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RESEARCH PAPER

Is the timing of scent emission correlated with insect visitor activity and pollination in long-spurred *Satyrium* species?

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ABSTRACT

Plants are expected to emit floral scent when their pollinators are most active. In the case of long-tubed flowers specialised for pollination by crepuscular or nocturnal moths, scent emissions would be expected to peak during dawn. Although this classic idea has existed for decades, it has rarely been tested quantitatively. We investigated the timing of flower visitation, pollination and floral scent emissions in six long-spurred *Satyrium* species (Orchidaceae). We observed multiple evening visits by pollinaria-bearing moths on flowers of all study species, but rarely any diurnal visits. The assemblages of moth pollinators differed among *Satyrium* species, even those that co-flowered, and the lengths of moth tongues and floral nectar spurs were strongly correlated, suggesting that the available moth pollinator fauna is partitioned by floral traits. Pollinarium removal occurred more frequently during the night than during the day in four of the six species. Scent emission, however, was only significantly higher at dusk than midday in two species. Analysis of floral volatiles using gas chromatography coupled with mass spectrometry yielded 168 scent compounds, of which 112 were species-specific. The scent blends emitted by each species occupy discrete clusters in two-dimensional phenotype space, based on multivariate analysis. We conclude that these long-spurred *Satyrium* species are ecologically specialised for moth pollination, yet the timing of their scent emission is not closely correlated with moth pollination activity. Scent composition was also more variable than expected from a group of closely related plants sharing the same pollinator functional group. These findings reveal a need for greater understanding of mechanisms of scent production and their constraints, as well as the underlying reasons for divergent scent chemistry among closely related plants.

INTRODUCTION

Floral volatiles play a key role as signals in pollinator attraction to flowers (Raguso 2008a,b). However, the same volatiles that attract mutualistic partners can also attract flower visitors that have a negative effect on plant fitness, including herbivores, nectar robbers and inefficient pollinators (see Miyake & Yahara 1998, 1999). There are several solutions to this problem. First, plants may emit volatiles that deter detrimental visitors simultaneously with volatiles that attract pollinators (Junker & Bluthgen 2010; Kessler *et al.* 2013). Alternatively, plants may restrict scent emission to those times of day when their pollinators are most active (Theis *et al.* 2007). This would limit the opportunities for flower detection by visitors that do not contribute positively to plant fitness. A further benefit of temporally restricted scent emission may be a reduction in cost associated with the production of energetically expensive scent compounds (Dudareva & Pichersky 2006).

In plants pollinated by crepuscular and nocturnal moths ('moths' in the remainder of the text, unless otherwise specified), floral scent is thought to be a particularly important cue for pollinator attraction, as visual cues alone are insufficient (Raguso & Willis 2002). The timing of scent emission in moth-pollinated plants is traditionally thought to be largely confined to the period between dusk and dawn (*e.g.* Faegri & van der Pijl 1979; Kaiser 1993; Knudsen & Tollsten 1993; Jürgens *et al.* 2002). Moth-pollinated plant species are characterised by scent compounds that could attract a wide range of insects (Knudsen & Tollsten 1993). By restricting the timing of emission of these compounds to a period in which moths, but not many other insects are active, the negative impact of attracting detrimental visitors can potentially be avoided.

Three lines of evidence are required to test the hypothesis that there is an association between moth pollination and nocturnal scent production. Visitor observations are required to confirm that moths are the most important flower visitors. However, not all visitors are effective pollinators (Ne'eman *et al.* 2010; King *et al.* 2013). By quantifying the timing of pollination, rather than visitation, additional evidence for evening pollination can be gathered. Experiments that manipulate the exposure of flowers in time can be used for this (Jürgens *et al.* 1996; Miyake & Yahara 1998; Young 2002; Giménez-Benavides *et al.* 2007; Morinaga *et al.* 2009; Martinell *et al.* 2010). Finally, scent emission needs to be measured during the peak activity of potential diurnal and nocturnal pollinators (typically late morning and early evening). Floral scent analysis based on gas

chromatography coupled with mass spectrometry (GC-MS) provides a powerful means for detailed studies of variation in diel patterns in scent production at the level of individual scent compounds (Balao *et al.* 2011; Dötterl *et al.* 2012). Few studies to date have integrated pollinator observations with an assessment of the timing of pollination and scent emission. Moreover, investigations are largely limited to a few established model systems, such as the nursery pollination system in *Silene* (*e.g.* Jürgens *et al.* 1996; Giménez-Benavides *et al.* 2007) or moth pollination in *Nicotiana* (Kessler *et al.* 2008). It is therefore largely unknown whether the purported association between moth pollination and evening scent emission exists across a broad range of species and systems.

The aim of this study was to assess the association between the timing and composition of floral scent emission and flower visitation and pollination in a comparative study of six long-spurred species of the orchid genus *Satyrium*. In almost all orchids, pollen is transferred as part of pollinaria, structures that consist of pollinia (the pollen mass which is either solid or, in the case of orchidoid genera such as *Satyrium*, composed of several hundred pollen units known as massulae), the viscidium (which sticks to flower visitors) and a caudicle (a structure that connects the viscidium to the pollinium; Johnson & Edwards 2000). These specialised structures make orchids a particularly useful model system to establish the timing of pollination, as pollinarium removal can be readily established through visual inspection of flowers in the field. The approach we used here was to inspect flowers for the removal of pollinaria at two intervals throughout a 24-h cycle. The six species selected here are putatively moth-pollinated, based on a previous study of one of the study species (Johnson 1997) or their possession of traits such as white flowers and a sweet and spicy scent (Hall 1982; Johnson 1997; van der Niet *et al.* 2009). To test the hypothesis that the timing of pollination and scent emission in the six study species would be correlated, we examined the following predictions: (i) moths would be the most important pollinators of the investigated *Satyrium* species; (ii) pollination levels would be higher during the night than during the day; and (iii) total scent emission and/or emission of single compounds would be highest during the night. As the pollination biology of several of the study species had not been studied previously, we also recorded other floral traits, such as spur length, nectar properties and spectral reflectance, to determine if these were also consistent with floral adaptation to moth pollinators.

MATERIAL AND METHODS

Study species

The six *Satyrium* species included in this study were *S. acuminatum* Lindl., *S. ligulatum* Lindl., *S. outeniquense* Schltr., *S. pallens* S.D. Johnson & H. Kurzweil, *S. situsanguinum* van der Niet & Liltved, *S. stenopetalum* Lindl. ssp. *brevicalcaratum* (Bolus) A.V. Hall and *S. stenopetalum* Lindl. ssp. *stenopetalum*. Detailed information about the morphology, distribution, classification and evolutionary relationships among these species is given in Hall (1982), Johnson & Kurzweil (1998) and van der Niet *et al.* (2009, 2011). We found no evidence of mechanical self-pollination in the study species and they are thus assumed to be entirely reliant on pollinator visits for seed production. All species are part of the 'satyrium clade' (van der Niet *et al.* 2005), which includes the long-spurred *Satyrium* species from the Cape Floristic Region of South Africa (Linder 2003). Details of study sites, including locality, observation hours and dates are given in (Appendix S1).

