of staminate and pistillate flowers of *C. uncatum* has shown no clear sexual dimorphism, either in the total amount or in the volatile bouquets, indicating that selection rather acts to reduce intersexual divergence, as previously suggested for other dioecious species (Ashman, 2009; Soler et al., 2012). In *Catasetum* species, the attraction of specific pollinators is assumed to be mediated by species-specific scent compositions (Hills et al., 1972). Thus, any substantial change in the scent pattern of flowers of one of the sexes could disrupt the interaction with their specific pollinators and lead to reproductive failure in the plant. In perfume-rewarding dioecious species in which scent composition is crucial for maintaining the attraction of specific pollinators, the selection for sexual divergence might act with respect to other cues that are not as crucial as floral scents for attracting pollinators. Euglossine males might therefore use floral cues other than scent to discriminate staminate from pistillate flowers.

In the present study, we have found evidence that short-range cues are involved in the sex discrimination of flowers. We have frequently observed that, after approaching a staminate flower, bees hover in front of it for a few seconds before leaving (or landing in a very few cases). In contrast, bees approaching pistillate flowers also hover in front of them but always land and collect scents. In bees, the short hovering in front of a flower before landing is generally associated with a short-range evaluation of visual and/or olfactory cues that lead to the decision to visit it or leave (Chittka and Raine, 2006; Dötterl and Vereecken, 2010; Lunau and Maier, 1995). Visual and olfactory cues are known to play a distinct role in attracting bees (Burger et al., 2012; Milet-Pinheiro et al., 2013; Schlindwein et al., 2014) but the interplay of the two cues is more effective in eliciting landing behaviour after approaching a flower than either cue alone (Burger et al., 2010; Milet-Pinheiro et al., 2012). In *C. uncatum*, we speculate that the highly pronounced morphological sex dimorphism (Fig. 1b and c) allows bees to discriminate staminate from pistillate flowers at short ranges. The potential role of visual cues (e.g., shape and/or colour) in the short-range discrimination of staminate and pistillate flowers of *Catasetum* by euglossine males has previously been suggested (Romero and Nelson, 1986) but experimental evidence is still missing. Alternatively to visual cues (or in addition to), bees may use long-chain heavy compounds (that are not detectable by using the methods that we have applied here) to discriminate flowers of different sexes after being attracted over long distances by volatiles. In the sexually deceptive orchid *Ophrys sphegodes*, for example, long-chain compounds such as esters and hydrocarbons have been found to play a role in the discrimination of individual flowers (Ayasse et al., 2000). Clearly, further studies are necessary to establish whether the discrimination of staminate and pistillate flowers of *Catasetum* by euglossine males indeed occurs at short-range and which floral cues are then involved. Independently of the cues involved in discrimination, the aversive behaviour of euglossine males to staminate flowers has a great significance for the reproductive success of the plants, i.e., if pollinarium-loaded bees avoid staminate flowers, the chance of visiting and pollinating a pistillate flower increases.

3.3. Daily fluctuation in scent emission and post-pollination events

In this work, we have quantified, by chemical-analytical techniques, the total amount of scent emitted by flowers of *C. uncatum* and have shown that scent emission decreases at the afternoon and ceases after pollination, as suggested by several authors studying perfume-producing orchids with measurements based on the human sense of smell (see, for example, Carvalho and Machado, 2002; Janzen, 1981; Martini et al., 2003). Non-manipulated flowers of *C. uncatum* emit more scent in the morning than in the afternoon and this coincides with the time at which *Euglossa* bees are most active in the flowers of *C. uncatum* (see also Milet-Pinheiro and Schlindwein, 2009b). In general, the activity of euglossine bees at natural perfume sources and at scent baits is greatest in the morning hours (Dressler, 1982). Thus, the co-ordination of scent emission with periods of higher activity of pollinators might be a strategy to save energy when the frequency of visits to flowers is expected to be low.

