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Circadian rhythm of a *Silene* species favours nocturnal pollination and constrains diurnal visitation

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• **Background and Aims** Traits related to flower advertisement and reward sometimes vary in a circadian way, reflecting phenotypic specialization. However, specialized flowers are not necessarily restricted to specialized pollinators. This is the case of most *Silene* species, typically associated with diurnal or nocturnal syndromes of pollination but usually showing complex suites of pollinators.
• **Methods** A *Silene* species with mixed floral features between diurnal and nocturnal syndromes was used to test how petal opening, nectar production, scent emission and pollination success correlate in a circadian rhythm, and whether this is influenced by environmental conditions. The effect of diurnal and nocturnal visitation rates on plant reproductive success is also explored in three populations, including the effect of the pollinating seed predator *Hadena sancta*.
• **Key Results** The result showed that repeated petal opening at dusk was correlated with nectar secretion and higher scent production during the night. However, depending on environmental conditions, petals remain opened for a while in the morning, when nectar and pollen still were available. Pollen deposition was similarly effective at night and in the morning, but less effective in the afternoon. These results were consistent with field studies.
• **Conclusions** The circadian rhythm regulating floral attractiveness and reward in *S. colorata* is predominantly adapted to nocturnal flower visitors. However, favourable environmental conditions lengthen the optimal daily period of flower attraction and pollination towards morning. This allows the complementarity of day and night pollination. Diurnal pollination may help to compensate the plant reproductive success when nocturnal pollinators are scarce and when the net outcome of *H. sancta* shifts from mutualism to parasitism. These results suggest a functional mechanism explaining why the supposed nocturnal syndrome of many *Silene* species does not successfully predict their pollinator guilds.

Key words: Flower scent, *Hadena*, nectar production, nursery pollination, nyctinasty, pollination syndrome.

INTRODUCTION

Plant biological rhythms influence the physiology of individuals and have evolved to enhance fitness in response to environmental changes (McClung, 2006; Sanchez *et al*., 2011). The circadian rhythms of flowers (mediated by light–dark cycles) include flower opening and closure movements (Linné's floral clock: Linneaus, 1783; van Doorn and van Meeteren, 2003; McClung, 2006), timing of nectar production (Cruden *et al*., 1983) and diel variation of flower scent (Dobson, 2006; Knudsen *et al*., 2006). Floral nyctinasty, one of the rhythmic movements of plant organs in response to the onset of darkness (Darwin and Darwin, 1880; Palmer and Asprey, 1958; Satter and Galston, 1981), is the repeated opening of flowers in the evening/night. It has long been presumed that flower nyctinasty as well as dynamics of nectar secretion and scent emission have evolved to match the time of activity of the most important pollinators (Dudareva *et al*., 2000*a*; van Doorn and van Meeteren, 2003; Pacini and Nepi, 2007). These flower traits are important components of pollination syndromes, defined as the suite of floral traits that have independently evolved in different plant lineages due to the convergent selection by specific groups of pollinators (Faegri and van der Pijl, 1979; Fenster *et al*., 2004; Vogel, 2006; Ollerton *et al*., 2009).

The typological nature of the pollination syndrome concept has been controversial since its formulation (see Waser *et al*., 2011). More recently, the criticisms of syndromes arose because phenotypic specialized flowers are not necessarily restricted to specialized pollinators (Waser *et al*., 1996; Ollerton *et al*., 2007; Armbruster, 2014), although recent works suggest that pollination syndromes accurately predict the most effective pollinators (Reynolds *et al*., 2009; Rosas-Guerrero *et al*., 2014; but see Ollerton *et al*., 2015 and subsequent responses). It has been suggested that the dilemma of specialized flowers with generalized pollination may be partially explained by the lack of fitness trade-offs (Aigner, 2001, 2004). Phenotypic specialization of flowers may incur fitness trade-offs when the positive effect of specialized flower traits on effectiveness of one group of pollinators is linked to reduced effectiveness of other pollinators (Galen and Newport, 1987; Hurlbert *et al*., 1996; Miller *et al*., 2014), or higher susceptibility to specialized herbivores and pathogens (Strauss and Whittall, 2006). When fitness

trade-offs are absent or weak, phenotypic specialization of flowers can be maintained without reducing the functional diversity of pollinators (Armbruster, 2014). These reasons, together with the spatio-temporal variation in the abundance and identity of the most effective pollinators, may explain the prevalence of generalized over specialized pollination systems from an evolutionary perspective (Herrera, 1996; Waser *et al*., 1996; Johnson and Steiner, 2000; Fenster *et al*., 2004; Gómez and Zamora, 2006; Waser and Ollerton, 2006).

The genus *Silene* L. (Caryophyllaceae) is a model system for studies in ecology and evolution (Bernasconi *et al*., 2009) and is characterized by its diversity of floral phenotypes. In *Silene*, two contrasting flower phenotypes have been traditionally described, namely nocturnal and diurnal (Lindman, 1897; Greuter, 1995). 'Diurnal' species usually have pink or red petals, and flowers are usually open during the day and night. These species do not show obvious changes of scent intensity between day and night as perceived by the human nose (Greuter, 1995; Jürgens, 2004, 2006). These correlated flower traits are indicative of both long-tongued bees and diurnal Lepidoptera syndromes (Fenster *et al*., 2004; Reynolds *et al*., 2009). 'Nocturnal' species have white or pale flowers that show repeated petal opening and intense scent emission in the evening/night (Greuter, 1995; Jürgens *et al*., 2002; Jürgens, 2006; Castillo *et al*., 2014; Buide *et al*., 2015), so they are suggestive of nocturnal moth syndrome (Faegri and van der Pijl, 1979; Jürgens *et al*., 2002; Giménez-Benavides *et al*., 2007; Reynolds *et al*., 2009; Martinell *et al*., 2010). However, many studies suggest that almost every *Silene* species is visited by diurnal and nocturnal insects (Jürgens, 2004; Jürgens *et al*., 2002; Kephart *et al*., 2006; Reynolds *et al*., 2009; Buide *et al*., 2015).