Visitor observations

Floral visitors were observed at peak flowering during several days and evenings, and in some cases over several years (Appendix S1). To avoid biasing observation time to the evenings, when most floral visitors were expected, the total duration of daytime observations exceeded, or was at least similar, to that of evening observations. Daytime observations were done during sunny and warm weather from mid-morning to mid-afternoon, when day-flying pollinators are typically active. Evening observations were carried out regardless of weather conditions, as it was found in the early stages of this study that moth visitors were active even during overcast and cold drizzly conditions. Evening observations started prior to dusk and were performed until a marked decrease in moth activity was observed, which typically occurred *ca.* 1 h after sunset. Further nocturnal observations were not performed. Behaviour and presence of orchid pollinaria was recorded for each floral visitor. A small sample of visitors was captured for identification and proboscis measurement. All insect identifications were carried out by Dr. Martin Kruger (Lepidoptera curator of the Ditsong National Museum of Natural History, Pretoria, South Africa).

Timing of pollination

In addition to direct observations of floral visitors, we used pollinarium removal from flowers as a measure of pollination between day [period between dawn and dusk, *ca.* between 06:00 and 18:00 h during this time of year for this area (Appendix S1)] and night (period between dusk and dawn). This allows for sampling a larger number of flowers than can typically be observed, and does not restrict pollination assessments to observation hours, which is particularly practical in systems where nocturnal pollinator activity is expected. We did not assess massulae deposition on stigmas as a measure of pollination, as a previous study of *Satyrium* showed that stigmas may be unreceptive during the first few days after anthesis due to protandry (Jersáková & Johnson 2007). We first marked flowers in which both pollinaria were intact on at least 17 plants per species (median = 26). Flowers were checked for pollinarium removal during each dusk and dawn for at least two consecutive 24-h periods (apart from *S. situsanguinum*, for which only 1 day period could be checked), but mostly for up to four consecutive 24-h periods. Once one or both pollinaria had been removed, it was no longer checked for consecutive pollination. To replace flowers from which pollinaria had been removed, newly opened flowers with both pollinaria intact (sometimes on newly added plants) were marked, such that the number of plants and flowers in the sample remained approximately similar throughout the experiment. Plants from which no pollinaria were removed from any of the marked flowers over the entire duration of the experiment were omitted from the analysis. Data were analysed for each species separately using generalised estimating equations in SPSS version 19

(SPSS, Chicago, IL, USA), as these models account for correlated responses when repeated measures are taken from the same subject. These analyses considered plants as the subject variable, incorporated a binomial error structure with a logit link function, and an autoregressive correlation matrix based on the date of sampling (within-subject variable). Significance was assessed using Score statistics. The predictor variable was time of day (day *versus* night) and the response variable was the proportion of flowers (treated as Bernoulli trials) with pollinaria removed (treated as events) during each 12-h sampling period. For graphical representation of marginal (adjusted) means and SE, we used values back-transformed from the logit scale, resulting in asymmetric SE.

Pollination success

To assess to what extent flower visitors contribute to overall pollination success, we quantified pollinarium removal and massulae deposition of recently wilted flowers. We sampled one recently wilted flower (*i.e.* which can no longer be pollinated) on each of 20–100 plants per species.

Nectar sampling and analysis

To assess rewards available in flowers of the six *Satyrium* species, we measured the standing crop of nectar, and sugar concentration and composition in flowers from three to 15 plants per species. Nectar was measured during the day. A preliminary analysis of bagged flowers indicated that nectar volume did not differ between morning and evening. Nectar quantity was measured after cutting the tip of the nectar spur and gently squeezing the nectar into 5-μl capillary glass tubes. Sugar concentration was determined for the same flowers using a Bellingham and Stanley hand-held refractometer (0–50%). Nectar from two to three randomly chosen flowers was stored on Whatman no. 1 filter paper, and kept at −20 °C in the laboratory until analysed. Sugar composition was determined using high-pressure liquid chromatography (HPLC). Samples were processed according to methods described in van der Niet *et al.* (2010). All samples were analysed on a LC-20AT (Shimadzu, Kyoto, Japan) HPLC equipped with a RID-10A refractive index detector (Shimadzu) and a Rezex RCM-Monosaccharide column (300 × 7.8 mm, 8-μm pore size; Phenomenex®, Torrance, CA, USA). Sugars were identified by comparing the retention times of peaks in the chromatograms of the nectar samples to the retention times of peaks in chromatograms of a standard solution containing sucrose, glucose, xylose and fructose.

We also measured the functional nectar spur length of each species, as the distance between the tip of the spur and the viscidia, from six to 55 randomly selected mature flowers taken from different individuals.

Spectral reflectance of flowers

To compare flower colour between the six species, light reflectance in the range between 300–700 nm was compared using an Ocean Optics S2000 spectrophotometer and fibre optic reflection probe (UV/VIS 400 μm) according to the method described in Johnson & Andersson (2002). Spectra were obtained for the labella, which represent the largest surface exposed to visitors. Additionally, to the human eye, colour contrast between the labellum and tepals is minimal in most species. For each species, results were averaged across three flowers, each sampled from different plants.

Scent sampling

To characterise variation in the quantity and composition of volatiles emitted by the six *Satyrium* species during peak activity times of potential diurnal and nocturnal visitors, we used headspace collection and gas chromatography-mass spectrometry (GC-MS) methods. We sampled scent of three to five individuals per population. To characterise differences in composition and quantity of volatiles emitted during day and night, sampling was performed at around noon and just after sunset. To control for between-individual variation in volatile emission, we sampled scent from the same individuals during day and night. To quantify intraspecific variation in scent emission, we sampled scent from one to four individuals from at least one additional population per species (apart from *S. outeniquense*, for which a second population could not be located; see Appendix S1 for the sites of the additional populations). For *S. ligulatum*, *S. situsanguinum* and *S. stenopetalum* we specifically selected populations that represent morphological variation within the species (Hall 1982; van der Niet *et al.* 2011).

Polyacetate bags (Kalle Bratschlauch, Wiesbaden, Germany) were placed over entire inflorescences of plants that were in peak flower (*i.e.* inflorescences with wilted flowers, fresh flowers and buds). Air from these bags was then immediately pumped through small thermodesorption cartridges filled with 1 mg Tenax® TA 60/80 and 1 mg Carbotrap® TM 20/40 mesh (both Supelco™ Analytical; Bellefonte, PA, USA) at a flow rate of 50 ml·min^{-1} for 10–20 min. Air flow through the bag was facilitated by cutting a minute hole in the bag. Traps were brought to the lab and stored at −20 °C until they were analysed. During each period in which flowers were sampled, we also sampled an empty bag as a control for volatiles present in the surrounding air.

