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REVIEW

Floral longevity and scent respond to pollen manipulation and resource status in the tropical orchid *Myrmecophila christinae*

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Abstract Floral longevity (FL) is a key aspect in plant reproductive ecology. Despite this, the effect of resource status on FL has usually been ignored, and never has resource status been linked to the effects of pollen manipulation on FL. In addition, immediate changes in floral scent characteristics subsequent to pollen addition/removal have not been looked at. Here we use the tropical orchid *Myrmecophila christinae* to address the following: (1) Does flower bud removal (resource status change) increase FL? (2) Does pollen manipulation (addition/removal) decrease FL, and do such effects interact with plant resource status? (3) Are there rapid changes in floral scent production and composition after pollen manipulation? To answer the first question, we removed 50% of the flower buds on 24 plants (24 more were controls). To test the second question, 1 month after removing buds, one of four flowers on each inflorescence received one of the following treatments: no manipulation, pollinia removal, pollination, or pollination + pollinia removal. Finally, to answer the third question, one of four flowers on each of 15 plants (different site) received one of the above-mentioned pollen treatments. Flowers were collected 2, 4, and 6 h after manipulation to measure scent production/composition. Results showed that flowers on bud-removed plants remained open significantly longer relative to those on control plants, and that pollination significantly decreased FL. Additionally, scent production increased throughout the morning and responded differently depending on the pollen manipulation treatment; scent composition on the other hand, remained relatively unchanged throughout the sampling period. By studying both floral scent and physical changes in *M. christinae*, this study intends to offer a more integrated view of floral senescence within the context of resource and pollen status conditions.

Keywords Floral longevity · Floral scents · *Myrmecophila christinae* · Pollen manipulation · Reproductive costs

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Introduction

Floral longevity is defined as the period of time from anthesis to floral senescence and has been recognized as a key process in plant reproductive ecology (Ashman and Schoen 1996). Flower life span can range from 1 day (e.g., *Ipomoea* spp.) to several months (e.g., Orchidaceae) depending on the plant species. Previous studies have proposed that such a wide range of variation in floral longevity responds to a trade-off between floral maintenance and construction under resource-limited conditions (Primack 1985; Ashman and Schoen 1994), which is conditioned by how quickly pollen is disseminated or received for any given flower (i.e., optimal floral longevity; Ashman and Schoen 1994, 1996; Schoen and Ashman 1995).

Numerous studies have shown that pollination of stigmas and/or pollen removal from stamens reduces floral longevity and/or flower attractiveness (e.g., Tollsten and Bergström 1989; van Doorn 1997; Evanhoe and Galloway 2002). However, not much attention has been paid to the relationship between plant resource status and floral longevity (but see Holtsford 1985; Ashman and Schoen 1997) or the effect of pollen addition and/or removal on flower longevity under different plant resource status conditions. In addition, pollen manipulation has been shown to affect not only floral longevity but also other floral traits important for pollinator attraction. For instance, floral scent production and composition have been shown to vary with flower age (Gregg 1983; Moya and Ackerman 1993), as well as before versus after pollination (Tollsten and Bergström 1989; Tollsten 1993; Schiestl et al. 1997; Theis and Raguso 2005). Specifically, pollination has been shown to cause shifts in fragrance composition and reduction in scent production, the latter proposed to be a mechanism to minimize flower maintenance costs (Schemske 1978; Arditti 1979; Vogel 1990; Harder and Barrett 1992; Tollsten 1993) and reduce the attractiveness of pollinated flowers (Arditti 1976; Gori 1983; Schiestl et al. 1997; Schiestl and Ayasse 2001; Muhlemann et al. 2006). Scent changes in response to pollination have typically been measured across several days, rather than over shorter time scales (e.g., hours). However, for species with low pollinator visitation rates and high reproductive costs, measuring floral changes across a finer temporal scale may be important as scent (and physical) responses following pollination are expected to occur rapidly in order to maximize reproductive assurance and minimize reproductive costs.

In tropical orchids, pollination is often followed by a fast change in flower color and cessation of scent and nectar production (Arditti 1979). Specifically, scent changes after pollination, which include a decrease in the total and relative amounts of certain compounds, appear to be widespread in Orchidaceae (Arditti 1979, 1992), with scent composition being mostly studied in sexually deceptive species (e.g., Schiestl et al. 1997). In addition, floral longevity has been shown to decrease in response to pollination for several orchid species (e.g., Ackerman 1989; Clayton and Aizen 1996; Martini et al. 2003). Pollen manipulation and reproductive resource status effects on floral longevity and floral scent production and composition might be expected to be greatest in tropical orchids, particulary in epiphytic species, because (1) tropical species are more pollen-limited than temperate species (Tremblay et al. 2005), and for this reason flowers would be expected to respond faster to pollen addition and/or removal because of a low chance of further pollinator visits and high flower maintenance costs (Castro et al. 2008), and (2) epiphytes undergo high levels of transpirational water stress (Zotz and Hietz 2001), which might translate to more limited resource budgets and greater reproductive costs.