Flower nyctinasty has been barely studied in *Silene* and, although there are many studies dealing with the flower specialization of *Silene* species, some questions remain unclear. First, few case studies have addressed whether presumed pollination syndromes of *Silene* species accurately predict the most effective pollinators (Giménez-Benavides *et al*., 2007; Reynolds *et al*., 2009; Martinell *et al*., 2010). Secondly, some flower traits have received less attention despite the fact that they may be also regulated by circadian rhythms, such as the dynamics of nectar secretion, anther dehiscence, pollen viability and stigmatic receptivity (Bassani *et al*., 1994; Witt *et al*., 1999; Buide and Guitán, 2002; Young and Gracvits, 2002). Thirdly, it is not clear whether the daily variation in these advertisement and reward traits affects the interaction between *Silene* species and their specialist nursery pollinators. The moths of the genus *Hadena* (Noctuidae) pollinate many *Silene* species, but also use the flowers and developing fruits as a food resource for their larval offspring. The outcome of this interaction may shift between mutualistic and antagonistic depending on the presence and importance of other pollinators (Giménez-Benavides *et al*., 2007; Reynolds *et al*., 2012). For these reasons, the *Silene*–*Hadena* system has emerged as a good model system to understand the evolution of mutualisms (Kephart *et al*., 2006).

In this study, we evaluate the functional coherence of flower traits exhibited by *S. colorata*, an interesting species with combined floral features of both diurnal and nocturnal syndromes. *Silene colorata* has flowers with pink petals, but shows a marked nyctinasty and emits flower scent during the night but not at mid-day (Prieto-Benítez *et al*., 2015). The closure of petals does not prevent visits by diurnal pollinators, as shown in other *Silene* species (Giménez-Benavides *et al*., 2007; Martinell *et al*., 2010). Our specific objectives were: (1) to characterize the diel variation of traits related to flower attractiveness to pollinators (petal opening, emission of scent and secretion of nectar); (2) to analyse its breeding system and to assess whether anther dehiscence and pollination success are synchronized with flower nyctinasty; (3) to determine whether floral specialization in *S. colorata* may incur fitness trade-offs between day and night pollination; and (4) to explore the effect of diurnal and nocturnal flower visitors on plant reproductive success in natural populations, including the interaction with its *Hadena* nursery pollinator.

MATERIALS AND METHODS

Plant material

Silene colorata Poiret (Caryophyllaceae) is an annual plant with a height of 15–60 cm. The calyx is 10–15 mm in length and petal limbs are 5–12 mm, bipartite and pink. Fruit capsules open at the top when ripe and hold 45–85 seeds of 1–1·5 mm in diameter (Talavera, 1990). Flowers are protandrous, and anthesis (first opening of the flower from the bud stage) is at sunset. The petal limbs remain open all night and close (rolling themselves up) early in the morning. Nonetheless, the sexual parts of flowers remain accessible when petals are completely rolled up (pers. obs.). This species inhabits croplands and roadsides of the Mediterranean region, north of Iran, Arabia and the Canary Islands (Talavera, 1990). In our area of study (Madrid, Spain) the flowering period usually spans from April to June.

Plants used in this study grew from seeds in the greenhouse of the Universidad Rey Juan Carlos (Móstoles, Madrid 40°20′02″N, 3°52′57″W, altitude 651 m). Seeds were obtained directly from natural populations in summer 2011 and 2012 (Supplementary Data Table S1) and stored in silica gel at ambient temperature until the following spring, when they were sown in 5 cm seedling trays. After 3 months, plantlets were transferred to 2 L pots until flowering. Plants grew outdoors in an insect exclusion cage from June to July in 2012 and 2013. Pollinator observations were done in the populations of origin.

Effect of light intensity and soil moisture on timing and duration of flower opening

Since petal nyctinasty is related to water content in limb cells (Halket, 1931), we expected that plants exposed to high light intensity and/or dry soil close their petals earlier in the morning, and open them later in the evening, compared with those exposed to low light intensity and/or wetter soil. To explore this, we subjected potted plants to a factorial experiment with two levels of light intensity and two levels of soil moisture. The initial number of plants was equal for all treatments but a failure in the irrigation system left the experiment as follows: 'Shade–Wet' ($n = 8$), 'Shade–Dry' ($n = 4$), 'Sun–Wet' ($n = 8$) and 'Sun–Dry' ($n = 8$). 'Wet' plants were supplied with 60 min of drip irrigation every day, and 'Dry' plants every 2 d. 'Sun' plants were exposed to direct solar radiation, whereas 'Shade' plants were placed under a shading net. The light intensity was 191·25 and 42·25 μmol photon/m^2/s in the 'Sun' and 'Shade'

treatments, respectively (mean of 2 d at 0800, 1630 and 2030 h, with a Field Scout Quantum Light Meter; Spectrum Technologies, Plainfield, USA). In the 'Sun' treatment, the temperature varied between 21·8 and 39·3 °C in the morning (0730 to 1100 h) and between 31·8 and 23·1 °C during the evening/night (2030–0000 h). In the 'Shade' treatment, the temperature varied between 21·9 and 32·6 °C in the morning and between 31·9 and 24 °C during the evening/night.

The dynamics of petal opening and closure at dusk and dawn were calculated by measuring the corolla diameter every 30 min, from 2030 to 0000 h and from 0730 to 1100 h, respectively. We measured 154 flowers (in total, 1731 measurements) with a digital caliper from 11 to 18 July 2013. The mean ± s.e. of flowers per plant used in each treatment were: 'Shade–Wet' 9·0 ± 2·7, 'Shade–Dry' 6·8 ± 2·8, 'Sun–Wet' 9·2 ± 1·0 and 'Sun–Dry' 9·6 ± 2·6. In each flower, the maximum diameter achieved was considered as 100 % of the corolla opening, and was used to calculate the percentage flower opening during each time interval.