Gas chromatography-mass spectrometry (GC-MS) analysis of floral scent

The GC-MS analysis of these scent samples was carried out using a Varian CP-3800 GC (Varian, Palo Alto, CA, USA) with a 30 m × 0.25 mm internal diameter and a film thickness of 0.25 μm Alltech EC-WAX column coupled to a Varian 1200 quadrupole mass spectrometer in electron-impact ionisation mode. Thermodesorption cartridges were placed in a Varian 1079 injector equipped with a Chromatoprobe thermal desorption device (Gordin & Amirav 2000; Dötterl *et al.* 2005). The flow of helium carrier gas was 1 ml·min^{-1}. The injector was held at 40 °C for 2 min with a 20:1 split and then increased to 200 °C at 200 °C·min^{-1} in splitless mode for thermal desorption. After a 3 min hold at 40 °C, the temperature of the GC oven was ramped up to 240 °C at 10 °C·min^{-1} and held there for 12 min. Compounds were identified using the Varian Workstation software with the NIST 2011 mass spectral library (NIST/EPA/NIH Mass Spectral Library, data version: NIST 2011; MS search software version 2.0 d) and verified, where possible, using retention times of authentic standards and published Kovats indices (*e.g.* references in the NIST 2011

library). For each compound we calculated its relative emission by dividing the peak surface area (total ion count) for that compound over the cumulative peak surface areas of all compounds that were not also found in the surrounding air.

Total absolute scent emission rates were calculated using the peak surface area of a known quantity of standards as a reference. Linalool and methyl nicotinate, the two compounds that dominated the scent of five out of six species, were used as standards. One microgram of each standard was injected into a thermodesorption cartridge, which was run on the GC-MS using the same programme as was used for the original samples; this procedure was repeated five times. For each run, the peak area representing 1 μg of standard was calculated by averaging the peak areas of linalool and methyl nicotinate. A grand average over five runs was calculated to arrive at the average total ion count per microgram of standard compound. The total ion count of compounds not found in the surrounding air of each sample was subsequently divided by the total ion count per microgram and by the sampling time in minutes to arrive at the absolute scent emission rate inflorescence^{-1}·min^{-1}. Absolute emission rates of individual compounds were calculated by dividing the peak area of that compound by the grand average given above. For calculation of the absolute emission rates of linalool and methyl nicotinate, we used the average ion count per microgram for both these compounds individually instead of their combined average.

Visualisation and statistical analysis of scent data

To visualise variation in the blends of volatiles emitted between samples, we applied dimension reduction with non-metric multidimensional scaling (NMDS) implemented in PAST version 2.15 (Hammer *et al.* 2001). To evaluate the effect of variation in emission rates among compounds on the grouping of samples, we performed NMDS based on Bray–Curtis similarities of square-root transformed volatile proportions (percentage amounts).

We first tested whether there was an overall difference in scent profiles between the six species studied, including the samples from all the populations. For this, we applied a one-way NP-MANOVA, as implemented in PAST version 2.15, with 'species' as factor. NP-MANOVA (non-parametric MANOVA, PERMANOVA) is a Mantel technique that operates directly on the similarity data in a randomisation-based univariate ANOVA (Anderson 2001).

For all remaining analyses described below, we only included the samples of the six populations for which day and night scent was sampled from the same plant. To explore temporal variation in scent emission, we compared day and night samples for differences in (i) the number of scent compounds, (ii) absolute scent emission rates per inflorescence, and (iii) the proportion of scent compounds in the entire blend. For all three analyses we tested whether there was a difference between day and night samples for each species individually.

For the comparison of the number of scent compounds emitted during the day *versus* night, we used generalised estimating equations in SPSS version 19, with plant as the subject variable for repeated measures. These models incorporated a Poisson error structure with a log-link function and an exchangeable correlation matrix. Time of day was the predictor variable and model significance was assessed using Score statistics.

For the comparison of absolute emission rates per inflorescence in each species, we used a paired *t*-test in SPSS version 19. We subsequently performed the same test for any compound that was emitted by more than 1 ng inflorescence^{-1}·min^{-1} during either day or night.

For the comparisons of scent composition between day and night samples, we used NP-MANOVA, as implemented in PAST version 2.15 (Hammer *et al.* 2001). Differences between day and night samples of each species were compared using a one-way NP-MANOVA based on Bray–Curtis similarities of square-root transformed proportion data.

Finally, we tested whether the timing of scent emission predicts the timing of pollination among populations. First, we calculated the proportion that the mass of emissions in the day scent sample made up of the combined mass of emissions in the day and night scent samples. These values were regressed against the proportion of total pollinarium removal contributed by daytime pollinators in each population, derived from the experiment described above. We used a reduced major axis regression (Warton *et al.* 2006) as implemented in PAST version 2.15 (Hammer *et al.* 2001), to account for the estimation of values on both axes.

RESULTS

Pollinator observations and pollination success

During a total of 140 h of observations, insects carrying pollinaria were observed for all six *Satyrium* species (Table 1). With the exception of a few bees (one carrying pollinaria) and butterflies on *S. outeniquense*, and a single sunbird visit to *S. pallens*, all visitors were moths, the vast majority of which were observed during the evening (Fig. 1). Moths captured on most *Satyrium* species comprised a variety of species and families (Table 1). *S. ligulatum* and *S. outeniquense* were exclusively visited by settling moths, while *S. acuminatum*, *S. situsanguinum* and *S. stenopetalum* ssp. *stenopetalum* were mainly visited by settling moths with occasional hawkmoth visits (Table 1, Fig. 1). *S. pallens* was mostly visited by hawkmoths (Fig. 1) and the few settling moths that visited this species mostly did not carry pollinaria (Table 1).

Pollinarium removal was higher at night than during the day for all species, although the difference was non-significant for *S. ligulatum* and *S. pallens* (Fig. 2). The percentage of flowers with pollinarium removal and/or massulae deposition was high for all species, resulting in a high overall percentage of flowers pollinated, ranging from 79% (*S. stenopetalum* ssp. *stenopetalum*) to 100% (*S. outeniquense*; Appendix S1).

Nectar

All species produced nectar, with volumes ranging between 0.21–0.86 μl per nectar spur (Table 2) largely depending on spur length (results not shown). Concentrations ranged from 27.4–38.9% (Table 2), largely depending on spur length (results not shown). There was a strong association between moth proboscis length and nectar spur length (Fig. 3). Nectar was mainly sucrose-dominant (Table 2).