In our chemical analyses, we found that manipulated flowers emitted less scent than non-manipulated ones but that the difference was not perceivable until one day after pollination. Based on this result, we expected that the difference in the frequency of visits by pollinators would also reflect this time interval. However, we observed that *Eg. nanomelanotricha* bees preferred non-pollinated pistillate flowers over pollinated ones as soon as 2 h after pollination. Therefore, the preference of pollinators for non-pollinated flowers might be related to other changes that are triggered more rapidly after pollination but that could not be assessed with the methods that we applied. Independent of the cues involved, the highly rapid loss in the attractiveness of flowers following pollination has an important consequence on plant fitness. In *Catasetum*, pollinarium removal and deposition represent the end of both the pollen donation (all male gametes are dispersed at once) and reception function (pistillate flowers normally only receive one pollinium of a pollinarium), a phase in which pollinators are no longer necessary. Thus, the diminished scent emission following pollinarium removal/deposition might have two main objectives:

(1) the direction of pollinators to flowers that have not yet been visited (see also Ayasse et al., 2000) and (2) the saving of energy for other physiological needs, such as fruit development.

4. Conclusions

In the present study, we provide an exhaustive investigation integrating the role of floral scents in various aspects of the pollination ecology of a dioecious perfume-producing orchid species. The use of the GC–EAD technique in this system has brought some interesting aspects of the interaction between perfume-producing plants and their euglossine pollinators to light. Historically, scientists have selected major compounds or compounds that are representative among different taxa for behavioural experiments. Ramirez et al. (2002) have listed a total of 69 compounds occurring in perfume-producing plants whose attractiveness to euglossine males has been confirmed in the field. In our GC–EAD analyses, we have detected 31 EAD-active compounds only in *C. uncatum*, among them 17 whose behavioural activity has not previously been tested. This illustrates the neurological complexity of euglossine bees and highlights the challenge of investigating aspects of the chemical communication between perfume-producing plants and their pollinators. We have found that floral scents play a pivotal role in the selective attraction of *Euglossa* species as pollinators of *C. uncatum*. Furthermore, we show that (1) pollinarium removal/deposition results in reduced scent emission and that (2) euglossine males visit preferentially pistillate to staminate flowers; we suggest that these two aspects are crucial for guaranteeing fruit set in *C. uncatum*. Unfortunately, we have not been able to show experimentally the way in which bees discriminate the flowers of the different sexes but we have found evidence that discrimination occurs at close rather than long distances. Thus, we speculate that, after being attracted over long distances by floral volatiles, which are not a sex dimorphic trait, bees use other cues at close range, such as shape and colour and/or low volatile compounds, to differentiate staminate from pistillate flowers. Indeed, visual cues are distinctive with regard to staminate and pistillate flowers of *Catasetum* species and, consequently, are good candidates for this discrimination. The collection and chemical analyses of flower solvent extracts are still necessary to determine whether low volatile compounds have a function in flower discrimination. Together, our results provide interesting insights into the evolution of floral signals in gender-dimorphic species and into its significance in plant reproductive biology.

5. Experimental

5.1. Study site

The Catimbau National Park is a nature reserve situated in the state of Pernambuco (NE-Brazil) and covers about 62,000 ha. The vegetation of the region is composed of evergreen shrubs and small trees that intermingle with widespread species of the surrounding *Caatinga*, the common deciduous vegetation of semi-arid NE-Brazil and some elements of cerrado vegetation (Andrade et al., 2004). Mean annual temperature and precipitation are 25 °C and 1100 mm, respectively. The rainy season is from January to March.

5.2. Study species

C. uncatum is endemic to the Brazilian northeast, with records for the states of Pernambuco, Alagoas, Ceará and Bahia (Oliveira et al., 2010). Individuals of this species produce inflorescences that normally bear either staminate or pistillate flowers (Fig. 1b and c). In extremely rare situations, an inflorescence might bear both staminate and pistillate flowers. Plant vouchers are stored in the Herbarium Geraldo Mariz (UPE), Recife, Brazil.

5.3. Flower biology

Staminate and pistillate inflorescences ($N = 5$ for each sex) of different individuals were bagged to describe the anthesis from flower opening to abscission. Inflorescences were observed daily from 0500 to 1700 h at intervals of 3 h. We recorded the time of flower opening, flower scent emission (to the human nose) and flower longevity. Stigma receptivity was determined once a day by using potassium permanganate (1:1000). The position of the osmophores was determined with neutral red (Dafni, 1992). The number of staminate and pistillate flowers per inflorescence and their ratio in the population were recorded in 43 inflorescences.

To evaluate the effect of pollinarium removal/deposition on flower longevity and scent emission, we removed the pollinaria of staminate flowers ($N = 77$ flowers of 5 inflorescences) and deposited pollinaria on the stigmas of pistillate flowers ($N = 24$ flowers of 5 inflorescences) by using insect tweezers (hereafter: manipulated flowers). All manipulated flowers were then assessed for visual (e.g., colouration, turgescence) and olfactory (to the human nose) changes at intervals of 3 h. For a more accurate depiction of changes in scent composition after pollinarium removal/deposition, scent was additionally collected by using dynamic headspace methods and was analysed by chemical-analytical techniques (see below).