Dynamics of flower scent emission

Previous analysis reported that *S. colorata* did not emit scent at mid-day, unlike the typical *Silene* species with diurnal pollination syndrome (Prieto-Benítez *et al.*, 2015). However, we wanted to assess whether *S. colorata* emits flower scent at the beginning of the day and, in that case, to compare the emission rate and composition with nocturnal samples. From 11 to 18 July 2013, we sampled flower volatile organic compounds (VOCs) from overall 11 plants using a dynamic head-space method. Inflorescences were enclosed in polyethylene oven bags for 5 min, and the emitted volatiles were then trapped for another 5 min in adsorbent tubes (Dötterl and Jürgens, 2005; Dötterl *et al.*, 2005) with a 9 V battery-operated pump (Giménez-Benavides *et al.*, 2007). The number of flowers per inflorescence ranged between two and ten, and the age of flowers was 1–4 d. Nine samples (from six plants) were taken during the night (between 2130 and 2315 h) and 11 samples (from the nine plants) during the day (between 0750 and 0930), when most of the flowers were at least partially open. Surrounding air samples were taken as negative controls to distinguish between floral compounds and ambient contaminants. Since we also wanted to assess whether flower VOCs are emitted from the petal limbs or from other parts of the flower, we sampled three plants during the night after removing all the petal limbs (hereafter 'no petal limbs' samples). After scent sampling, the flowers in each bag were counted and clipped. To control for the emission of green leaf volatiles (GLVs; Visser *et al.*, 1979; Light *et al.*, 1993), we took one sample from vegetative parts (leaves and stems) and the volatiles detected were deleted from the matrix of flower scent compounds.

The volatiles trapped were analysed by gas chromatogrphy–mass spectrometry (GC-MS) using an automatic thermal desorption (TD) system (TD-20, Shimadzu, Japan) coupled to a Shimadzu GCMS-QP2010 Ultra equipped with a ZB-5 fused silica column (5 % phenyl polysiloxane; 60 m, i.d. 0·25 mm, film thickness 0·25 µm, Phenomenex). The samples were run with a split ratio of 1:1 and a constant helium carrier gas flow of 1·5 mL min^{-1}. The gas chromatograph oven temperature started at 40 °C, then increased by 6 °C min^{-1} to 250 °C and held for 1 min. The MS interface worked at 250 °C. Mass spectra were taken at 70 eV (EI mode) from *m/z* 30 to 350. GC-MS data were processed using the GCMSolution package, Version 2·72 (Shimadzu Corporation 2012). Identification of the compounds was carried out using the NIST 11, Wiley 9, FFNSC 2 and Adams (Adams, 2007) databases as well as the database available in MassFinder 3. Some of the compounds were confirmed by comparing mass spectra and retention times with those of synthetic reference compounds. Total scent emission was estimated by injecting known amounts of monoterpenoids, aromatics and aliphatics (added to adsorbent tubes). The mean response of these compounds (mean peak area) was used to determine the total amount of each compound extracted from the adsorbent tubes (Dötterl *et al.*, 2005). For each sample and compound, we calculated the absolute amount emitted (ng) by flower (number) and time (min).

Nectar secretion dynamics

To characterize the temporal variation of nectar production, we took samples from 464 flowers of 23 plants (20 ± 3·1 flowers per plant) available in the insect exclusion cage, from 12 June to 16 July 2013. Before the initiation of anthesis, several cohorts of flower buds were randomly marked with colour codes to control for flower age and sexual stage. Flowers were sampled until 3 d after the beginning of anthesis. Nectar was sampled in three time intervals, morning (1000–1300 h), late afternoon (1700–1900 h) and night (2100–2300 h), with 0·25 µL calibrated microcapillaries (Drummond Scientific Co.). The length of the nectar column was measured with a digital caliper to calculate the extracted volume (µL). The calyx tube of *S. colorata* is deep and narrow, so the nectar extraction involved opening of this tube. We quantified the nectar accumulated from anthesis to each measurement time (Witt *et al.*, 1999). Sample size was large (n = 30–70 at each time interval) to cope with the intrinsic variation of nectar measurements and with the high frequency of nectarless flowers (Witt *et al.*, 1999).

Anther dehiscence, breeding system and pollination success throughout the day

To assess whether nyctinastic flower opening is coupled with the release of pollen grains and the elongation of the style, we carried out direct observations from initial flower opening until the fourth day of each flower. We observed 2–3 flowers each from five plants in June 2012, and captured a long series of photographs every 15 min with a 90 mm macro lens to make a time-lapse sequence (Bieleski *et al.*, 2000).

To test for variation in pollination success after manual pollen supply throughout the day, we performed a hand pollination experiment on June–July 2012. We randomly assigned 263 flowers from 117 plants (2·2 ± 0·2 flowers per plant) to one of the following time intervals, 'Morning' (0900–1100 h) 'Afternoon' (1530–1900 h) and 'Night' (2100–2300 h). Pollinated flowers were in the second or third day of the female state. Pollen was collected immediately before pollination. All hand pollinations were performed with pollen from another plant of the same population (intra-population xenogamy), assigned randomly.

Additionally, to investigate the breeding system of *S. colorata* (i.e. its dependence on pollinators), we randomly assigned 296 flower buds from 137 plants (3·0 ± 0·1 buds per plant) to one of the following treatments: (1) spontaneous autogamy (flowers were individually bagged at the bud stage); (2) geitonogamy (hand pollination with pollen from another flower of the same plant); (3) intra-population xenogamy; and (4) inter-population xenogamy, with pollen from a population located >4·5 km away (Table S1). Pollen donors were randomly assigned within treatments. Pollinations were carried out only at night (2100–2300 h) because it was the time when the highest pollination success was achieved in the previous experiment (see the Results).

In both experiments, pollen was supplied by taking one anther of the donor flower with forceps and brushing it on the stigmas of the recipient flower. Flowers were bagged at the bud stage with organza bags (4 × 3 cm), opened for hand pollinations (if appropriate), and re-bagged thereafter until fruit ripening to avoid pollen contamination from other flowers. Flowers were not de-anthered, but the possible self-contamination was controlled with the spontaneous autogamy treatment. After 2–4 weeks, we sampled all fruits to calculate fruit set (proportion of flowers setting fruit) and the number of seeds per fruit. To describe the breeding system, we calculated a modification of the self-incompatibility index (ISI; Zapata and Arroyo, 1978). The ISI was calculated both with fruit set and number of seeds, dividing the success of geitonogamy by the success of intra-population or inter-population xenogamy. Values ≤0·25 indicate self-incompatibility (Sobrevilla and Arroyo, 1982; Faria *et al*., 2012). Finally, we tried to explore the variation in pollen viability throughout the day by germination tests. Pollen collected at the same time intervals was placed onto Petri dishes containing agar with 30 % sucrose (Buide and Guitán, 2002). However, the culture medium used to test the pollen viability did not work in this species, despite the fact that it was the best medium for a related species, *S. acutiflora* (Buide and Guitán, 2002).