Table 1. Captured floral visitors of the six *Satyrium* species, identified to the lowest taxonomic rank possible. The presence of pollinaria on the proboscis is indicated, as well as the proboscis length.

plant species	insect species (N individuals)	mean number of pollinaria/individual (individuals with pollinaria)	mean proboscis length (mm)
S. acuminatum	Noctuidae: Cuculliinae		
	Cucullia extricata (Walker, 1867) (2)	1 (2)	25.29
	Cucullia ruptifascia (Hampson, 1909) (1)	3	22.86
	Cucullia terensis (Felder & Rogenhofer, 1874) (1)	1	19.88
	Sphingidae: Macroglossinae		
	Theretra capensis (Linnaeus, 1764) (1)	2	27.38
S. ligulatum	Arctiidae: Lithosiinae (1)	0	1.66
	Hypagoptera rufeola (Hampson, 1900) (1)	0	3.02
	Geometridae: Ennominae (1)	0	5.98
	Idiodes saxaria (Guenée, 1858) (1)	1	6.00
	Psilocerea sp. (1)	1	5.8
	Crambidae: Spilomelinae		
	Piletocera lanceolalis (Guenée, 1854) (1)	0	2.49
	Pyraloidea (1)	0	3.75
	Noctuidae: Hadeninae		
	Tycomarptes inferior (Guenée, 1852) (1)	0	6.72
S. outeniquense	Noctuidae: Heliothinae		
	Helicoverpa armigera (Huebner, 1809), (1)	0	7.18
	Crambidae: Pyraustinae		
	Loxostege frustalis (Zeller, 1852) (4)	0	3.81
	Pyraloidea (1)	1	3.76
S. pallens	Noctuidae: Heliothinae		
	Heliothis scutuligera (Guenée, 1852) (1)	0	5.45
	Noctuidae: Ophiderinae		
	Pandesma robusta (Walker, 1858) (1)	7	7.45
	Sphingidae: Macroglossinae		
	Hyles malgassica (Denso, 1944) (1)	>7	24.59
	Temnora p. pylas (Cramer, 1779) (2)	1 (1)	19.92
	Theretra cajus (Cramer, 1777) (1)	>1	25.84
S. situsanguinum	Noctuidae: Plusiinae		
	Cornutiplusia circumflexa (Linnaeus, 1767) (1)	4	13.33
S. stenopetalum ssp. *stenopetalum*	Noctuidae: Cuculliinae		
	Cucullia extricata (Walker, 1867) (4)	0.66 (1)	22.65

Spectral reflectance of flowers

Apart from differences in brightness, spectral reflectance of labella was similar across species (Fig. S1). There was no reflectance in the UV range, and all spectra showed a sharp increase in reflectance at 400 nm. Reflectance was equal between 400–700 nm, apart from that of *S. outeniquense*, which showed a slight peak between 500–600 nm.

Floral scent composition

The six species produced a total of 168 scent compounds. Of these, 112 (67%) were species-specific (for a detailed list of compounds, see Appendix S2). The total number of compounds emitted per species ranged from 17 in *S. outeniquense* to 75 in *S. ligulatum*. Blends of most species were dominated by a few compounds, and only seven compounds comprised 10% or more of the blends of species included here (Table 3). Two of these compounds were shared among species. Linalool occurred in relatively high relative amounts in *S. acuminatum* and *S. ligulatum*, while methyl nicotinate occurred in high relative amounts in the four remaining species (Table 3). Species that shared dominant scent compounds clustered closely together in a multivariate analysis based on the relative proportion of scent compounds (Fig. 4). Scent profiles were significantly different among species (NP-MANOVA $F = 105.9$, $P < 0.0001$).

Day–night comparisons of scent profiles

Scent profiles differed significantly between day and night sampling periods in terms of the composition of scent for *S. acuminatum*, *S. outeniquense* and *S. situsanguinum*, but not for the remaining species (Table 4). There was no difference in the number of compounds produced during day or night, apart from *S. acuminatum*, which produced more compounds during the day period (Table 4). Absolute scent emission rates were significantly higher during the night sampling period only for *S. acuminatum* and *S. ligulatum* (Fig. 5). For the majority of individual compounds that were emitted in more than 1 $ng \cdot min^{-1}$ $inflorescence^{-1}$ there was no difference in emission rates between day and night sampling periods, the exception being several compounds in *S. ligulatum*, which were emitted at significantly larger rates during the night period than during the day period, in line with an overall increase in night emission rates (Appendix S3). In addition, linalool was emitted at

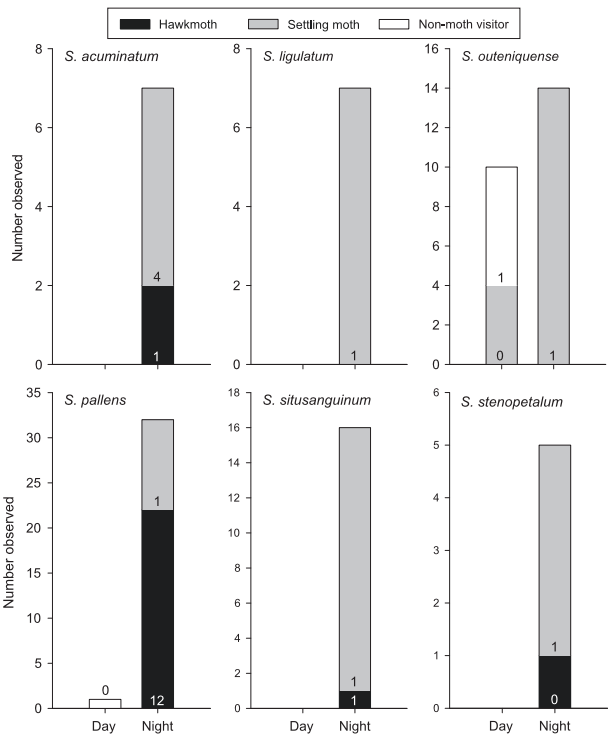

Fig. 1. Observed floral visitors of the six *Satyrium* species during daytime and evening by functional pollinator group. Number of visitors of each category carrying pollinaria is indicated inside bars.

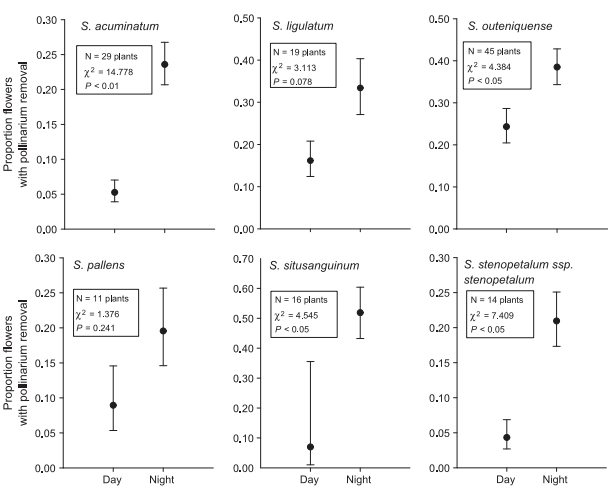

Fig. 2. Mean (±SE) proportion of flowers with pollinarium removal during day and night for the six *Satyrium* species.

