PLANT ANIMAL INTERACTIONS

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Temporal and sexual variation of leaf-produced pollinator-attracting odours in the dwarf palm

Abstract Information on intra-specific variation in pollinator-attracting floral traits provides clues to selective pressures imposed by pollinators. However, these traits also reflect constraints related to floral phenology or morphology. The specific weevil pollinator *Derelomus chamaeropsis* of the dioecious Mediterranean dwarf palm *Chamaerops humilis* is attracted by volatile compounds that leaves, and not flowers, release during anthesis. Production of these olfactory cues is thus probably not constrained by any other floral function. This provides the opportunity to study variation of a "floral" trait that is not produced by a floral organ. We studied volatile compounds emitted by leaves of 12 individual *C. humilis* over the whole flowering season. The quantity of volatile compounds emitted by leaves reached a maximum when plants required pollinator visits. The relationship between odour emission and floral phenology was slightly different between male and female plants, probably reflecting differences in the exact time at which females and males benefit from pollinator visitation. Male plants produced higher quantities of volatile compounds than females. Odour composition was highly variable among individuals but did not differ between male and female plants. In this system, female *C. humilis* are pollinated by deceit and pollinators should be selected to avoid visiting them. The absence of sexual difference in blend composition may thus prevent pollinators from discriminating between male and female plants.

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Introduction

Signals that attract pollinators influence many aspects of sexual reproduction in animal-pollinated plants. Pollination biologists have mainly focused on visual traits of flowers due to their wide diversity in colour, size and shape (Faegri and Van der Pijl 1979) and the consequences of such variation for pollinator behaviour (Peakall and Handel 1993; Møller and Sorci 1998; Schemske and Bradshaw 1999). More recent studies have analysed olfactory signals produced by flowers and established their role in pollinator attraction (Pellmyr and Thien 1986; Dobson 1994; Knudsen and Tollsten 1995; Dobson et al. 1996).

Traits involved in pollinator attraction affect both the identity and the number of pollinators. Because potential pollinators respond differently to floral signals (Menzel and Shmida 1993; Schemske and Bradshaw 1999), the combination of attractive traits (e.g. colour, shape, chemical composition of fragrances) provides an indication of the most likely pollinators (Dobson 1994; Johnson and Steiner 2000). Besides the combination of qualitative floral traits, the intensity of the signal (e.g. quantity of volatile compounds emitted, floral size) often influences attractiveness and directly affects individual fitness (Young and Stanton 1990; Johnson et al. 1995). Recent studies have emphasised that such floral traits may vary considerably both among individuals (e.g. Galen and Kevan 1980; Fry and Rauscher 1997; Schiestl et al. 1997; Ayasse et al. 2000; Subramaniam and Rauscher 2000; Grison-Pigé et al. 2001; Knudsen 2002) and over the flowering season (e.g. Shaver et al. 1997; Grison-Pigé et al. 2001; Schiestl and Ayasse 2001). Therefore, to fully understand plant/pollinator communication, it is necessary to study attractive signals on several individual plants, throughout the complete flowering period.

Table 1 Phenological stages of the flowering period for male and female inflorescences. Six detailed stages can be defined. The whole flowering period can also be separated into three more general phases. Stage length was measured for the 12 plants from which volatile compounds were collected. There were no significant differences between sexes for stage length [General Linear Models procedure (SAS1999)]

Stage	Male inflorescence	Female inflorescence	Stage length (days)	General phase
A	Bracts closed, enveloping the developing inflorescence		15.4±9.9	Floral bracts closed
B	Bracts closed, the inflorescence occupying more than half of the space provided by the prophyll		2.8±2.5	
C	Floral bracts opening, the inflorescence emerging less than 2 cm		2.0±1.9	Anthesis
D	Pollen production	Female receptivity: white and wet stigmas	7.5±3.9	
E	End of pollen production, flowers start to dry	End of receptivity: brown stigmas	8.6±6.3	
F	Dry inflorescence: flowers fall down	Fruit development: ovaries swollen green	7.6±3.9	End of flowering

Variation in floral attractive traits over time or among individuals, populations or sexes in a given species is usually interpreted as the consequence of variation in pollinator-mediated selection (Shaver et al. 1997; Schiestl et al. 1997; Grison-Pigé et al. 2001; Schiestl and Ayasse 2001). However, flowers are also the sites of gamete production. Floral structures must also protect gametes against desiccation and herbivory. Variation in floral traits may thus alternatively be related to other functions performed by floral organs and might be generated by factors such as variation in herbivory pressure or in abiotic conditions (Delph et al. 1996; Galen 1999).