Flower visitation rates and reproductive success in natural populations

To assess whether the floral phenotype of *S. colorata* is adapted to a particular type or functional group of pollinators (*sensu* Fenster *et al*., 2004), we conducted a field test from 5 to 22 May 2012. Censuses of flower visitors were made in three natural populations (2 Paso, Mostoles and Xanadu), for a total of 15–20 h of observation per population. To collect visitation data, we established five 1 × 1 m sampling plots in each population. The density of plants per plot varied between populations (13·66 ± 2·3, 26·83 ± 6·9 and 22·45 ± 5·89 plants m^{-2} in 2 Paso, Mostoles and Xanadu, respectively) and the number of flowers per plot ranged between 25 and 320 at the flowering peak. Diurnal observations were made on sunny days without wind, at different time intervals from 1000 to 1900 h. At each time interval, we made observations of 5–10 min in each plot and noted the identity and number of contacts of insect species with the reproductive structures of the flowers. We made 146 censuses, corresponding to 11·8 h of observation. Nocturnal observations were conducted with customized digital video cameras equipped with near-infrared light. On each census date, we placed one camera 15–30 cm in front of each sampling plot and counted the number of open flowers the camera was framing. We recorded each plot continuously from 2100 to 0100 h. We visualized a total of 40·1 h to note the identity and frequency of flower visitors. We could not accurately distinguish among moths species in the night video records. All visiting insects touching sexual organs were considered pollinators regardless of the efficacy of the visit. Insects were grouped into functional groups to calculate visitation rates (visits per flower h^{-1}) per time interval (morning, 0900–1500 h; afternoon, 15:00–1900 h; and night, 2100–0100 h). The minutes of observation per population ranged between 150 and 200 in the morning, between 50 and 100 in the afternoon and between 672 and 1078 in the night.

Two to three weeks after pollination census (25 May–4 June 2012), we randomly sampled ten plant individuals that had completed the full life cycle in each plot. We counted the total number of flowers (dried or aborted) and fruits produced per plant to calculate the natural fruit set in the populations. The rate of fruit predation (number of predated fruits/total number of fruits) by the *Hadena* nursery pollinators was also estimated because the larvae of these moths leave a characteristic hole in the capsules (pers. obs). Non-predated fruits were dissected to count the number of seeds. Outside the plots, we also collected green fruits that were carried to the laboratory. After some days, the *Hadena* larvae that emerged from the parasitized fruits thereof were reared until the adult stage to identify the species.

Statistical analysis

To explore the effect of light intensity and soil moisture on the dynamic of flower opening, a LMM (linear mixed model) was carried out with the following explanatory variables: light (Shade and Sun), moisture (Wet and Dry), time (every 30 min) and the interaction light × moisture × time. These factors were computed as fixed effects. Flower and plant were computed as random effects. The percentage of petal opening was arcsin [square root(X)] transformed before analysis to achieve normality. To explore the nectar dynamics, another LMM was applied with day (first, second and third), time of sampling (morning, late afternoon and night) and the interaction day × time of sampling as the explanatory variables. Nectar volume was square root transformed to achieve normality. These factors were computed as fixed effects and plant as random effect.

To analyse the variation of flower scent depending on the treatment (morning, night and flowers without petals), a set of generalized linear model (GLM) analyses were made for total scent production and the production of each compound independently. For these GLMs we used a tweedie error structure because of the zero-inflated distribution of the data (Dunn and Smyth, 2005; Tascheri *et al*., 2010). To depict variation in floral scent composition among samples, we used non-metric multidimensional scaling (NMDS). To test the differences in the complete scent (relative scent composition) among the treatments, a permutational multivariate analysis of variance (PERMANOVA) was made. A Bray–Curtis pairwise matrix of similarities (Clarke and Gorley, 2006) based on the percentage amount of the compounds was used for NMDS and PERMANOVA. To avoid that NMDS and PERMANOVA

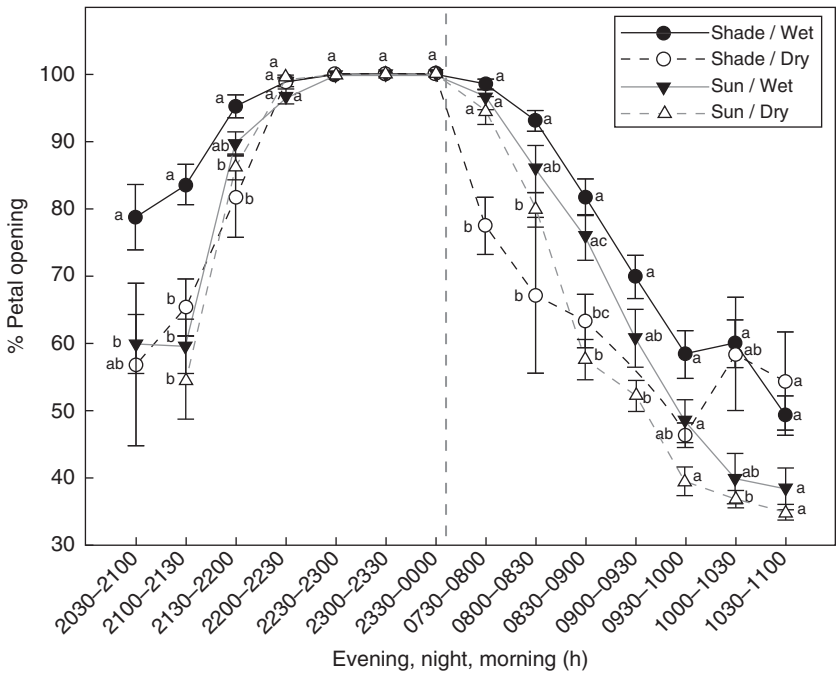

FIG. 1. Dynamic of flower opening and closure throughout the day (mean ± s.e. of percentage petal opening) of plants subjected to four combinations of light intensity and soil moisture. Different letters indicate significant differences among treatments during the same time period. The vertical dashed line denotes the transition from night to morning.