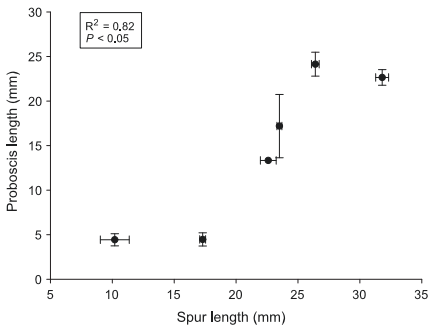

Fig. 3. Reduced major axis regression of mean spur length (±SE) against mean proboscis length (±SE) of caught floral visitors of the six *Satyrium* species.

significantly higher rates during the night period in *S. acuminatum* (Appendix S3), in line with overall elevated night emission rates. In *S. situsanguinum* methyl nicotinate was emitted at significantly higher rates during the day period, while 6-methyl-5-heptene-one was emitted at significantly higher rates during the night period (Appendix S3).

There was no significant relationship between the proportion of total pollinarium removal and the proportion of scent emitted during the day sampling period ($t = 0.59$, $P = 0.63$).

DISCUSSION

In this study, we confirmed that each of the six *Satyrium* species studied is predominantly pollinated by moths during the evening, consistent with previous observations and predictions based on morphological features (e.g. Johnson 1997; van der Niet *et al.* 2009, 2011). Our observations of *S. acuminatum*, *S. outeniquense*, *S. pallens*, *S. situsanguinum* and *S. stenopetalum* ssp. *stenopetalum* represent the first pollinator records for these species. Diurnal flower visitors were rarely observed. The predominance of nocturnal over diurnal pollination was further confirmed from our analysis of the timing of pollination in four out of six species. Multivariate analyses showed differences in scent profiles between day and night sampling periods. However, using regression analysis, we found no direct association between the timing of scent emission and pollination. Furthermore, total emission rates were significantly higher during the night sampling periods in only two species. This result was also reflected in an analysis of emission rates at the level of individual compounds. We therefore reject the hypothesis that these moth-pollinated flowers are predominantly evening-scented (*e.g.* Faegri & van der Pijl 1979).

There are different possible explanations for the lack of concordance between moth pollination and evening scent emission

Table 2. Nectar spur length, and nectar characteristics (Mean ± SD, where appropriate) of the six *Satyrium* species, samples sizes indicated between brackets.

species	spur length (cm)	volume (μl)	concentration (% sugar)	sucrose (%)	glucose (%)	fructose (%)
S. acuminatum	26.43 ± 2.24 (53)	0.86 ± 0.58 (15)	30.6 ± 4.5 (15)	96.9 (2)	0.0	3.1
S. ligulatum	10.16 ± 0.64 (15)	0.21 ± 0.12 (3)	27.4 ± 14.0 (3)	75.0 (2)	12.1	12.8
S. outeniquense	17.31 ± 0.84 (12)	0.53 ± 0.12 (12)	36.3 ± 4.6 (12)	78.0 (3)	9.0	13.0
S. pallens	23.51 ± 1.47 (55)	0.57 ± 0.25 (13)	36.4 ± 8.8 (13)	100.0 (3)	0.0	0.0
S. situsanguinum	22.59 ± 2.02 (15)	0.56 ± 0.32 (10)	38.9 ± 6.5 (10)	85.8 (2)	10.6	3.5
S. stenopetalum ssp. *stenopetalum*	31.77 ± 2.02 (15)	0.53 ± 0.46 (3)	28.4 ± 1.9 (3)	98.8 (2)	0.0	1.2

Table 3. Average relative amounts (%) of main floral scent compounds (>1.0% in any species, 66 samples; see Appendix S2 for a table with all compounds listed) of six *Satyrium* species (*S. acuminatum* = *S. acu*; *S. ligulatum* = *S. lig*; *S. pallens* = *S. pal*; *S. outeniquense* = *S. out*; *S. situsanguinum* = *S. sit*; *S. stenopetalum* = *S. ste*).

A.				*S. acu*	*S. acu*	*S. acu*	*S. lig*	*S. lig*	*S. lig*	*S. pal*	*S. pal*	*S. pal*
population				J	C	C	RH	C	C	J	J	MEI
sample type (day *versus* evening)				e	d	e	d	d	e	d	e	e
number of compounds				36	26	23	69	52	65	30	24	28
compound	CIC	KRI	CAS #									
aliphatics												
alcohols												
Hexan-1-ol	b	1360	111-27-3	0.1	tr	tr	—	0.1	tr	0.4	0.2	0.7
(*E*)-2-Hexen-1-ol	b, $	1400	928-95-0	0.4	–	–	–	–	–	0.4	–	0.1
aldehydes												
Pentanal	b	975	110-62-3	–	–	–	–	–	–	1.3	1.2	–
(*Z*)-11-Hexadecenal	b, $	2159	53939-28-9	–	–	–	–	–	–	0.5	1.3	0.8
Methyl (9Z)-9-hexadecenoate	b, $	2242	1120-25-8	–	–	–	–	–	–	2.1	2.9	1.8
benzenoids & Phenylpropanoids												
benzaldehyde	c	1525	100-52-7	0.3	–	–	0.1	9.0	6.5	–	–	–
3,4-Dimethoxytoluene	b	1806	494-99-5	–	–	–	–	–	–	–	–	–
benzyl alcohol	c	1865	100-51-6	1.5	0.4	0.1	tr	37.4	16.5	–	–	–
phenylethyl alcohol	c	1925	60-12-8	tr	tr	tr	tr	0.1	tr	–	–	–
eugenol	c	2141	97-53-0	0.6	0.3	0.1	–	–	–	–	–	–
(*e*)-Cinnamyl alcohol	b	2300	4407-36-7	0.2	tr	tr	0.1	tr	0.1	–	–	–
monoterpenes												
acyclic												
β-Myrcene	b	1145	123-35-3	1.4	0.7	0.2	0.7	0.9	1.5	–	–	–
(*Z*)-Ocimene	b	1228	3779-61-1	0.4	0.2	0.1	1.6	1.6	0.4	–	–	–
(*E*)-Ocimene	b	1245	3338-55-4	0.1	0.2	tr	39.3	19.9	42.9	–	–	–
Linalool	c	1551	78-70-6	92.2	96.1	98.7	37.0	7.5	4.3	–	–	–
cyclic												
Eucalyptol	c	1213	470-82-6	–	–	–	–	–	–	–	–	–
(*E*)-Linalool oxide (furanoid)	b	1451	23007-29-6	0.0	0.1	tr	1.7	3.7	3.8	–	–	–
(*Z*)-Linalool oxide (furanoid)	b	1500	1365-19-1	0.1	–	–	1.8	2.3	2.8	–	–	–
α-Terpineol	b	1704	98-55-5	0.1	0.1	0.1	tr	–	–	–	–	–
(*E*)-Linalool oxide (pyranoid)	b	1731	41720-62-1	–	–	–	7.7	11.7	14.1	–	–	–
Epoxylinalol	b	1734	14049-11-7	–	–	–	7.5	3.6	5.4	–	–	–
irregular terpenes												
apocarotenoids												
Dihydro-β-ionone	b	1838	17283-81-7	–	–	–	–	–	–	10.9	18.3	3.3
Dihydroactinidiolide	a	2324	17092-92-1	–	–	–	–	–	–	1.3	1.8	1.7
other irregular terpenes												
6-Methyl-5-heptene-2-one	b	1335	110-93-0	0.2	–	–	0.1	0.2	0.1	0.7	0.7	–
nitrogen containing compounds												
methyl nicotinate	c	1779	93-60-7	–	–	–	–	–	–	78.6	68.6	79.6
indole	c	2365	120-72-9	–	–	–	–	–	–	–	–	tr
sesquiterpenes												
(*e*,*e*)-α-Farnesene	b	1725	502-61-4	–	–	–	–	–	–	–	–	0.5
(*e*,*e*)-Farnesyl acetate	b	2194	4128-17-0	–	–	–	–	–	–	–	–	–
(*e*)-Caryophyllene	c	1618	87-44-5	–	–	–	–	–	–	0.5	1.3	7.2
miscellaneous cyclic compounds												
m/z: 43,84,81,41,67,110		1922		–	–	–	–	–	–	–	–	–