Myrmecophila christinae is an epiphytic orchid found growing in the coastal shrub vegetation of the Yucatan Peninsula, where it has been suggested to use a food-deception pollination system based on floral scents that attract recently emerged bees (i.e., naive pollination; Rico-Gray and Thien 1987). Reproductive success has been shown to be extremely low for this orchid species (Rico-Gray and Thien 1987). Flowers are large and remain open for up to 10 days (Rico-Gray and Thien 1987; Malo et al. 2001), which combined with elevated temperature levels and reduced precipitation found along the Yucatan coast (summer monthly averages can reach 30°C; García 1988), suggests high flower construction and maintenance costs (Primack 1985; Galen 2000). Given all of these characteristics we chose to study *M. christinae* as we addressed the following questions: (1) Does experimental removal of flower buds increase floral longevity in *M. chirstinae* (does resource status change lead to reduced flower construction costs)? (2) Does pollen addition and/or removal decrease floral longevity, and are these responses influenced by plant reproductive resource status? (3) Does pollen addition and/or removal change floral scent production and composition, and how is this change expressed over a short time scale? Overall, the present work contributes to the understanding of phenotypic plasticity in floral longevity and scent characteristics under changing pollination and plant resource status conditions.

Materials and methods

Study species and sites

Myrmecophila christinae Carnevali and Gómez-Juárez var. *christinae* (Orchidaceae; previously *Schomburgkia tibicinis* and *M. tibicinis*) is a self-compatible epiphytic orchid endemic to the Yucatan Peninsula (Carnevali et al. 2001). It is found growing on *Cocothrinax readii* and *Thrinax radiata* palm trunks in the coastal dune vegetation along the coast and has been shown to present a non-model food-deception pollination system mediated by two large solitary bee species, *Eulaema polychroma* and *Xylocopa* sp., which are necessary vectors for fruit formation (Rico-Gray and Thien 1987). *Xylocopa* bees have been shown to be the most common pollinators for *M. christinae* on the Yucatan coast, while *Eulaema* bees are much less abundant and were only observed to visit *M. christinae* once throughout several flowering seasons (Rico-Gray and Thien 1987; Parra-Tabla and Vargas 2004).

The structure of the column (fused androecium, style, and stigmas) results in a mechanism whereby the pollinia

are glued to the bee's thorax as the insect exits the flower (Rico-Gray and Thien 1987; Malo et al. 2001). *Xylocopa* flower visits are extremely fast and bees barely come into contact or manipulate the flower. After visiting a given flower, they generally will not visit other flowers on the same plant. Flowers are large (8–9 cm in diameter) and open at dawn (~0500 h), being fully receptive at this moment. They remain open for 1 week on average and are located at the tip of ca. 2-m-long inflorescence stalks (1.8 ± 2.1 inflorescences per plant, 6–15 flowers per inflorescence). The flowering season extends from March to June, while fruits are produced from May to July (Rico-Gray and Thien 1987). Pollinator limitation has been shown for this species, and percent fruit set can be as low as 2.3% (Rico-Gray and Thien 1987). In addition, rates of pollinia removal have shown to be equally low (Parra-Tabla and Vargas 2007).

We used two sites for the field experiments reported in this study. The first one, called Telchac (Yucatan, Mexico; N 21°19′47″, W 89°22′16″), was used to study the effects of flower construction costs (i.e., reproductive resource status) and pollen manipulation on floral longevity in *M. christinae*. This site's vegetation was composed mainly of coastal dune shrubs, a characteristic type of vegetation found along the northern coast of the Yucatan Peninsula, between 100 and 500 m from the shore and up to 10 m a.s.l. (Malo et al. 2001). Telchac climate is tropical dry and warm (Bs_o; modification of Köpen by García 1988). Average temperature is 25.7°C, and mean annual precipitation is 469 mm, most of which falls between July and October.

The second site, named Puerto Cancun, was located within the city limits of Cancun (Quintana Roo, Mexico; N 21°10′30″, W 86°48′40″) and was used to study the effects of pollen manipulation and time after treatment application on floral scent characteristics in *M. christinae*. Puerto Cancun is a nursery area devoted to restoration of native flora of the Yucatan Peninsula and includes a portion of land with mangrove and coastal dune vegetation where *M. christinae* naturally grows. This site is 10 m a.s.l., and climate is tropical warm humid and subhumid (Ax; modification of Köpen by García 1988). Average temperature is 27.4°C, and the mean annual precipitation is 1,041 mm.