5.4. Pollination ecology

Flower visitors were collected with entomological nets, prepared, dried and stored in both the Entomological Collection of the Federal University of Pernambuco (UFPE) and the Entomological Collection of Federal University of Minas Gerais (UFMG). The frequency of flower visitors was determined in 6 staminate ($N = 74$ flowers) and 6 pistillate inflorescences ($N = 29$ flowers) of different individuals. Bees were collected as soon as they landed on a flower. Inflorescences were observed on six non-consecutive days, from 0600 h to 1700 h, corresponding to 66 h of observation. To assure that an eventual difference in frequency of visits to staminate and pistillate flowers did not reflect stochastic factors (e.g., weather, among-day fluctuation in visitor availability), a staminate and a pistillate inflorescence of individuals growing close to each other were observed simultaneously each day. The behaviour of floral visitors in flowers was recorded and documented photographically in further observations during the whole period of field work. Fruit set under natural conditions was determined in 23 pistillate inflorescences (135 flowers).

5.5. Field bioassays

To evaluate the effect of post-pollination events on pollinator attraction, choice bioassays were performed in the field with free-flying bees. Frequency of visits by euglossine pollinators was recorded only on pistillate inflorescences ($N = 3$); in each inflorescence, flowers were divided (randomly) into non-pollinated or manually pollinated treatments. The number of non-pollinated and pollinated flowers in each inflorescence was the same. Two of the inflorescences had eight flowers and one had six flowers. Pollinaria were deposited into the stigma by using a tweezer but without contacting flowers. Thus, we consider that the handling of the flowers has no effect on the frequency of visits by pollinators in the bioassays. Visits to flowers were recorded continuously for 2 h after pollination. Bees were collected as soon as they landed on flowers, in order to avoid pollination. The bees were stored in an icebox for the duration of the experiment and then released

again. Bioassays were repeated three times on three different days between 0930 h and 1130 h by using three different inflorescences.

5.6. Sampling of floral volatiles

Floral volatiles were collected in the field, by using standard dynamic headspace methods, for three different purposes: (1) to establish the scent bouquet of staminate and pistillate flowers of *C. uncatum*; (2) to verify the effects of pollinarium removal or deposition on floral scent emission; (3) to obtain scent samples for electrophysiological measurements.

To obtain a floral scent sample for GC–MS analyses, an individual flower was enclosed in a polyester oven bag (8 × 7 cm; Toppits®) for 5 min after which the volatiles were trapped for 3 min in an adsorbent tube by using a membrane pump (G12/01 EB, Rietschle Thomas, Puchheim, Germany). The pump worked at a flow rate of 200 ml/min. The adsorbent tubes consisted of ChromatoProbe quartz microvials from Agilent Inc. (length: 15 mm; inner diameter: 2.5 mm), cut at the closed end and filled with 3 mg of a 1:1 mixture of Tenax-TA (mesh 60–80, Supelco) and Carbotrap (mesh 20–40, Supelco). The mixture was fixed in the tubes by using glass wool.

To characterise the scent bouquet of staminate and pistillate flowers of *C. uncatum*, we collected volatile samples in non-manipulated flowers ($N = 9$ for each sex) from different plant individuals. To avoid the influence of daytime and flower age on scent emission, all samples were collected on sunny days between 0900 h and 1000 h from flowers that had opened 3 days previously. Additionally, in some of these flowers ($N = 6$ for staminate, $N = 5$ for pistillate flowers), soon after sampling of volatiles, we manually removed or deposited pollinaria with insect tweezers to verify the effects of these events on floral scent emission. Volatiles were then collected 2 h, 6 h and 24 h after flower manipulation (between 1100–1200 h and 1500–1600 h of the same day and 0900–1000 h on the following day). Scent emission in *Catasetum* flowers is known to vary during the day (Hills et al., 1999). Because of this, simultaneously to each extraction, we sampled volatiles from neighbouring (i.e., flowers at the same inflorescence and age) non-manipulated flowers ($N = 6$ for staminate, $N = 5$ for pistillate flowers) as a control.