The dioecious dwarf palm (*Chamaerops humilis* L., Arecaceae) offers an interesting case where pollinator attraction is distinct from other causes of variation in attractive floral traits. The volatile compounds that attract the specific pollinator to this species are produced by leaves, not by flowers, and the odour is only produced during the flowering season (Dufa et al. 2003). Thus, pollinator attraction is likely to be its major function. This provides the opportunity to examine variation of a trait that performs a usually "floral" function but is not produced by a floral organ.

In this study, we document patterns of variation in signals that attract pollinators of the dwarf palm. We quantified variation in volatile compound emission: (1) among individuals and (2) over the flowering season for each individual. We used this information to compare emission of olfactory cues between male and female plants, focussing on: (1) the relationship between odour emission and floral phenology, (2) the composition of the chemical bouquet, and (3) the quantity of volatile compounds released.

Materials and methods

Study species

The dwarf palm is a dioecious palm and grows near the Mediterranean coasts of Europe and North Africa. It is pollinated by a host-specific palm flower weevil (*Derelomus chamaeropsis* F., Curculionidae), which obtains several types of rewards on the plants, including shelter, egg-laying sites and food for adults and larvae (Anstett 1999). Pollinators lay eggs in the rachises of both male and female inflorescences (Dufa and Anstett 2004). However, larvae only grow in the rachises of male inflorescences, and female plants are pollinated by deceit (Anstett 1999). Because larval development occurs after male flowering has ended, pollinators do not impose a high cost for male plants. The following spring, larvae pupate within rachises and a new generation of weevils emerge from old male rachises (Anstett 1999).

Collection and identification of volatile compounds

We collected the volatile compounds emitted by one fully expanded leaf from each of 12 potted *C. humilis* (six males and six females), every two days, over their whole flowering period. The first collection occurred when the bracts of inflorescences started to emerge from the stem but were still totally enclosed around the developing inflorescence. Collections ended when all flowers had wilted. Plants were kept outdoors in the experimental garden of the Centre d'Ecologie Fonctionnelle et Evolutive in Montpellier, France, where they experienced natural conditions of sunlight and temperature. However, odour collections were performed in a greenhouse to control for environmental conditions.

A preliminary study indicated that leaves emitted volatile compounds mostly during the day (Dufa et al. 2003). Each day, from 16 March to 29 May 2001, two sessions of odour collection were conducted, each for three plants. The morning session was conducted from 0900 hours to 1200 hours (solar time); the afternoon session from 1200 hours to 1500 hours (solar time). For each plant, we alternated morning and afternoon sessions. At each collection, we recorded the phenological stage of the plants (see Table 1 for the definition of phenological stages).

Volatile compounds were collected by headspace adsorption (Heath and Manukian 1994; Grison-Pigé et al. 2001). One leaf was enclosed in a polyethylene terephtalate (Nalophan) bag (Kalle Nalto, Wursthüllen, Germany). Pure air was blown into the bags at 400 ml min^{-1} and extracted at 300 ml min^{-1} through a trap containing 30 mg of Alltech Super Q absorbent (ARS, Gainesville, Fla.). The difference in flux ensured that the system was continuously purged through the inevitable leaks and that no outside air could enter the system. For each session, blanks were collected in parallel using an empty bag. After 3 h of collection, trapped volatiles were eluted with 150 µl of dichloromethane and preserved at −18°C until analysis. Two internal standards (nonane and dodecane, 200 ng $µl^{-1}$) were added to each sample for quantification purposes.