were greatly influenced by the most abundant compounds, the data (percentage contribution of the compounds to the total scent) were fourth root transformed (Clark and Warwick, 2001). To evaluate the breeding system, and the differences in pollination success throughout the day, we used generalized linear mixed models (GLMMs) for fruit set and LMMs for number of seeds. Treatment (geitonogamy, intra-population xenogamy and inter-population xenogamy) and pollination time (morning, afternoon and night) were used as explanatory factors for breeding system and pollination success, respectively, and plant identity was computed as a random effect. For fruit set GLMMs, we used Binomial error structure for the presence or absence of fruits (1 when a flower set fruit; 0 when did not). Autogamic hand crossings were excluded from the analyses because they did not produce any fruit (see the Results). To explore differences in flower visitation rate, we carried out a GLM with population, time and population × time. Fruit set, fruit predation (LM) and seed number (GLM) comparisons between populations were performed with population as the explanatory factor. To test the effect of the visitation rate on the pollination success, we performed four (total, morning, afternoon and night rates) LM and four GLM regressions for fruit set and seed number, respectively. For these GLM regressions we used Poisson error structure because the seed number had positive integer values. Post-hoc analyses were performed with the Tukey HSD test. Analysis were implemented with the 'tweedie', 'nlme', 'lme4', 'car' and 'argricolae' packages (Fox and Weisberg, 2011; Pinheiro *et al*., 2013; Dunn, 2014; Mendiburu, 2014; Bates *et al*., 2015) in R software (R Core Team, 2014), except the NMDS and PERMANOVA that were implemented in PRIMER 6·1·11 (Clarke and Gorley, 2006).

RESULTS

Effect of light intensity and soil moisture on flower nyctinasty

There were significant effects of light intensity ($F_{1,24} = 8{\cdot}73$, $P = 0{\cdot}007$) and time of measurement ($F_{13,1525} = 507{\cdot}9$, $P < 0{\cdot}001$) on the percentage of petal opening. Soil moisture ($F_{1,24} = 4{\cdot}13$, $P = 0{\cdot}053$) was marginally significant, and the interaction moisture × light × time was significant ($F_{13,1525} = 4{\cdot}01$, $P < 0{\cdot}001$). In the evening at 2100–2130 h, petals of the Shade–Wet treatment were more unrolled than petals of other treatments (Fig. 1). However, all treatments reached 100 % opening at 2200–2230 h. Petals of all treatments remained open until dawn. In the morning, petals of the Shade–Dry treatment were the first to close at 0730–0800 h, followed by plants under the Sun–Dry conditions at 0830–0900 h. Plants under the Shade–Wet treatment maintained the petals more open than those under Dry treatments until 1000–1030 h. The most fully closed petals at the end of the morning were those under Sun treatments, although differences were not significant.

Dynamic of flower scent emission

The GC-MS analyses showed that two of the night samples did not emit any scent. These plants had flowers with petals not completely open when volatiles were trapped (2110 h). These two samples were not taken into account in the GLM analysis. Another night sample with the petals excised also did not emit scent. These three samples were not taken into account in the NMDS analysis and PERMANOVA. The scent composition differed among the treatments (pseudo $F_{1,17} = 8{\cdot}48$; $P < 0{\cdot}001$). Night samples had a different composition from

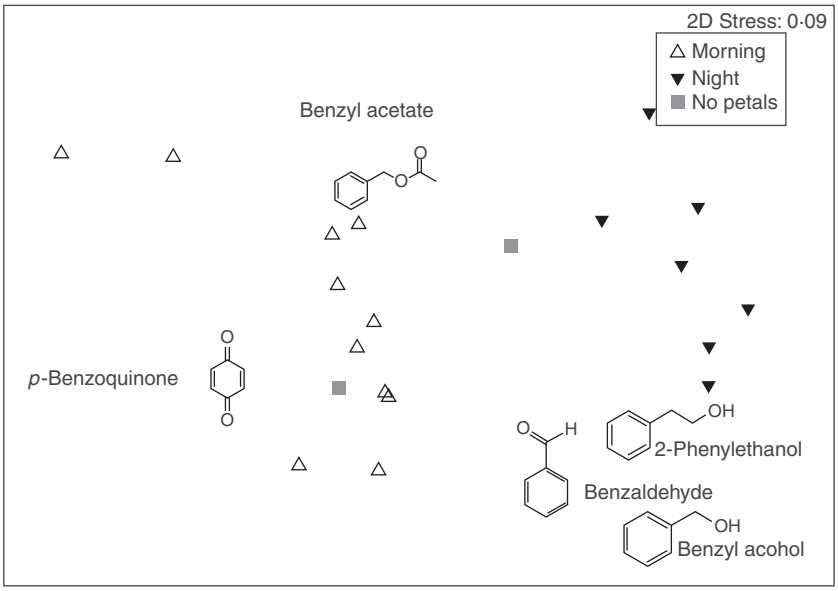

FIG. 2. Non-metric multidimensional scaling (NMDS) of the flower scent released by *S. colorata* in the morning (0750–0930 h), at night (2130–2315 h) and at night with petal limbs excised. The NMDS is based on the percentage amount of the compounds. Main volatile compounds are placed following correlations of each compound with the ordination axis.

morning samples and flowers with no petals limbs. There was no difference between morning samples and the scent of flowers with no petal limbs (Fig. 2). The standardized total scent production (ng flower min^{-1}) was higher in the night than in the day and no petal limbs samples ($F_{2,18} = 48{\cdot}62$ $P < 0{\cdot}001$) (Fig. 3). There were significant differences among treatments (morning, night and flowers with no petal limbs) with any production in some compounds (benzaldehyde $F_{1,8} = 9{\cdot}57$ $P = 0{\cdot}015$; benzyl alcohol $F_{1,16} = 16{\cdot}99$ $P < 0{\cdot}001$; *p*-benzoquinone $F_{2,18} = 6{\cdot}23$ $P = 0{\cdot}009$), but not in 2-phenylethanol ($F_{2,18} = 0{\cdot}74$ $P = 0{\cdot}49$), benzyl acetate ($F_{2,18} = 0{\cdot}67$ $P = 0{\cdot}52$) and E-caryophyllene ($F_{1,8} = 0{\cdot}9$ $P = 0{\cdot}37$) (Fig. 3). Benzylaldehyde and E-caryophyllene were emitted in high amounts at night but not during the day. The emission of benzyl alcohol was higher at night than in the day. Conversely, three unknown compounds were only emitted in the morning, and the emission of *p*-benzoquinone was higher in the day than at night. The excision of the petal limbs reduced the emission of benzaldehyde and eradicated the emission of benzyl alcohol, but increased the amount of *p*-benzoquinone, and did not affect E-caryophyllene, 2-phenylethanol and benzyl acetate (Fig. 3).