Floral longevity: flower bud removal and pollen manipulation treatments

In early April of 2001, at the onset of *M. christinae's* reproductive season (late March), we visited Telchac where we selected 48 adult plants and marked one inflorescence on each plant; inflorescences were matched for approximately the same number of flower buds. Because plants typically grow in groups, one inflorescence was marked per clump, with clumps being 10 m or more apart to ensure the selection of different genets. To study the effect of reproductive resource status on floral longevity, we manipulated flower construction and maintenance investment (i.e., costs) by conducting a flower bud removal experiment for which we randomly selected 24 of the previously mentioned plants and removed 50% of the developing flower buds on each marked inflorescence (buds randomly chosen) during the earliest possible developmental stage in order to minimize damage. In addition, inflorescences were followed throughout the field season to verify that flower bud removal did not result in pathogen attack of affected tissue (which it did not). The remaining 24 plants were controls, and their inflorescences were not subject to flower bud removal. Each inflorescence in *M. christinae* has a predetermined number of flower buds, which means that inflorescences that were subjected to flower bud removal could not compensate by producing more buds later on. We expected that bud removal would reduce flower construction costs and increase resource allocation to flower maintenance (i.e., longevity), as has been shown in related studies that have used such methodology to evaluate resource reallocation strategies in plant reproductive structures (see Silvertown 1987; Herrera 1991; Ashman and Hitchens 2000; Abdala-Roberts et al. 2007). This is a reasonable assumption for *M. christinae*, which is probably resource-limited (water, nutrients) and should respond to manipulation by investing more on flower maintenance (i.e., longevity) of remaining flowers on an inflorescence.

One month after removing flower buds, using the same 48 plants, one of four intact newly opened flowers on each flagged inflorescence was randomly selected to receive one of the following pollen manipulation treatments: control or no manipulation (C), pollinia removal (R), pollination without pollinia removal (POL), and pollination with pollinia removal (POL + R); the latter two involved cross-pollination. Pollinia were manipulated using tweezers, and treatments were applied between 0500 and 0600 h (i.e., when flowers open). All marked inflorescences were bagged after pollen treatment application, and plants were visited daily until all sampled flowers had closed. The response variable measured was the number of days each flower remained open (i.e., longevity).

Statistics

A nonconventional survival analysis was conducted using a generalized linear model in SAS (PROC GENMOD; SAS Institute, Cary, NC, USA, SAS 2002) for which pollen and bud removal treatments, as well as their interaction, were treated as main effects influencing the number of days until a flower closed. The interaction term might provide potential insights into changes in floral longevity responses

to pollen manipulation depending on plant reproductive resource status (see Abdala-Roberts et al. 2007). Every flower was visited on a daily basis, and each visit represented an observation (flower open = 0) up until the target event took place (flower closed = 1), resembling a classic logistic regression analysis (binomial distribution, and logit link function), which in this case modeled the probability of a flower closing through time. For this analysis, time was assumed to be a discrete variable (number of classes = number of observations until the target event took place), which differs from a classical survival analysis which considers time as a continuous variable (Allison 1999). Nonsignificant factors were removed from the model in a backward fashion. Type III analysis was used, and preplanned contrasts were conducted in a pairwise manner between treatments for significant main effects using the ESTIMATE option.

Measures of plant size were not used as covariates because it is difficult to distinguish between *M. chrisinae* genets as plants usually grow in clumps (Rico-Gray and Thien 1987). In addition, Parra-Tabla and Vargas (2004, 2007) failed to find an effect of pseudobulb size and leaf number on reproductive effort, which might suggest a weak relationship between measures of plant size and reproductive investment for the studied species.

Floral scents: pollen manipulation treatment and changes through time

The following year, in May of 2002, we visited Puerto Cancun where we marked one inflorescence on each of 15 adult *M. christinae* plants; all inflorescences had approximately the same number of flower buds. Subsequently, one of four newly opened flowers on each marked inflorescence was randomly selected to receive any one of the previously described pollen manipulation treatments (C, R, POL, or POL + R). Treatment application started when flowers opened, at 0500 h. Inflorescences were not bagged, although they were monitored throughout the morning to make sure pollinator visits did not occur. Three groups of five plants each had their treated flowers collected 2, 4, and 6 h (0700, 0900 and 1100 h, respectively) after pollen manipulation for scent analysis (i.e., each group was subjected to one of the three flower collection time intervals). Although previous studies on floral scents have analyzed chemical changes across one or more days, here we chose much shorter sampling time intervals because *M. christinae* flowers close within 24 h of being pollinated. This directly constrains the measurement of scent changes across a larger time frame and also suggests that such changes might take place very rapidly following pollination and/or pollinia removal. In addition, although a previous study reported changes in floral scent production and composition due to flower removal (e.g., *Ophrys sphegodes*; Schiestl et al. 1997), in this study, flowers were fixed in hexane immediately after being collected (see description below), and thus, such changes are assumed to be minimal. Furthermore, clipped labella from other orchid species have been shown to elicit the same pollinator responses when compared to intact flowers (Schiestl et al. 1999).