Volatile compounds were analysed by injection in a Varian CP-9003 gas chromatograph (Alltech column EC-1, non-polar, length 30 m, internal diameter 0.25 mm, film thickness 0.25 µm, carrier gas He), in which the oven temperature was regulated by a multirise program (3 min at 50°C; 50–100°C at 2°C min^{-1}; 100–180°C at 4°C min^{-1};180–250°C at 6°C min^{-1}). The relative quantities of volatile compounds emitted by the leaves were estimated for each sample using as a scale the areas of the peak of the two internal standards. Compounds were identified using a gas chromatograph (Hewlett Packard)-mass spectrometer (model 5870, Hewlett Packard) (DB5

column, length 30 m, internal diameter 0.25 mm, film thickness 0.25 μm, carrier gas He, oven temperature program: 50–180°C at 3°C min^{-1}). Component identification was based on computer matching of the mass spectra with Wiley 138 and NBS 75 K libraries and on retention indices reported in the literature (Adams 1995). If needed, we used additional libraries (Adams, Joulain, Casabianca, NTO) for the identification of the mass spectra.

Data analysis

Initially, we analysed variation in the quantity of volatile compounds collected. We first performed graphical observations and defined a phase of high production for each individual plant (plants were considered to have entered this phase when they produced at least 30% of the quantity of volatile compounds collected on the day of maximum production). With an ANOVA [General Linear Models procedure (proc GLM) (SAS 1999)] we tested for a sex effect on: (1) the duration of the high production phase, and (2) the delay between the opening of floral bracts and the day of maximum production. We then focused on the samples collected during the high production phase and analysed the quantity of volatile compounds released by plants during this phase, using proc GLM (SAS 1999; type III SS). Prior to this analysis we log-transformed the variable to obtain normally distributed residuals. The explanatory variables tested were sex of the plant, plant individual (nested in sex and included in the model as a random effect), phenological stage and extraction session (morning or afternoon).

Subsequently, we focused on three volatile compounds, E-β-ocimene (monoterpene), E-β-farnesene (sesquiterpene) and one unidentified sesquiterpene (not found in the available mass spectrometer libraries), which together represented on average 85% of the quantity of odour produced at the maximum of odour emission (Dufaÿ et al. 2003). We built qualitative variables based on the presence or absence of each of the three compounds within the collected blends. We analysed these variables using a logistic regression [binomial distribution and logit link function, proc GENMOD (SAS 1999)] and corrected for the overdispersion of data

[dscale option, proc GENMOD (SAS 1999)]. We tested for effects of the phenological phase and plant sex.

To study the chemical composition of blends collected during the high production phase, we analysed the proportions of the three major chemical compounds within each blend collected during this phase for all plants, with a multiple ANOVA [proc GLM (SAS 1999), type III SS]. We tested for effects of plant sex, plant individual (nested in sex and included in the model as a random effect), phenological stage and extraction session (morning or afternoon). We verified that sphericity was respected. For all ANOVAs, residuals were normally distributed (Shapiro-Wilk $P > 0.1$).

Results

Quantity of volatile compounds released

Temporal dynamics of odour production by leaves

Temporal patterns of the emission of volatile compounds by leaves consistently showed one major peak and sometimes another peak, but of lower intensity (Fig. 1). The day of maximum production varied among plant individuals from April 1 to May 8 2001, but always occurred during plant anthesis, i.e. after the opening of floral bracts. Female and male plants reached their maximum in emission, respectively at 3.6±1.5 and 9.3 ±5.1 days after floral bracts opened; this delay was significantly shorter in females than in males ($F_{1,10}$=6.66, P=0.03).

High production (i.e. when the quantity of volatiles collected exceeded 30% of the quantity collected at the maximum production) lasted 8.2±2.3 days, encompassing