Additionally to these samples collected for thermal desorption, we also collected solvent samples ($N = 3$ for each sex) for electrophysiological analyses with the three euglossine species known to occur in the Catimbau National Park, namely *Eg. nanomelanotricha* Nemésio, 2009 (former *Eg. melanotricha* Moure, 1967), *Euglossa carolina* Nemésio, 2009 [former *Eg. cordata* (Linnaeus, 1758)] and *El. nigrita* Lepeletier, 1841. Adsorbent tubes (length 100 mm; inner diameter 4 mm) filled with 50 mg of the same adsorbent mixture were used. The protocol used to collect solvent samples was the same as that used for thermal desorption samples (see above), with the exception that volatiles were sampled for 48 h (from 9:00 to 9:00 h) with a flow rate of 100 ml min^{-1}. Volatiles in the adsorbent tubes were then eluted with 300 µl of a 9:1 mixture of hexane (99.5%, Merck) and acetone (99.8%, Merck).

To detect ambient contaminants, negative controls (empty bags; $N = 3$) were collected by using adsorbent tubes and the same methods described above. All headspace samples were stored in 2 ml screw cap vials at −20 °C until the chemical analyses.

5.7. Chemical analysis

To identify the floral volatiles of *C. uncatum*, including those that elicited antennal responses in the bees, headspace samples were analysed on a mass spectrometer (Quadrupole 5972, Agilent, Santa Clara, CA, USA) coupled to an Agilent gas chromatograph (HP 6890) fitted with a ChromatoProbe kit (AvivAnalytical, Hod Hasharon, Israel). A quartz microvial was loaded into the probe, which was then inserted into the modified GC injector. The injector split vent was opened and the injector heated to 40 °C to flush any air from the system. The split vent was closed after 2 min and the injector was heated at 200 °C/min and then held at 200 °C for 4.2 min, after which the split vent was opened and the injector cooled down. We used an HP-5 fused-silica capillary column (50 m long, inner diameter 0.25 mm, film thickness 0.25 µm, Agilent Technologies, Santa Clara, USA). Electronic flow control was employed to maintain a constant helium carrier gas flow of 1.5 ml min^{-1}. The GC oven temperature was held for 2 min at 50 °C, then increased by 6 °C per min to 240 °C and held for 7 min. The MS interface worked at 260 °C and the ion trap at 175 °C. Mass spectra were taken at 70 eV (in EI mode) with a scanning speed of 1 scan s^{-1} from m/z 30 to 350. The GC–MS data were processed by using Agilent MSD ChemStation Software. Component identification was carried out by using the NIST 08 and the Essential oils mass spectral data bases and confirmed by a comparison of retention times with published data (Adams, 2007). Identification of individual components was confirmed by a comparison of both mass spectrum and GC retention data with those of authentic standards available in our compound collection.

To quantify the absolute amount of each floral compound in the samples, a known amount (100 ng) of each of three external standards belonging to different compound classes [Aromatics: methyl salicylate; Monoterpenes: eucalyptol; and Sesquiterpenes: (*E*)-β-caryophyllene] were injected into the thermal desorption cartridges and analysed in the same manner as described previously. The mean peak area found in five runs was used to determine the total amount of each compound in the floral samples. Volatiles detected in the samples obtained from the empty bag samples were considered as ambient contaminants and excluded from the floral scent list.

5.8. Electrophysiology

Electrophysiological responses of all three euglossine bee species occurring in the Catimbau National Park were tested with respect to the floral scents of *C. uncatum* in GC–EAD analyses. The analyses were performed on a gas chromatograph (Thermo GC Ultra, Thermo Scientific, Milan, Italy), equipped with a flame ionisation detector (FID) and coupled with an EAD setup (heated transfer line, two-channel universal serial bus acquisition controller) provided by Syntech (Kirchzarten, Germany), and a VB-5 column (30 m long, 0.25 mm i.d., 0.25 µm film thickness, ValcoBond) (see also Milet-Pinheiro et al., 2014). Electronic flow control was used to maintain a constant helium carrier gas flow of 1 ml/min. An aliquot of the solvent headspace samples (1 µl) was injected in splitless mode at an oven temperature of 60 °C and an injector temperature of 200 °C, followed by opening the split valve after 1 min and increasing the oven temperature at a rate of 7 °C/min to 200 °C. The final temperature was held for 5 min. The column was split at the end by a splitter tee (SGE Analytical Science) into two pieces of deactivated capillary (length 40 cm, i.d. 0.25 mm). A make-up gas (nitrogen) was added before the splitter. One capillary was led to the FID and the other outside the GC oven, into a glass tube in which the effluent was mixed with a clean and humidified airflow. The airflow was directed over the antenna of the bees.