Immediately after being collected, flowers were placed in a vial containing 20 ml of hexane for 1 min and then stored at 10°C. This direct solvent extraction method allowed the detection of compounds present at the moment of flower collection and has been used to quantify both volatile and nonvolatile compounds in flowers (e.g., Shaver et al. 1997; Flach et al. 2006). Additionally, results from direct solvent extraction methods have been shown in some cases to be consistent with results from other techniques (e.g., headspace sampling), although differences have also been reported (Zhang et al. 2000; Song et al. 2003). After the hexane treatment, 1 ml of floral solvent extract solution was taken out of each sample and then concentrated by nitrogen flux until a volume of 100 µl was reached, from which a 1-µl subsample was taken and analyzed by coupled gas chromatography–mass spectrometry (GC–MS) using a Varian model Star 3400 CX GC coupled to an MS and an integrated data system (Varian Saturn 4D, Palo Alto, CA, USA). Ionization was conducted by electron impact at 70 eV and 230°C. Helium was used as the carrier gas at a constant flow of 1 ml/min. A DB5-MS column (30 m × 0.25 mm ID, film thickness 0.25 µm, J and W Scientific, Folsom, CA, USA) was temperature-programmed for 50°C for 2 min, rising 15°C/min up to 280°C, and held for 10 min, with the injector at 250°C and transfer line at 280°C. Individual components were identified by comparison to standards based on both mass spectra and GC retention time. Other identifications were made by comparison to mass spectra found in system libraries (NIST 2001, Gaithersburg, MD, USA) and those cited in the literature (Adams 1995). The abundance of each compound was estimated by calculating the GC peak area of that compound, while the amount of scent produced per flower was taken as the sum of GC peak areas for all compounds found in that sample.

Statistics

Scent production was analyzed with a generalized linear model in SAS (PROC GENMOD; SAS Institute 2002) for which pollination treatment (four levels) and time (three levels), as well as their interaction, were used as main (fixed) effects. The data were not normally distributed, even after transformation, and for this reason we assumed a Gamma distribution, which is suitable for continuous data, and used a log link function based on the assumption that

the effect of the predictor variables was multiplicative (Crawley 1993). Type III analysis was used, and pre-planned contrasts were conducted in a pairwise manner between levels for significant main effects using the ESTIMATE option (*P*-values were corrected based on the Bonferroni adjustment).

Using principal component analysis (PCA) in SYSTAT (SYSTAT 2000), we explored the effect of pollen manipulation and amount of time after treatment application on the abundance of the scent compounds detected for sampled flowers (i.e., changes in scent composition). The resulting PCA matrix was composed of 60 objects (flowers, tagged with a particular pollen and collection time treatment) and 18 variables (scent compounds). PCA derives multivariate axes of variation [called principal components (PCs)], the first two or three of which are expected to explain most of the observed variation in the data. It is possible to determine which compounds of floral scent are structuring each component (based on their loadings or correlations with the latter), and thus, which of the former are most likely to be responsible for the observed separation between flowers under each treatment and sampling time in multivariate space (Quinn and Keough 2002). In order to satisfy the assumption of normality, all of the data were log + 1 transformed prior to analysis. In addition, the analysis was carried out based on a correlation matrix and using VARIMAX axis rotation. Finally, a post-hoc two-way ANOVA (GLM, type III sums of squares) was performed to test for differences in standardized *z*-scores for PC 1 due to pollen treatment and time of sampling. Prior to statistical analysis, the data showed a normal distribution. Bonferroni tests were used to test for differences between pairs of pollen treatment and sampling time level means (*P*-values were corrected).

All the analyses conducted for floral scents excluded long-chain alkenes detected in samples, as these compounds have been shown to play a more important role for sexually deceptive rather than for food-deceptive orchids (e.g., Schiestl et al. 2000; Mant et al. 2005); furthermore, including these compounds in the analysis would have obscured the detection of patterns for more volatile compounds relevant to the study system.