Anther dehiscence, breeding system and pollination success throughout the day

Anther dehiscence of *S. colorata* took place immediately after the flower bud burst. The two whorls of five stamens dehisced sequentially on the first and second night, and they withered at dusk each. The style elongation began at dusk of the third day. Both anther dehiscence and style elongation are synchronous with nyctinastic flower opening (Supplementary Data Video S1). The pollen was of fresh and dusty appearance from anther dehiscence until mid-day, but then it turned dry and clumpy (pers. obs.).

In the breeding system experiment, the spontaneous autogamy treatment did not produce any fruit. There were no differences between geitonogamy, intrapopulation xenogamy and interpopulation xenogamy in fruit set ($\chi^2_2 = 5{\cdot}2$ $P = 0{\cdot}07$) and in the number of seeds ($F_{2,156} = 0{\cdot}7$ $P = 0{\cdot}5$) (Table 1). The ISI values indicated that *S. colorata* is self-compatible (fruit set ISI = 0·86 and seed number ISI = 0·91 for interpopulation xenogamy; fruit set ISI = 1·01 and seed set ISI = 0·95 for intrapopulation xenogamy). The number of seeds was lower in the afternoon than in the morning and night pollinations ($F_{2,144} = 21{\cdot}96$, $P < 0{\cdot}001$, Table 1). Fruit set was lower in the afternoon than in the morning, but fruit set at night was no different from that during the morning and afternoon ($\chi^2_2 = 9{\cdot}08$, $P < 0{\cdot}012$, Table 1).

Nectar dynamic

Nectar production was very low in *S. colorata* (range = 0–0·21 μL) and there was a high proportion of flowers that did not produce nectar (Fig. 4). Nonetheless, there were significant differences in nectar volume between time intervals ($F_{2,433} = 5{\cdot}05$, $P = 0{\cdot}007$) and in the interaction day × time of sampling ($F_{4,433} = 3{\cdot}1$, $P = 0{\cdot}02$) but not between days ($F_{2,455} = 2{\cdot}61$, $P = 0{\cdot}07$). The first morning after flower opening, nectar volume was high, and then decreased during the course of the day (Fig. 4). On the second day, flowers showed the same pattern but there were no significant differences among times of the day. On the third day (first in the female stage) there were also no significant differences (Fig. 4).

Flower visitation rates, reproductive success and fruit predation by the nursery pollinator

Field results showed that *S. colorata* was visited by both diurnal and nocturnal insects (Fig. 5). In the daytime (morning

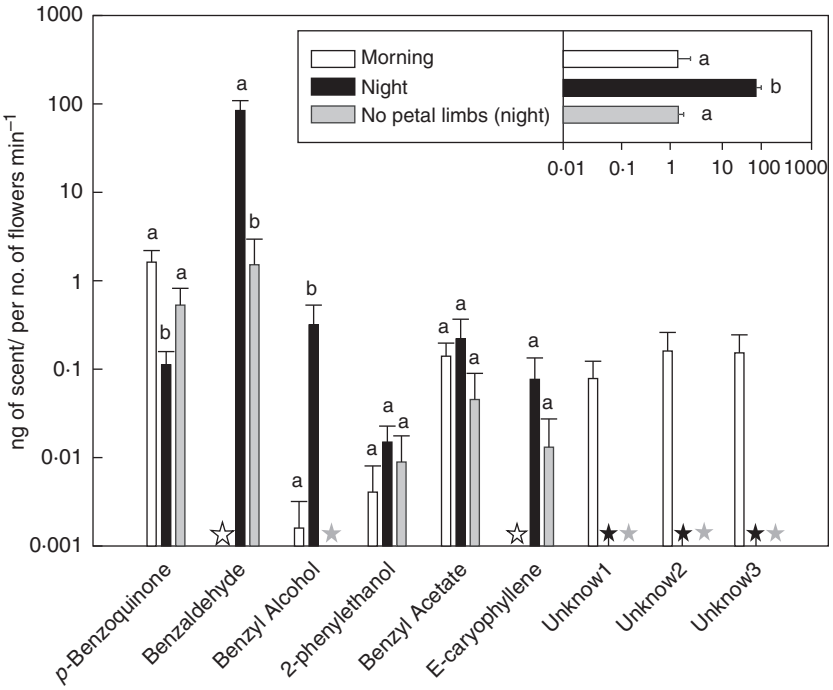

FIG. 3. Mean ± s.e. of floral emission rates of each volatile compound. The insert denotes the total floral scent emission rates. Stars denote no production of a compound. Different letters indicate significant differences among treatments. Both graphs are in log scale.

TABLE 1. *Number of seeds per fruit and fruit set in the breeding system experiment and the experiment on the pollination success throughout the day*

	No. of seeds (mean ± s.d.)	Fruit set (%)
Breeding system		
Geitonogamy	37·03 ± 2·8	77
Intra-population xenogamy	38·95 ± 2·2	87
Inter-population xenogamy	40·64 ± 4·0	75
Pollination success		
Night	39 ± 2·2[a]	87[ab]
Morning	44·8 ± 2·3[a]	93[a]
Afternoon	18·4 ± 3·0[b]	74[b]

Different letters indicate significant differences among treatments.