Fig. 1 Quantity of volatile compounds (ng 3 h^{-1}) released by 12 *Chamaerops humilis* (*1–6* female individuals, *7–12* male individuals) every 2 days during the flowering season in spring 2001. The *horizontal bars* indicate anthesis
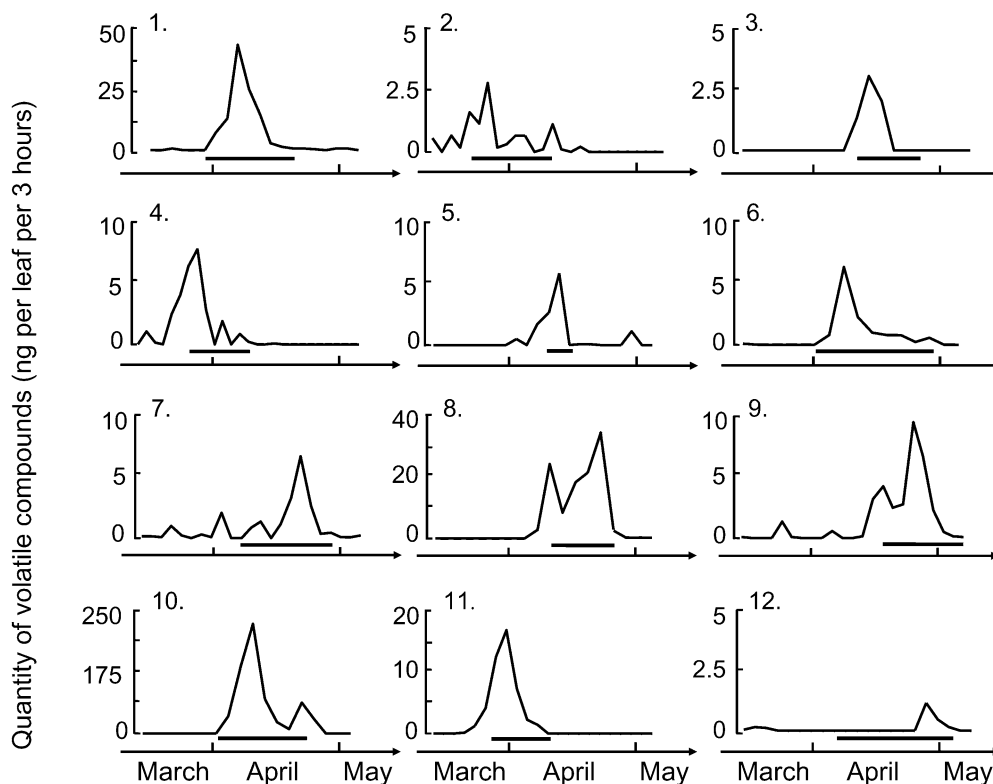
the day of maximum production, and did not differ between females (8.0 days) and males (8.3 days; $F_{1,10}=0.06$, $P=0.82$). Five (three males and two females) out of 12 plants began the high production phase before anthesis, at the end of stage B, on average 3.6 days before the floral bracts opened. All plants had a high production of volatiles during anthesis and no individual continued to produce amounts of volatiles after flowers started to fade.

Quantity of volatile compounds produced during the high production phase

The quantity of volatile compounds was extremely variable among individual plants. At maximum production, the quantity collected ranged from 1.13 ng to 224.54 ng leaf^{-1} 3 h^{-1}, with a mean of 30.14 ng leaf^{-1} 3 h^{-1}. An ANOVA built on the quantity of volatile compounds collected for the different sessions during the high production phase showed that this quantity depended both on the individual plant (nested in sex: $F_{10,40}=15.14$, $P<10^{-4}$) and on the sex of the plants (marginally significant: $F_{1,10}=4.34$, $P=0.06$). On average, male plants produced more than 4 times more volatiles than did females (respectively 30.64 ng 3 h^{-1} and 6.54 ng 3 h^{-1}). We found no effect of the collection session (morning or afternoon) nor of the phenological stage (stages C, D or E) on the quantity of volatile compounds collected.

Blend composition

We focused on the three major compounds found within blends, one monoterpene (*E*-β-ocimene) and two sesquiterpenes (*E*-β-farnesene and an unidentified sesquiterpene).

Temporal dynamics in the production of the three major compounds

The presence of each of the three compounds within blend collections varied with plant phenology (with three levels: closed bracts/anthesis/end of flowering).

The presence of *E*-β-ocimene in leaf blends depended on floral phenology (*df* =1, $\chi^2=19.76$, $P=0.0002$). *E*-β-ocimene was mostly released during the last two phases (78.6% and 83.3% of blends collected, respectively, during anthesis and at the end of flowering contained *E*-β-ocimene). A significantly lower proportion (25.6%) of blends collected before floral bracts opened contained *E*-β-ocimene (Fig. 2). We found no effect of plant sex nor of the interaction between sex and phenology on the presence of *E*-β-ocimene in blends.