Results

Floral longevity: flower bud removal and pollen manipulation effects

After removing the nonsignificant flower bud removal by pollination treatment interaction ($\chi^2 = 1.15$, $df = 3,403$, $P = 0.7$), the logistic analysis showed significant flower bud removal ($\chi^2 = 16.93$, $df = 1,406$, $P < 0.0001$) and pollen manipulation treatment effects ($\chi^2 = 66.03$, $df = 3,406$, $P < 0.0001$) on the probability of a flower closing through time. Results showed that flowers on inflorescences with previously removed flower buds had a lower probability of closing through time (i.e., closed later) than control inflorescences (Table 1). On the other hand, pre-planned contrasts between pairs of pollen manipulation treatments showed the following differences (Table 1): the probability of a flower closing through time did not differ between flowers subject to POL and POL + R treatments ($\chi^2 = 0.02$, $P = 0.89$), and flowers under these treatments showed the greatest probabilities (fastest-closing flowers), differing significantly from control ($\chi^2 \geq 51$, $P < 0.0001$, in both cases) and removed pollinia flowers ($\chi^2 \geq 13$, $P < 0.0001$, in both cases). The next fastest-closing flowers were those with removed pollinia, which had a significantly lower probability of flower closure compared to POL and POL + R flowers as mentioned previously, although still much greater than controls ($\chi^2 = 39.90$, $P < 0.0001$; Table 1). Finally, control flowers showed the lowest probability of flower closure through time and differed significantly from flowers subjected to all other treatments.

Table 1 Results from the log-linear model analysis with pollen manipulation and flower bud removal as main effects influencing the amount of time individual flowers remained open in *M. christinae*

Effect	Probability	LCL	UCL
Flower bud removal			
0%	0.48a	0.38	0.59
50%	0.32b	0.21	0.47
Pollen manipulation			
C	0.15b	0.06	0.31
R	0.59a	0.40	0.74
POL	0.95c	0.82	0.98
POL + R	0.95c	0.84	0.98

Values reported are mean predicted probability of closure through time, followed by lower and upper confidence limit mean values for each treatment (LCL and UCL, respectively)

Different letters indicate significant differences between treatments for each main effect ($P < 0.05$)

Floral scents: changes through time and pollen manipulation effects

Scent production

The generalized linear model showed significant effects of time of sampling ($F_{2,47} = 51.67$, $P < 0.0001$) and time of sampling by pollen manipulation ($F_{6,47} = 31.74$, $P < 0.0001$; Fig. 1); the pollen manipulation effect was not significant ($F_{3,47} = 5.763$, $P = 0.12$). Preplanned contrasts showed an increase in scent production during the

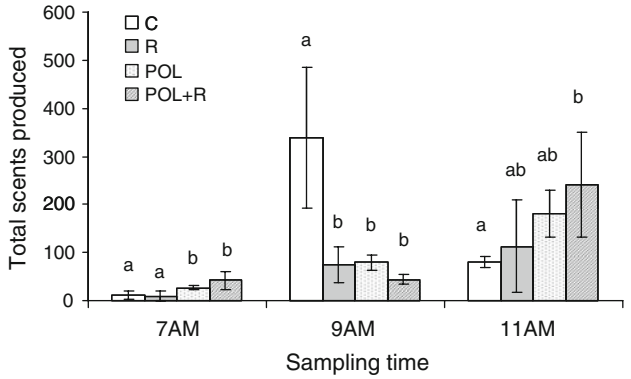

Fig. 1 *Myrmecophila christinae* flower scent production for pollen manipulation treatments across flower collection intervals (values are mean GC peak areas ± SE). Note that on the *Y*-axis values are $\times 10^3$. *C* No manipulation, *R* removed pollinia, *POL* pollination, no pollinia removal, *POL* + *R* pollination and pollinia removal. *Different letters* indicate significant differences between pollen treatments for each time interval

sampling period, as flowers collected at 0900 and 1100 h had significantly greater average values compared to those collected at 0700 h (0700 vs. 0900 h: $\chi^2 = 46.49$, $P < 0.0001$; 0700 vs. 1100 h: $\chi^2 = 71.36$, $P < 0.0001$). Although a treatment effect on scent production was not observed, the significant interaction term indicated that the effects of each pollen treatment varied through time (see Fig. 1). Specifically, control flowers showed a substantial increase in scent production at 0900 h, differing significantly from all other pollen treatments at this moment ($\chi^2 \geq 11.32$, $P < 0.001$ in all cases). Additionally, POL flowers showed an increase in scent production at 1100 h and differed significantly from C flowers ($\chi^2 = 6.11$, $P = 0.01$; see Fig. 1).

Abundance per compound and scent composition

Overall, the most abundant compounds found in flower extracts were *p*-cresol, benzenoids such as methyl salicylate and benzothiazole, and an unidentified compound (see Table 2). PCA showed that the first three axes of variation explained 42.67, 13.30, and 10.89% of the variation in compound abundance in floral scents, respectively. Most compounds showed a strong loading on PC 1 suggesting qualitative and quantitative similarities in scent profiles across pollen treatments and sampling times. Compounds structuring PC 1 were benzothiazole, methyl salicylate, calamenene, tetradecane, pentadecane, iso-amyl isovalerate, and an unidentified compound (Table 2). On the other hand, unidentified compound no. 2 and α-curcumene structured PC 2, both with negative values (Table 2). Based on *z*-scores for PC 1 and PC 2, a relatively clear separation was observed between flowers collected at 0700 h and those collected later (Fig. 2a). Pollen