B.				*S. out*	*S. out*	*S. sit*	*S. sit*	*S. sit*	*S. sten*	*S. sten*	*S. sten*	*S. sten*
population				J	J	MP	MP	HK	C	C	GWH	HK
sample type (day *versus* evening)				d	e	d	e	e	d	e	e	e
number of compounds				17	11	42	42	36	29	35	32	41
compound	CIC	KRI	CAS #									
aliphatics												
alcohols												
Hexan-1-ol	b	1360	111-27-3	tr	0.2	0.3	0.4	0.1	0.7	0.1	1.3	0.3
(*E*)-2-Hexen-1-ol	b, $	1400	928-95-0	0.4	1.7	–	–	–	–	–	–	–

Table 3. Continued.

B.				*S. out*	*S. out*	*S. sit*	*S. sit*	*S. sit*	*S. sten*	*S. sten*	*S. sten*	*S. sten*
population				J	J	MP	MP	HK	C	C	GWH	HK
sample type (day *versus* evening)				d	e	d	e	e	d	e	e	e
number of compounds				17	11	42	42	36	29	35	32	41
compound	CIC	KRI	CAS #									
aldehydes												
Pentanal	b	975	110-62-3	–	–	–	–	–	–	–	–	–
(*Z*)-11-Hexadecenal	b, $	2159	53939-28-9	–	–	–	–	–	–	–	–	–
Methyl (9Z)-9-hexadecenoate	b, $	2242	1120-25-8	–	–	–	–	–	–	–	–	–
benzenoids & Phenylpropanoids												
benzaldehyde	c	1525	100-52-7	1.3	1.3	0.4	0.8	0.2	0.2	0.1	tr	0.1
3,4-Dimethoxytoluene	b	1806	494-99-5	–	–	–	–	–	0.1	0.1	31.0	15.6
benzyl alcohol	c	1865	100-51-6	–	–	0.3	0.2	0.3	–	–	–	0.1
phenylethyl alcohol	c	1925	60-12-8	0.7	1.2	–	–	3.3	0.1	tr	0.2	1.6
eugenol	c	2141	97-53-0	–	–	22.1	20.8	29.5	–	–	–	–
(*e*)-Cinnamyl alcohol	b	2300	4407-36-7	–	–	4.7	6.6	4.5	–	–	–	–
monoterpenes												
acyclic												
β-Myrcene	b	1145	123-35-3	tr	–	–	–	–	0.2	0.3	0.6	tr
(*Z*)-Ocimene	b	1228	3779-61-1	–	–	–	–	–	–	–	–	–
(*E*)-Ocimene	b	1245	3338-55-4	–	–	–	–	tr	tr	tr	0.1	–
Linalool	c	1551	78-70-6	0.1	–	tr	tr	tr	–	tr	tr	tr
cyclic												
Eucalyptol	c	1213	470-82-6	–	–	–	–	–	7.6	22.0	36.6	1.5
(*E*)-Linalool oxide (furanoid)	b	1451	23007-29-6	–	–	–	–	–	–	–	–	–
(*Z*)-Linalool oxide (furanoid)	b	1500	1365-19-1	–	–	–	–	–	–	–	–	–
α-Terpineol	b	1704	98-55-5	–	–	–	–	–	0.3	0.2	1.5	1.0
(*E*)-Linalool oxide (pyranoid)	b	1731	41720-62-1	–	–	–	–	–	–	–	–	–
Epoxylinalol	b	1734	14049-11-7	–	–	–	–	–	–	–	–	–
irregular terpenes												
apocarotenoids												
Dihydro-β-ionone	b	1838	17283-81-7	–	–	–	–	–	–	–	–	–
Dihydroactinidiolide	a	2324	17092-92-1	–	–	–	–	–	–	–	–	–
other irregular terpenes												
6-Methyl-5-heptene-2-one	b	1335	110-93-0	0.2	1.7	0.7	4.7	1.0	4.3	0.7	0.8	3.0
nitrogen containing compounds												
methyl nicotinate	c	1779	93-60-7	96.5	92.4	48.3	28.0	50.0	84.7	73.4	25.0	67.9
indole	c	2365	120-72-9	–	–	8.7	16.9	3.8	–	–	–	–
sesquiterpenes												
(*e*,*e*)-α-Farnesene	b	1725	502-61-4	–	–	11.2	17.4	5.8	tr	tr	tr	tr
(*e*,*e*)-Farnesyl acetate	b	2194	4128-17-0	–	–	–	–	–	0.1	1.0	0.1	4.9
(*e*)-Caryophyllene	c	1618	87-44-5	–	–	tr	–	tr	–	–	–	–
miscellaneous cyclic compounds												
m/z: 43,84,81,41,67,110		1922		–	–	–	–	–	0.1	tr	tr	1.6