The presence of *E*-β-farnesene in leaf blends also depended on floral phenology (*df*=1, $\chi^2=12.79$, $P=0.0051$). *E*-β-farnesene was mostly released during anthesis (48.6% of blends collected at anthesis contained *E*-β-farnesene). The proportion of blends containing *E*-β-
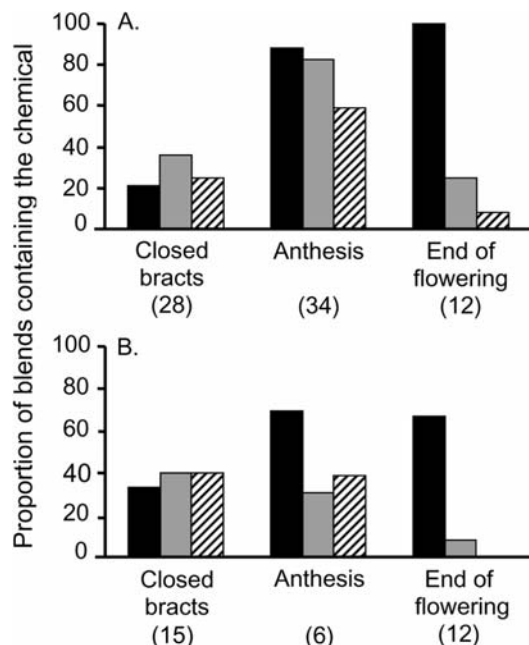

Fig. 2 Percentage of blends in which the three major compounds, *E*-β-ocimene (*black bar*), the unidentified sesquiterpene (*grey bar*) and *E*-β-farnesene (*hatched bar*) were detected, as a function of the phenological phase at which blends were collected, for **A** female and **B** male plants. *Numbers in brackets* indicate the number of chemical blends analysed

farnesene at the end of flowering (4.2%) was significantly lower, while the proportion of blends collected before floral bracts opened (30.2%) was intermediate (Fig. 2). We found no significant effect of the sex of the plant nor of the interaction between sex and phenology.

Presence of the unidentified sesquiterpene depended on both sex (*df*=1, $\chi^2=5.71$, $P=0.02$) and phenology (*df*=2, $\chi^2=6.85$, $P=0.03$). This compound was found significantly more often in leaf blends collected on male plants than on female plants. It was mostly released during anthesis (55.7% of blends collected at this phase contained this compound); the proportion of blends containing this sesquiterpene at the end of flowering (16.6%) was significantly lower and the proportion of blends collected before floral bracts opened (37.2%) was intermediate (Fig. 2). We found no interaction between sex and phenology.

None of these compounds was produced during the first phenological stage (stage A). Samples containing these compounds before floral bracts opened were always collected during stage B, shortly before the opening of floral bracts. *E*-β-ocimene was commonly detected in volatile collections performed after the high production phase, while detection of the two sesquiterpenes quickly stopped.

Variation of the chemical signature

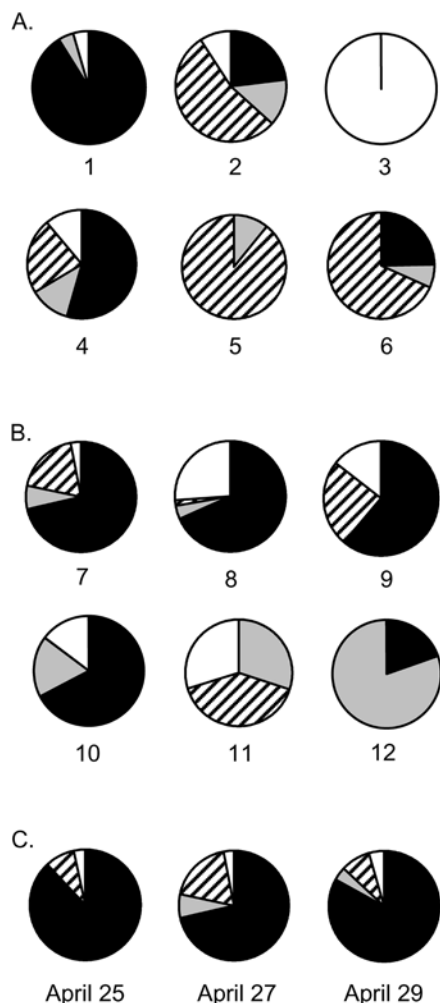The proportions of the three major compounds within leaf blends were extremely variable among individuals. At