Table 2 Relative abundance (mean percentage of total scent production and standard error of the mean) of each scent compound detected in *M. christinae* flower extract samples, as well as compound loadings on each of the first two principal components from the PCA analysis

Compound	Abundance		Loadings	
	Mean	SE	PC 1	PC 2
p-Cresol	35.41	4.31	0.235	−0.257
NI 1	20.01	2.02	0.707	0.237
Benzothiazole	10.02	1.71	0.778	0.332
Methylsalicylate	8.04	2.26	0.908	0.333
Undecane	7.57	1.57	0.595	−0.045
Iso-amyl isovalerate	4.27	0.5	0.805	0.204
Dodecane	2.88	0.84	0.528	0.024
Pentadecane	2.77	0.5	0.825	0.376
Tetradecane	2.32	0.46	0.866	0.059
Cadinene	2.02	0.5	0.633	−0.312
Calamenene	1.86	0.3	0.928	0.182
Longifolene	0.95	0.22	0.414	0.106
Tridecane	0.91	0.19	0.607	−0.044
α-Cubebene	0.24	0.08	0.522	−0.581
α-Curcumene	0.24	0.08	0.493	−0.739
p-Benzoquinona	0.24	0.08	0.612	−0.062
NI 2	0.14	0.08	0.269	−0.705
α-Calacorene	0.05	0.02	0.470	−0.579

NI Unidentified compounds

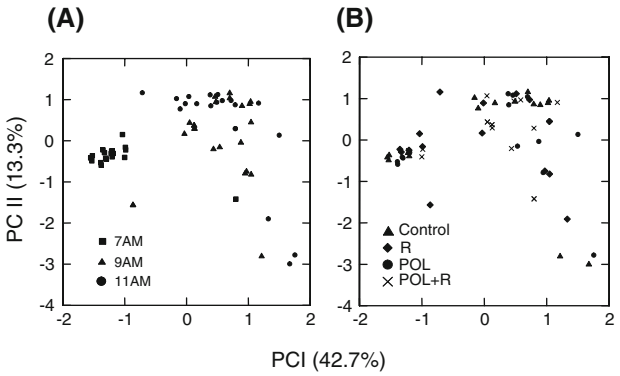

Fig. 2 Comparison of floral scent composition (using compound abundances) in *M. christinae* flowers across flower collection time intervals (**a**) and pollen treatments (**b**) based on standardized *z*-scores for PC 1 and PC 2. *C* No manipulation, *R* removed pollinia, *POL* pollination, no pollinia removal, *POL* + *R* pollination, with pollinia removal

treatments did not exhibit as clear of a separation between manipulated flowers and controls (Fig. 2b).

The ANOVA for PC 1 standardized *z*-scores showed significant differences due to time of sampling ($F_{2,47} = 70.72$, $P < 0.0001$) and a marginally significant time of sampling by pollen manipulation interaction ($F_{6,47} = 2.01$,

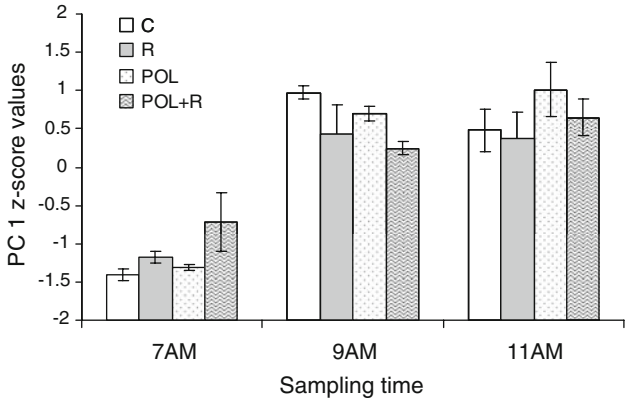

Fig. 3 Standardized *z*-score values for PC 1 (means ± SE) for each pollen treatment across flower collection time intervals. *C* No manipulation, *R* removed pollinia, *POL* pollination, no pollinia removal, *POL* + *R* = pollination and pollinia removal

$P = 0.08$). *Z*-scores did not differ significantly between pollen manipulation treatments ($F_{3,47} = 0.61$, $P = 0.61$). Bonferroni tests between sampling time intervals showed that the lowest mean *z*-score value was for flowers collected at 0700 h (-1.15 ± 0.11), followed by those collected at 0900 h (0.58 ± 0.11), and finally those at 1100 h (0.59 ± 0.14); both 0900 and 1100 h flowers had significantly greater values relative to control flowers ($P < 0.0001$ in both cases). The marginally significant interaction term showed a relatively similar pattern (although not as clear) to that observed for scent production (POL and C flowers showed a tendency towards greater mean values at 0900 and 1100 h, respectively) (see Fig. 3).