and afternoon), small bees were the most frequent visitors (range from 81 to 100 % of diurnal visits), followed by bombilid flies (0–18 %) and hoverflies (0–1 %). At night, all visitors were moths (Noctuidae and Geometridae). The visitation rate varied between time intervals ($F_{2,43} = 5{\cdot}50$, $P = 0{\cdot}008$), and between populations ($F_{2,43} = 7{\cdot}85$, $P = 0{\cdot}002$) (Fig. 5). Also there was a significant effect of the interaction between population and time interval ($F_{4,43} = 5{\cdot}26$, $P = 0{\cdot}002$) (Fig. 5). Fruit set and number of seeds were not different among populations ($F_{2,12} = 0{\cdot}41$, $P = 0{\cdot}67$ and $F_{2,12} = 0{\cdot}59$, $P = 0{\cdot}57$, respectively). Fruit set was not affected by visitation rates ($F_{1,13} = 0{\cdot}67$, $P = 0{\cdot}43$; $F_{1,13} = 0{\cdot}02$, $P = 0{\cdot}89$; $F_{1,13} = 1{\cdot}59$, $P = 0{\cdot}23$; $F_{1,12} = 2{\cdot}45$, $P = 0{\cdot}14$; for the total, morning, afternoon and night visitation rate, respectively). There was a positive influence of the total ($Z_{1,12} = 4{\cdot}04$, $P = 0{\cdot}04$), night ($Z_{1,12} = 2{\cdot}16$, $P = 0{\cdot}031$) and afternoon ($Z_{1,11} = 2{\cdot}03$, $P = 0{\cdot}042$) visitation rate on the seed number, but no effect of the morning visitation rate ($Z_{1,11} = -1{\cdot}84$, $P = 0{\cdot}14$). There were differences among populations on the fruit predation rate by *Hadena sancta* ($F_{2,11} = 4{\cdot}51$, $P = 0{\cdot}037$) (Fig. 5), the only species that emerged from fruits of *S. colorata* ($n = 23$). The fruit predation rate was higher at the 2 Paso population, which also had the highest number of nocturnal visits (Fig. 5).

DISCUSSION

Flower traits and nyctinasty

The first observation of the floral phenotype of *S. colorata* is an interesting contradiction. The species has bright pink petals which points towards a diurnal pollination system (Jürgens, 2006), but flowers are fully opened only at night. Flower colour has been one of the classical features for definition of pollination syndromes (Faegri and van der Pijl, 1979; Proctor *et al.*, 1996), but it must be treated with caution as a key trait (Waser *et al.*, 1996). In Sileneae, the flower colour is not very reliable as a predictor trait for diurnal or nocturnal pollination (Prieto-Benítez *et al.*, 2015).

Our results suggest that the nyctinastic petal folding in *S. colorata* is influenced by changes in light intensity and accelerated or delayed by soil water content. In shadow microhabitats (e.g. beneath trees or shrubs), the light intensity decreases earlier at dusk and increases later at dawn, and the soil retains more water. In consequence, the flowers open earlier and close later, extending the period of flower display, resulting in an increase in the visibility for evening and early morning flower visitors. In sunny and dry microhabitats, petal closure is accelerated at dawn and the petals remain closed (rolled) during the whole day, reducing

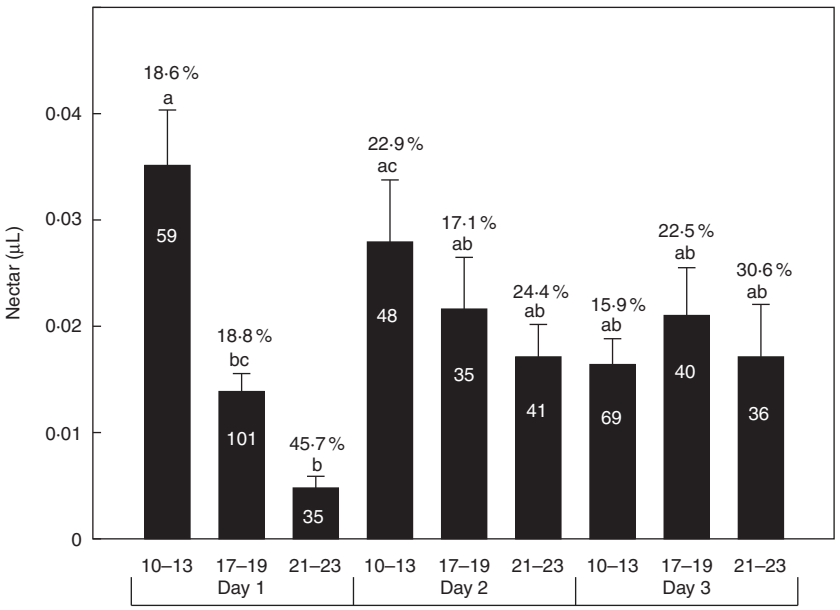

FIG. 4. Each bar represent the mean nectar volume (µL) accumulated from anthesis to the time of measurement (h) in flowers of *S. colorata*. Note that the first bar corresponds to the morning after flower bud opening at dusk. On day 1 and 2, flowers are in the male stage; on day 3 flowers reach the female stage. Error bars denote the s.e. Different letters indicate significant differences among time intervals. Numbers above each bar denote the percentage of nectarless flowers. The number within each bar denotes the sample size.

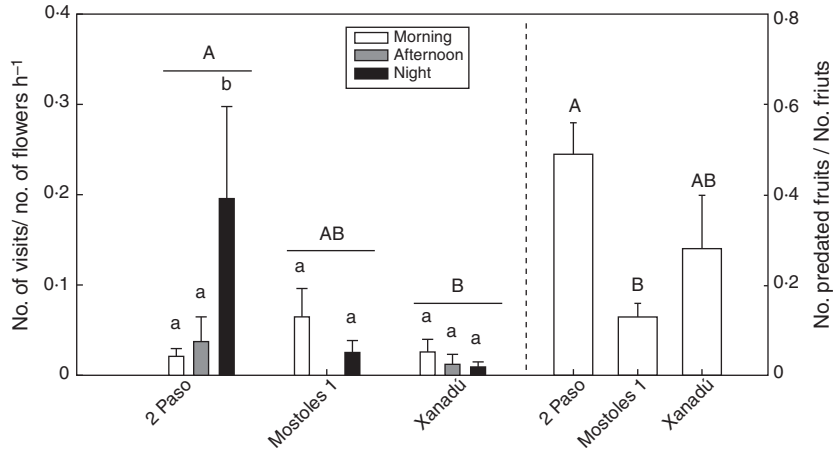

FIG. 5. Variation among *S. colorata* populations in flower visitation rates at each time interval (left panel), and fruit predation rate (right panel). Vertical bars denote means ± s.e. Different upper case letters indicate significant differences among populations. Different lower case letters denote differences among time intervals within each population.

the temporal range of floral display. The closing and opening of petals was also influenced by light intensity and air humidity in *Silene saxifraga* (Halket, 1931). Halket showed in this pioneer work that closing of petals is due to the loss of cell water content by transpiration, and opening is due to cell refilling probably in response to a combination of sugar uptake and degradation of polysaccharides (van Doorn and van Meeteren, 2003). The maintenance of turgor in the petals requires a constant input of water from vegetative parts (Ram and Rao, 1984), and this demand may involve a high cost especially in dry environments (Nobel, 1977; Galen *et al*., 1999; Teixido and Valladares, 2013, 2014). Since light is the most important input signal to the circadian clock (McClung, 2006; Sanchez *et al*., 2011), an increase in light intensity may regulate the flower closure to signal to the plant the high evapotranspirational demand in the middle hours of the day. Minimizing floral water loss by nocturnal flowering may be an advantageous strategy in hot and dry ecosystems (Teixido and Valladares, 2014), and is found in other Mediterranean plants such as *Capparis* species (Rhizopoulou *et al*., 2006), and in most desert cacti (Valiente-Banuet *et al*., 1997; Fleming *et al*., 2001).