Fig. 3 Relative quantities of *E*-β-ocimene (*black area*), the unidentified sesquiterpene (*grey area*) and *E*-β-farnesene (*hatched area*), and other compounds (*white area*) in blends emitted by **A** the six female plants, **B** the six male plants at the peak of release and for **C** one male individual (individual no. *7*) for all volatile collections performed during the high production phase, on 25, 27 and 29 April 2001. The proportions of the major compounds at the maximum production were highly variable among individuals (**A** and **B**). In contrast, the odour blend emitted by a given individual exhibited low variation across time (**C**)

Table 2 Multiple ANOVA explaining the proportions of the three major compounds during the high production phase. Individual plant and phenology had significant effects on the bouquet composition, while sex had no any significant effect on the bouquet composition

Variable	*df*	MS	*F*	*P*
Sex	1	209	0.28	0.6
Individual (sex)	10	789	52.46	<0.0001
Phenology	4	48	3.20	0.03
Error	25	15		

within individuals. This variance was low compared to that among plants (Table 2), showing that each plant had its own chemical signature (Fig. 3c). We found no significant effect of sex of the plant, extraction session (morning or afternoon) or interactions between any tested factors.

Discussion

This study of attractive compounds emitted by *Chamaerops* leaves during the flowering season highlights a precise relationship between odour production and floral phenology: plants reached their maximum of odour release at a moment when pollinator visitation was beneficial, the exact moment of this maximum being earlier in female plants than in males. Following several individual plants also allowed us to reveal an extreme variation among individuals in both quantity of odour released and blend composition. Finally, we documented a marginally significant sex difference, male plants releasing more attractive volatiles than conspecific females. All these results provide clues to the selective pressures imposed by the specific pollinator on the attractive signals emitted by male and female *C. humilis*.

Attractive scents and phenology

Attractive scent production by *C. humilis* leaves occurs only during the flowering period (Dufa et al. 2003). By studying this synchrony in more detail, we have shown a strong phenological pattern for both odour blend composition and quantity of volatile compounds released by leaves. All plants produced scent during a short period (6–12 days) during anthesis. The day of maximum odour release varied among plants from April 1 to May 8 but always coincided with anthesis.

The opening of floral bracts coincided with the release of the three major volatile compounds. The production of *E*-β-ocimene continued, however, well after the high production phase, while release of sesquiterpenes stopped. This difference could (1) be due to different properties of the pathways involved [monoterpenes and sesquiterpenes are synthesised in two different biogenetic pathways in different parts of the cell (Bohlman et al. 1998)], or (2)

maximum production, the relative quantities of *E*-β-ocimene, *E*-β-farnesene and the unidentified sesquiterpene varied, respectively, from 0 to 91%, from 0 to 89% and from 0 to 80% (Fig. 3a, b).

A multivariate ANOVA built on the proportions of the three compounds within blends collected for all sessions during the high production phase showed that the bouquet depended on plant individual and phenological stage (Table 2). The individual plant effect was the factor explaining the highest part of the variance (Table 2). Phenology had an overall effect on the relative proportions but we found no statistical difference for pairwise comparisons between phenological stages (Tukey-Kramer: $P>0.1$ for all tests). The sum of squares associated with the error in the model included the intra-individual variation, i.e. the variance of the chemical bouquet among dates

reflect different roles of the three compounds in pollinator attraction.

Female plants reached their maximum scent emission early, 3–4 days after floral bracts opened, while for males this was later, at about 9 days. Male and female reproductive success may be maximised by different timing or duration of pollinator visitation. A better knowledge of flowering phenology and receptivity of male and female plants in natural populations is now needed to correctly interpret these differences in the kinetics of odour release between sexes.