Compounds are listed in order of increasing retention time within each compound class. KRI = Kovats retention index; CAS# = unique numerical identifiers of compounds assigned by the Chemical Abstracts Service; tr = trace amount (<0.1% of total sample); d = day sample, e = evening sample. CIC = compound identification criteria and notes: a = comparison of MS with published data; b = comparison of MS and retention time with published data; c = comparison of MS and retention time with published data and authentic standard; $ = position of double bonds have not been characterised in detail. Populations: J = Joubertina; C = Coldstream; RH = Red Hill; MEI = Meiringspoort; MP = Middelberg Pass; HK = Hans se Kop; GWH = Groot Winterhoek.

found in our study. One possibility is that the headspace collection and GC-MS methods used here are poor at detecting variation in emission rates (if, for example, the adsorbent filters used to collect scent or the GC column itself become overloaded). However, this possibility can be excluded as identical methods used in the same lab and by others have previously revealed very strong diel patterns of scent emission (Dötterl *et al.* 2005, 2012; Peter *et al.* 2009). Although we may have failed to observe some diurnal pollinators, our measures of the timing of pollination (Fig. 2) support the general conclusion that these are moth-pollinated taxa. We used a comparatively small number of scent sampling points during the 24-h cycle, but these sampling periods coincided with the activity peaks of diurnal and crepuscular pollinators. Sampling periods thus did not limit our ability to test the hypothesis of associations between evening scent emission and moth pollination. We therefore think that our findings are robust and reflect a lack of predominant evening scent emission in these moth-pollinated

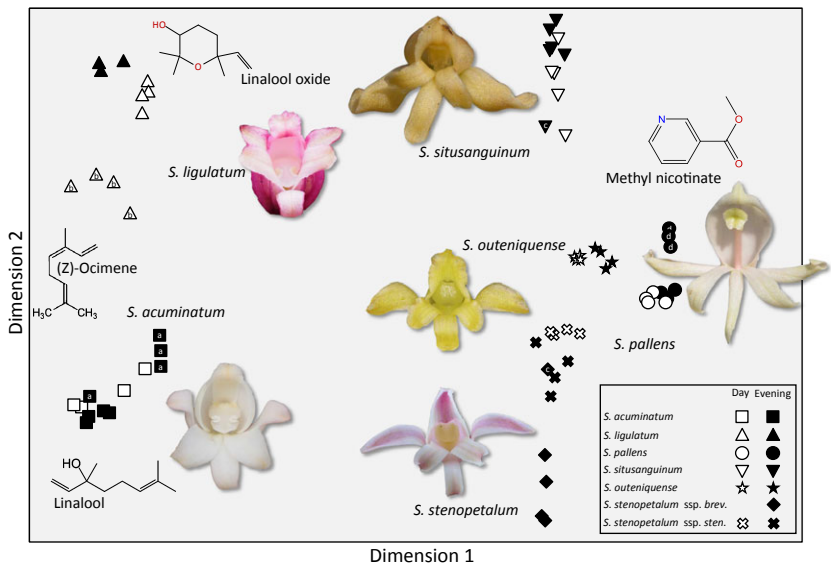

Fig. 4. Multidimensional scaling of scent samples of the six *Satyrium* species based on Bray–Curtis similarities of square-root transformed proportions of compounds in the blend (2D stress = 0.06). Letters in symbols represent the origin of the additional populations sampled for scent (see also Appendix S1). a = Joubertina, b = Red Hill, c = Hans se Kop, d = Meiringspoort.

Table 4. Day–night differences in scent composition and the number of scent compounds of six *Satyrium* species (*S. acuminatum* = *S. acu; S. ligulatum* = *S. lig*; *S. pallens* = *S. pal*; *S. outeniquense* = *S. out*; *S. situsanguinum* = *S. sit*; *S. stenopetalum* = *S. ste*). If the variable could be quantified as being larger during day or night, the time of day with the highest value is indicated. See text for details of statistical analyses.

	S. acuminatum	*S. ligulatum*	*S. outeniquense*	*S. pallens*	*S. situsanguinum*	*S. stenopetalum* ssp. *stenopetalum*
scent composition	*	n.s.	*	n.s.	**	n.s.
number of compounds	day*	n.s.	n.s.	n.s.	n.s.	n.s.

Significance level: *$P < 0.05$; **$P < 0.01$; n.s. = not significant.

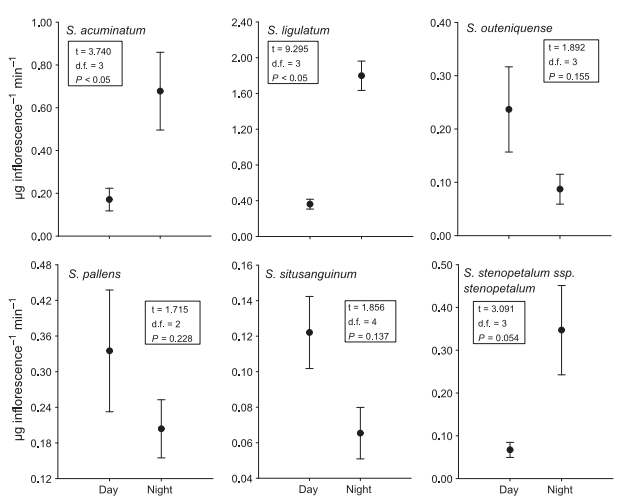

Fig. 5. Mean (±SE) scent emission rates (µg·inflorescence^{-1} min^{-1}) during day and evening of the six *Satyrium* species.

orchids. One possibility that we cannot exclude based on our data is that besides the observed activity peak of moths at dusk, a second activity peak at dawn may occur (*e.g.* Braun *et al.* 2012). Further observations and scent sampling are required to assess whether such an activity peak occurs, and whether scent plays a different role during this activity peak compared to what we measured for the activity peak in the evening.

The variation in timing of scent emission does not likely reflect site-specific variation in ambient temperatures at the time of sampling. For all species, including those for which we found elevated evening emission, ambient temperatures during the time of sampling were likely higher at noon than at dusk, and sampling protocols were similar. Additionally, the observed patterns for the three species without enhanced evening emission were obtained at different sites and on different dates. As such, the scent sampling could be interpreted as representing independent replicates, controlling for potential site and day effects.