Discussion

Results from this study revealed important floral responses to pollen and reproductive resource status manipulations in the tropical orchid *M. christinae*. Some of these findings (e.g., pollination effects on floral longevity) have been previously reported for several species including orchids. Others such as resource status effects on floral longevity have received much less attention. Moreover, we found potentially important short-term patterns in scent production for control and pollinated flowers, which might impact pollinator visitation rates. Nonetheless, analyses of floral scents remain exploratory and warrant further investigations of the consequences for pollinator attraction and plant reproductive success. Overall, by looking at both physical and scent chemical changes in flowers of *M. christinae*, we offer a more integrated view of floral senescence with relation to pollination and resource status conditions.

Pollen manipulation and resource status effects on floral longevity

The relationship between plant reproductive resource status and floral longevity represents an important mechanism that might impact plant reproductive assurance and overall fitness (Primack 1985; Rathcke 2003), especially in species with low pollinator visit rates and high reproductive costs. Most studies have failed to make this connection and never have its consequences on plant fitness been assessed. Exceptions are related studies by Holtsford (1985) and Ashman and Schoen (1997) who manipulated floral longevity by controlling the amount of time before pollination in *Calochortus leichtlini* and *Clarkia tembloriensis*, respectively. Both studies found a trade-off between floral longevity and fruit and seed production given that a delayed pollination treatment (i.e., greater longevity) caused a significant decrease in fruit and seed production. In addition, a study by Abdala-Roberts et al. (2007) with the orchid *Cohniella ascendens* found a significant increase in floral longevity due to flower bud removal. Likewise, in this study we also observed a trade-off of this kind for *M. christinae* as floral longevity increased significantly (i.e., lower probability of closing in time) in inflorescences with previously removed flower buds, compared to control inflorescences. Presumably, this could be taken as evidence of a resource reallocation mechanism in which more resources are being designated to keeping flowers open in bud-removed inflorescences due to a reduction in flower construction costs. Another way of testing for resource status effect on floral longevity would be to manipulate bottom-up effects and measure changes in flower longevity. We consider that the relationship between resource status and floral longevity, as well as trade-offs between the latter and other reproductive components, remains understudied and further investigations should take the next step and measure plant fitness consequences of such effects.

At the same time, the pollen manipulation treatment also showed a significant effect on floral longevity. Specifically, pollinated flowers (POL and POL + R) closed much faster than control flowers or even removed pollinia flowers, and this is a typical response that has been reported for other orchids (e.g., Ackerman 1989; Proctor and Harder 1995; Clayton and Aizen 1996; Martini et al. 2003; Stpiczynska 2003). Likewise, flowers with removed pollinia closed significantly faster than control flowers, although this reduction in longevity was not as strong as that observed for pollinated flowers. This milder response of pollinia removal on floral longevity compared to pollen deposition has also been found in other orchids (e.g., *Chloraea alpina*, Clayton and Aizen 1996; *Mistacidium venosum*, Luyt and Johnson 2001), and in some cases no effect of pollina removal was observed at all (*Gongora quinquenervis*;

Martini et al. 2003). The reason for such differences in floral longevity responses to pollinia removal compared to deposition might be that the senescence-inducing signals provided by the former are weaker and less numerous than those caused by pollination (Clayton and Aizen 1996). Another explanation might be that the fitness benefit of remaining open is greater if the male function has been fulfilled compared to if the female function is fulfilled, given that in the latter case, the benefit of closing sooner is greater in order to secure fruit formation (see discussion in Clayton and Aizen 1996). Unexpectedly, flowers subject to both pollinia removal and insertion (POL + R) did not show a greater reduction in longevity compared to flowers that were only pollinated (Table 1), which confirms the nonadditive nature of these effects (Clayton and Aizen 1996). Finally, despite the fact that the pollen treatment by bud removal interaction was not significant, we suggest that future studies evaluate such an effect, as pollination (or pollinia removal) effects on floral longevity might change across plant resource status conditions and might represent an important source of variation in reproductive assurance and overall plant fitness.

Pollen manipulation effects on floral scents

In addition to studying the effects of pollen deposition and/or addition on floral longevity, a considerable amount of research has been devoted to understanding the effects of such manipulations on floral scent emissions. In general, studies have found that pollination causes a decrease in scent production and changes in composition after one or more days of treatment application (e.g., Arditti 1979; Tollsten 1993; Schiestl et al. 1997). However, in contrast to such studies the present work measured pollen manipulation effects on floral scents across a shorter time period. The reasons for this are (1) *M. christinae* flowers close within 24 h of being pollinated (results in this study), and (2) short-term floral scent responses to pollen manipulation have not been described before and might reveal patterns that are relevant to pollinator attraction and/or deterral.