Blend polymorphism

The chemical compounds produced by *C. humilis* varied greatly among plants both in quantity and blend composition. However, for a given individual plant the composition of the bouquet showed very low variation across time during the high production phase. Because this was observed on potted plants in controlled conditions, environmental factors are unlikely to be a major cause of odour variability. Therefore, each plant seems to have its own chemical signature. Because few studies have examined intraspecific variation in chemical signals, it is not known whether such qualitative and quantitative variation in floral traits is common in pollination mutualisms or whether *C. humilis* constitutes a particular case. However, as suggested by the few other studies on intraspecific variation in olfactory cues (Gouinguené et al. 2001; Grison-Pigé et al. 2001; Knudsen 2002), our results demonstrate that even in the context of an obligate, species-specific interaction, chemical components of floral/leaf scents must be analysed on several individuals to obtain an accurate picture of a species' chemical signals. Moreover, intraspecific variation in odour composition could explain the limited utility of floral fragrance composition for deducing phylogenetic relationships among species (Levin et al. 2003).

The causes of this variation in attractive traits remain unclear. Further studies will establish whether chemical signal in *C. humilis* varies mainly among populations, as has been shown in many cases, variation being generated by inter-population differences in the guild of pollinators or in the abundance of natural enemies (Groth et al. 1987; Bergström and Bergström 1989; Robertson and Wyatt 1990; Galen 1999); or within populations, as in some systems where intraspecific variation is generated by particular selective pressures, such as frequency-dependent selection (Eckert et al. 1996; Ayasse et al. 2000; Subramaniam and Rausher 2000; Gigord et al. 2001).

Sexual variation of the chemical signal

We compared both qualitative (blend composition) and quantitative (quantity of volatile compounds) aspects of the chemical signal between male and female plants. We detected no difference between male and female plants in composition of the bouquet, as is expected in dioecious species in which one sex is pollinated by deceit by mimicking the attractive traits of the other (Bawa 1980; Dafni 1984; Dukas 1987).

With regard to the quantity of volatile compounds emitted during the high production phase, we recorded a marginally significant male/female difference ($P=0.06$). This was probably due to both the very high individual variation in the quantity of fragrance and the small number of individual plants that were followed. Because the average difference between males and females was very large (respectively 30.64 ng 3 h^{-1} and 6.54 ng 3 h^{-1}), we considered it to be biologically significant. The necessity to follow all plants throughout the flowering season limited the number of individuals we could study. Further work will be done on a larger number of plants, each studied only during its peak of odour production. Although the question has not been experimentally tested in this system, the quantity of odour released is likely to reflect the attractiveness of the plants, because it influences the distance at which pollinators perceive the attractive signal. This assertion is consistent with what is generally observed in dimorphic plant species where males (or hermaphrodites in gynodioecious species) are more attractive than conspecific females (Bawa 1980; Ågren et al. 1986; Delph et al. 1996; Armstrong 1997; Eckart 1999; Williams et al. 2000).

The classic hypothesis to explain sexual dimorphism is that male and female plants are under different selective pressures to attract their pollinators, which is usually interpreted as a consequence of sexual selection, male reproductive success being limited by access to mates while females are limited by resources (Charnov 1979; Willson 1979). This view has been contested by several authors who have pointed out that differences in floral traits may not necessarily be linked with their role in pollinator attraction but with constraints acting on the floral organ, which differ between male and female flowers (reviewed by Delph et al. 1996; Eckart 1999). For instance, the presence of stamens sometimes directly induces a larger petal size through hormonal correlation (reviewed in Delph et al. 1996).

In *C. humilis*, males emitted greater quantities of volatile compounds attractive to the pollinator and these were not produced by a floral organ. Moreover, both the absence of fragrance emission outside the flowering period and the fine synchrony between the exact flowering phenology and the quantity of scent emitted strongly suggest that the major, and perhaps the only, function of the volatile compounds is pollinator attraction. Finally, to our knowledge, nothing is known about possible sex-specific constraints on leaf physiology or morphology, but such questions can now be examined. Together, these results suggest that male *C. humilis* are under stronger selective pressure than conspecific females to attract their pollinator. These differences in selective pressures may be due either to a higher benefit of pollinator attraction for male fitness or to a higher cost to female fitness (for instance through damage caused by pollinators once they are within the female inflorescences, or by negative correlation between fruit production in one year and the subsequent survivorship or fruit production, resulting in reduced lifetime fitness).

This system opens interesting perspectives and shows that pollinator attraction can sometimes be distinguished from selection acting on other floral functions. Finding similar patterns of pollinator attraction in other plant species will be of great help in better understanding how pollinator-mediated selection has acted on floral traits.

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