Antennae were cut at the base and tip and mounted between two electrodes, which were filled with insect Ringer solution (8.0 g/l NaCl, 0.4 g/l KCl, 04 g/l $CaCl_2$) and connected to silver wires. Electrophysiological measurements were performed with one antenna per male individual ($N = 8$ for each species). Bees were collected at the Catimbau National Park and at the Água Fria Farm,

Chã Grande, NE-Brazil, where *Eg. nanomelanotricha*, *Eg. carolina* and *El. nigrita* also occur abundantly (Milet-Pinheiro and Schlindwein, 2009a). The bees, collected either on flowers or scent baits (filter paper impregnated with ß-ionone, eucalyptol, and skatole), were stored inside an icebox until the GC–EAD analyses, which were performed in the Laboratory of Chemical Ecology (Federal University of Pernambuco, Brazil). A floral scent compound was considered to be EAD-active when it elicited a depolarisation response in at least four mounted antennae.

5.9. Statistical analyses

The mean number of flowers in staminate and pistillate inflorescence and the mean longevity of staminate and pistillate flowers were compared by using a Student *t*-test. Data were transformed to their square roots to achieve normality (Kolmogorov–Smirnov test). Homogeneity of variances was assessed with a Levene's test. All analyses were performed in Statistica 7.0 (StatSoft, 2004).

A chi-square expected vs. observed test was used to assess differences in bee responses between pollinated vs. non-pollinated pistillate flowers in the choice bioassays. The null hypothesis was that bees visit the same numbers of pistillate and staminate flowers. The responses of bees in the three different bioassays (three different days) to pollinated and non-pollinated pistillate flowers were pooled.

Frequency of visits to staminate and pistillate flowers during the day was compared by permutational analyses. Similarities in frequency of visitors between individual flowers were determined by calculating Euclidean distances by using PRIMER 6.1.11 (Clarke and Gorley, 2006). To test for differences in frequency of visits to staminate and pistillate flowers and among different daytimes, we used a three level nested PERMANOVA analysis [factors: sex, individual (nested in sex), and daytime], again in PRIMER 6.1.11, based on the Euclidean distances. PERMANOVA is a technique for testing the simultaneous response of one or more variables to one or more factors in a ANOVA experimental design on the basis of a (dis)similarity matrix with permutation methods (Anderson et al., 2008). We used 10,000 permutations for the analysis.

Possible differences in scent emission between staminate and pistillate flowers, between manipulated and non-manipulated flowers and among different daytimes were assessed by comparing: (1) the total amount of volatiles (quantitative) and (2) the pattern of scent emission for either the relative ratio of compounds (semi-quantitave) or the presence/absence of compounds (qualitative). All comparisons were performed only with compounds that were biologically active, in the EAD measurements, with regard to the three euglossine species.

Repeated measures ANOVA was used to test for quantitative variability in the total amount of scent emitted at various daytimes, also considering sex and pollination status of the flowers as factors. Data were transformed to their square roots to achieve normality. Post-hoc tests were performed by using the Tukey test. All tests were performed in Statistica 7.0 (StatSoft, 2004).

Semi-quantitative and qualitative similarities in floral scent patterns among samples were determined by calculating the Bray-Curtis and the Sørensen similarity indices, respectively, by using PRIMER 6.1.11 (Clarke and Gorley, 2006). The relative ratios of compounds were transformed to their fourth root for the semi-quantitative analysis. Based on the similarity matrices, nonmetric multidimensional scaling (NMDS) was used to depict variation in floral scent among samples (Clarke and Gorley, 2006). To test for differences in scent profiles between staminate and pistillate flowers, between manipulated and non-manipulated flowers, and among the different daytimes, we used a four level nested PERMANOVA analysis [factors: sex, individual (nested in sex), treatment, and daytime], again in PRIMER 6.1.11, based on Bray-Curtis or Sørensen similarity matrices. We used 10,000 permutations for the analysis.

PERMDISP (Anderson et al., 2008) was used in Primer 6.1. 6 to test for differences in within-scent variability (dispersion) in pistillate and staminate flowers (based on qualitative and on semi-quantitative species-based matrices) (10,000 permutations). In addition to providing more information about the scent variation *per se*, the outcomes of these tests also helped to interpret a potential influence of dispersion on the PERMANOVA results.

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