The timing of scent emission appears to differ among compounds that dominate the scent profiles of these species. Scents of the two species for which we found higher emission rates in the evening were dominated by linalool and (*E*)-ocimene, respectively. Both of these compounds have been shown to trigger electrophysiological responses and behavioural responses in moths (Dötterl *et al.* 2006; Sun *et al.* 2012), but they are also common compounds of flowers that are not moth-pollinated (Knudsen *et al.* 2006). The elevated evening emission of these compounds may thus lead to floral specialisation for more efficient pollination of flowers with otherwise specialised floral traits through limited emission in time. The blends of all four species without elevated evening emission were dominated by methyl nicotinate. The function of methyl nicotinate is not well studied, although several species in which it has been recorded as scent component are also moth-pollinated (Levin *et al.* 2001; Raguso *et al.* 2003). Its potential role as a moth attractant is further indicated by the apparent absence of methyl nicotinate in the scent of *Satyrium* species pollinated by vectors other than moths. For instance, the bee-pollinated *S. erectum*, which is sister to *S. situsanguinum* (van

der Niet *et al.* 2011), produces methyl nicotinate only in trace amounts 1000-fold smaller than in the scent of moth-pollinated satyriums in this study (van der Niet, unpublished data). Similarly, the shift from moth to bird pollination (in *S. coriifolium*) in the clade that otherwise only contains *S. outeniquense* and *S. stenopetalum* was associated with the complete loss of scent production (van der Niet, unpublished data). If methyl nicotinate functions as defence against florivores, its absence in non-moth-pollinated taxa would not be expected. If we assume that methyl nicotinate mainly functions as a moth attractant, there are two explanations for the high daytime emission. Selection by moths for daytime emission may have occurred on account of noctuid species that in some years are highly active during the day (cf. van der Niet *et al.* 2014), or may have occurred at a site different to that at which the experiments were performed. More work is clearly needed to establish the function of methyl nicotinate in floral scent, for instance by testing behavioural responses of various insect groups, including moths, to methyl nicotinate.

The lack of higher evening scent emission does not necessarily mean that floral scent is unimportant in mediating plant–pollinator interactions. While floral scent emission may not peak during the evening, all species emit considerable amounts of scent during the evening. Many of the scent compounds identified in our study have been shown to elicit positive antennal and/or behavioural responses in moths. These compounds include linalool (Dötterl *et al.* 2006; Meagher & Landolt 2008), α-farnesene (Sutherland *et al.* 1974), (*E*)-ocimene (*e.g.* Sun *et al.* 2012) and β-myrcene (Meagher & Landolt 2008). The combination of the presence of these compounds and the patterns of spectral reflectance of the flowers of the species studied here might lead to specialisation for moth pollination. In four out of six species the presence of relatively long nectar spurs restricts the potential visitor assemblage to birds and insects with mouthparts long enough to reach the nectar. In the range where the studied *Satyrium* species occur, the only potential visitors with such long mouthparts are moths, sunbirds, long-tongued flies (Goldblatt & Manning 2000), some butterfly species and day-flying hawkmoths (Johnson 2010). Apart from moths, all of these visitor categories typically pollinate unscented flowers, most of which are bright pink or red. The specific suite of floral traits, including floral scent, in the study species may therefore play a key role in attracting only a subset of potential pollinators and hence lead to specialisation (Raguso & Willis 2002).

Our observations of specialised moth pollination constitute novel pollinator data in four out of six species, adding to a growing body of work on *Satyrium* pollination (*e.g.* Johnson 1997; Johnson *et al.* 2011). Some key traits are shared among the species studied here, and those previously identified as moth-pollinated (Johnson 1997; Johnson *et al.* 2011): a medium-sized to long floral spur containing sucrose-dominated nectar, a 'sweet' floral scent, dull colours and (with the exception of *S. pallens*) lateral viscidia that facilitate pollinarium placement along the proboscis of a visitor probing the spurs for nectar. Despite this broad convergence, there was variation among the study species in their functional pollinator assemblages, spur lengths and floral scents. Although further pollinator observations are necessary to account for spatial and temporal variation in pollinators, we argue that interspecific variation is not random. First of all, we found a significant pattern of association between the lengths of moth mouthparts and floral nectar spurs (cf. Johnson *et al.* 2011; Boberg *et al.* 2014). This, together with the finding that pollination success was high across all species, is indicative of adaptive fit between plant and functional pollinator group (*e.g.* hawkmoths *versus* small settling moths). Furthermore, in areas where species co-flowered, floral visitors differed among species. Although hybrids can form even between distantly related *Satyrium* species (*e.g.* Ellis & Johnson 1999), these were rare or absent. This suggests that co-flowering species attract and are pollinated by a largely different subset of the local moth community.

One of the most surprising findings of this study was the profound variation in scent between species, despite their close phylogenetic relationship (van der Niet *et al.* 2011). Interspecific variation seems to be an extension of intraspecific variation, which was found in species for which multiple samples from several populations were available (cf. van der Niet *et al.* 2010). Variation is not only caused by differences in relative proportion among dominant scent compounds, but also by the large number of species-specific compounds. The combination of distinct scent profiles and distinct moth visitor assemblages suggests that pollinators characterised by different scent preferences act as selective agents (Schiestl & Johnson 2013). The current data, however, do not allow for testing this hypothesis. More pollinator observations, in combination with behavioural responses of moths to different volatiles, are required.

CONCLUSIONS

In this study we have provided evidence that is at odds with the classic paradigm that moth-pollinated plant species emit scent mainly during the evening. This finding does not mean that specialised interactions between flowers and moth pollinators are not mediated by scent. Rather, the findings highlight the need to understand additional factors, such as phylogenetic inertia or a role for scent volatiles in herbivore deterrence, which can shape diel patterns of scent production.

Our study design did not allow for a more detailed investigation of the function of individual scent compounds. However, our comparative approach involving six species revealed extensive interspecific variation in scent emission, despite similar pollination systems and close phylogenetic affinity. This underlines that findings from single-species studies cannot always be generalised, even to the level of a genus.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Pollination success of the six *Satyrium* species.

Appendix S1. Localities of study sites including species studied, dates of fieldwork, hours of diurnal and nocturnal pollinator observations, and nature of data collected for each species.

Appendix S2. Scent characteristics of the six *Satyrium* species analysed, including all populations. Compounds are listed in order of increasing retention time within each compound class. KRI represents Kovats Retention Indices, CAS are the unique numerical identifiers of compounds assigned by the Chemical Abstracts Service. Abbreviations of sites and time of day of sampling are explained on the right of the Table. Cells coloured red identify the presence of a compound in the blend of a species. Numbers indicate the mean proportion of the total blend represented by that compound. Mass fragments for unknowns are listed with the molecular ion first, followed by the base peak and other fragments in decreasing order of abundance. Compound identification criteria are as follows: a = comparison of mass spectra with published data; b = comparison of mass spectra and retention time with published data; c = comparison of mass spectra and retention time with published data and authentic standard.

Appendix S3. Comparison between day and night emission of compounds emitted at more than 1 ng·inflorescence^{-1} min^{-1} in any of the six *Satyrium* species. Compounds are listed by increasing retention time. For compounds indicated with n.s. no significant difference was found. Compounds coloured dark grey were emitted significantly more strongly at night ($\alpha < 0.05$), compounds coloured light grey were emitted significantly more strongly during the day ($\alpha < 0.05$).

Figure S1. Mean spectral reflectance of the labella of the six *Satyrium* species.

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