Scent production

Although direct solvent extraction with hexane has been used frequently to quantify long-chain alkenes in sexually deceptive orchid species, it has also been used to measure the production of volatile compounds (e.g., Shaver et al. 1997; Flach et al. 2006). Nonetheless, we do caution on potential differences in results based on the use of other techniques such as headspace sampling. Based on the hexane extraction method, we found a significant increase in scent production from 0700 to 1100 h, and although the pollen treatment effect was not significant, the significant treatment by time interaction indicated differences in the response of flowers through time depending on the pollen treatment. The observed increase in scent production of C flowers at 0900 h might represent a natural peak in scent production by *M. christinae* plants adapted to early morning pollination by *Xylocopa* bees. Subsequent to this increase, scent production most likely starts decreasing in the late morning as temperature and flower maintenance costs increase (Arditti 1979) and pollinator activity decreases. On the other hand, pollinated flowers (especially POL + R) showed an increase in scent production for the latest sampling time (1100 h), differing significantly from control flowers, which suggests that pollination can have an effect on floral scent production (see below on scent composition). Pollen removal, however, seemed not to have any affect on scents, which coincides with a weaker effect of this treatment on longevity compared to pollination. These results are particularly appealing since flower color and shape have traditionally been the only variables used to explain variation in reproductive success in food-deceptive orchids. Further studies are needed, however, that look at (1) the loyalty or predictability of floral scent production short-term responses to pollen addition and/or removal, and (2) pollinator electrophysiological responses and choice tests using individual compounds found in *M. christinae's* floral scent.

Scent composition

PCA provided a more detailed description of chemical responses to pollen manipulation and time of sampling effects. Specifically, PC 1 was structured (positively) by benzenoids such as methyl salicylate and benzothiazole, as well as other less abundant compounds such as iso-amyl isovalerate, undecane, and an unidentified compound. In addition, *p*-cresol was the most abundant volatile compound found but was not strongly loaded to any of the first three PCs. The overall pattern observed from both the PCA and ANOVA for PC 1 scores indicated an increase in abundance for most compounds throughout the morning (as well as a more subtle increase for pollinated flowers at 1100 h), and not so much a change in scent composition.

Some of the compounds detected in *M. christinae*'s scent (e.g., benzenoids) might play a role in pollinator attraction by mimicking nectar (Andersson et al. 2002), while others may work as pollinator scent marks. An example of the latter is *p*-cresol, which has been reported as a pheromone in *Xylocopa* (Hefetz 1983). These findings suggest that although previous studies have reported a food-deceptive system for *M. christine* (Rico-Gray and Thien 1987), the plant may also include pheromone-like attraction that makes it different from traditional food-deceptive systems in orchids (see Johnson et al. 2005).

Regardless of the specific mechanism of attraction, the observed effect of pollen addition on scent chemistry (or the abundances of some of these compounds) may have important consequences on pollinator attraction and plant reproductive success, considering that other flowers from the same plant or nearby plants have not been pollinated yet. Additional experiments are needed in which floral scent responses to pollination as well as pollinator responses to these compounds are fully characterized in order to evaluate their role as attractants or repellents.

A final note worth discussing has to do with the euglossine bee *Eulaema polychroma*, which has been previously reported as a pollinator of *M. christinae*. It is well-known that male euglossine bees collect floral fragrances from certain orchid species and in so doing, pollinate flowers (Dressler 1982; Roubik 1989). Furthermore, *E. polychroma* males have been shown to respond electrophysiologically to compounds such as methyl salicylate (Schiestl and Roubik 2003, but see Ackerman 1983), which in this study strongly structured PC 1. Nonetheless, based on data from several reproductive seasons, *E. polychroma* was shown to be virtually absent at the study area, leaving *Xylocopa* bees as *M. christinae*'s only pollinator (Rico-Gray and Thien 1987; Parra-Tabla and Vargas 2004). Therefore, from an evolutionary perspective, the fact that *Xylocopa* bees do not collect fragrances to obtain these compounds (and thus are probably not as attracted to them) might result in *Xylocopa* selecting for a different floral scent composition in *M. christinae* populations in Yucatan compared to other regions where *Eulaema* is abundant. This idea, although appealing, remains speculative and in order to be tested would require a multi-population approach at sites with and without *Eulaema*, as well as long-term experimental manipulations of pollinator species abundances.

Conclusions

Findings from this study have shown that reproductive resource status is an important factor driving natural variation in floral longevity, as well as overall plant reproductive success via changes in the latter. In addition, although our analyses only offer an exploratory view of floral scent dynamics and its consequences for pollinator attraction, we found that potentially important scent production changes occur across short time scales (i.e., hours) in *M. christinae*, and these changes depend to a certain extent on pollen manipulation. Such short-term responses might be particularly important in pollen-limited species such as orchids, for which pollination-induced flower senescence is most rapid. We consider that simultaneously studying resource status and pollen status effects on floral longevity and scent characteristics offers a more integrated view of floral senescence and pollinator attraction and/or deterral mechanisms.

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