The significance of flower closure response to the abiotic environment may be considerable for several reasons. First, plastic responses of floral attractiveness to the environmental

conditions may influence the suite of pollinators and how they forage flowers during the day and season, affecting the plant fitness. This is important because the actual climate change is affecting the abiotic factors regulating floral attractiveness. Secondly, if plant genotypes vary in their sensitivity to abiotic factors, some of the variation in flower opening and closure among individuals may be caused by heritable differences, and this may be subject to selection. Moreover, selection of flower traits imposed by the abiotic environment and by pollinators may conflict (Carroll *et al*., 2011). The maintenance of flower nyctinasty may be constrained by a trade-off between water economy and attractiveness to diurnal pollinators (despite the fact that morning pollinators may select for longer periods of flower opening, drought stress may favour genotypes with rapid flower closure).

Nectar secretion, flower scent and sexual phases

We have found that nyctinastic petal opening is synchronized with nectar secretion dynamics in *S. colorata*. Nectar production took place only at night, at least on the first and second day after anthesis. On the third day (first day of the female phase), this pattern was lost. Since insect visits were excluded in our experiment, the reduction in nectar volume from night to afternoon could be due to evaporation or resorption. Evaporation of nectar may be limited in this species due to the high osmolarity provided by the high hexose (glucose and fructose) content (Witt *et al*., 2013) and the availability of nectar within a long floral tube, which might reduce the evaporative effects of a low relative humidity of the air (Pacini and Nepi, 2007). However, nectar resorption is a widespread strategy in unvisited flowers, presumably to recover the resources invested in nectar production (Burquez and Corbet, 1991; Pacini and Nepi, 2007). Nocturnal nectar production also occurs in other *Silene* species with nocturnal pollination syndrome (Witt *et al*., 1999; Reynolds *et al*., 2009; Castillo *et al*., 2013), and similar diel patterns have been previously described in other species pollinated by nocturnally active animals (Cruden *et al*., 1983; Tschapka and von Helversen, 2007; Amorim *et al*., 2013). The high number of nectarless flowers in this species is in accordance with previous findings in several plant species and also in *Silene* (Brink, 1982; May, 1988; Gilbert *et al*., 1991; Witt *et al*., 1999).

Nyctinastic flower opening and nectar secretion are also synchronized with the emission of high amounts of scent at night. The petals usually produce most of the VOCs of the flower scent (Dobson *et al*., 1990; Bergström *et al*., 1995). When petal limbs were abscised at night, scent amount and composition decreased significantly, and was similar to those of intact flowers at morning. This suggests that emission of the most abundant flower VOCs at night, benzaldehyde and benzyl alcohol, takes place mainly in the expanded petals, whereas other compounds (i.e. *p*-benzoquinone, E-caryyophyllene, 2-phenylethanol and benzyl acetate) are released from other floral organs. Petals are also responsible of the benzenoid emission in *S. latifolia* (Dötterl and Jürgens, 2005) and are also the main scent producer in *Petunia* and *Antirrhinum* flowers (Dudareva *et al*., 2000*b*; Verdonk *et al*., 2003). When petals are folding in the morning, the scent production ceases, and in the afternoon *S. colorata* do not produce any flower scent (Prieto-Benítez *et al*., 2015) until the petals open again at night.

Anther dehiscence and style elongation were also synchronized with petal opening. Anther dehiscence at dusk may provide a fresh atmosphere for pollen during the night, but in the morning temperature increases and the pollen grains get dry and clump together (pers. obs.), decreasing their viability (Nepi *et al*., 2001). In the nocturnal *S. latifolia*, *in vitro* pollen germinability reaches the maximum at midnight and then decreases (Aonuma *et al*., 2013). In contrast, in the diurnal *S. acutifolia*, pollen germinability declined progressively after dehiscence in the daytime (Buide and Guitán, 2002). Unfortunately the culture medium used by these authors did not work in *S. colorata*, so we cannot prove the loss of viability in the daytime. In any case, our experiment showed that hand pollination yielded higher reproductive success (both fruit set and number of seeds) at night and in the morning than in the afternoon. These differences may indeed be due to the reduction of pollen viability, but may also be due to a decrease in stigma receptivity, since it is known that high temperatures at mid-day can reduce stigmatic receptivity (Hedhly *et al*., 2005).

Breeding system, pollinators and predators

Silene colorata is self-compatible as are many other *Silene* species (Bocquet, 1968). Spontaneous autogamy is not viable due to protandry (the anthers mature and wilt before the elongation of styles), so this species depends on flower visitors even for geitonogamous pollination. The opening of petals at dusk may attract moths visually and additionally provide a landing platform for settling moths. At the same time, the production of nectar reward increases in parallel with the emission of high amounts of benzaldehyde, benzyl alcohol, 2-phenylethanol and benzyl acetate, all of them related to the attraction of moths (Heath *et al*., 1992; Meagher, 2002; Dobson, 2006; Giménez-Benavides *et al*., 2007). These flower VOCs are also present in the nocturnal scent of other *Silene* species with moth pollinators, such as *S. subconica*, *S. viscosa*, *S. latifolia* and *S. ciliata* (Jürgens *et al*., 2002; Dötterl *et al*., 2005; Giménez-Benavides *et al*., 2007; Castillo *et al*., 2014). This combination of traits may lead the moths to remove the fresh pollen just after dehiscence and to deposit it on the young receptive styles, increasing their efficiency as pollinators.

In the morning, other insects such as bees, bombilids and hoverflies also visit *S. colorata* and may pollinate the flowers with the remaining pollen. The interplay of colour and scent is essential for diurnal insects for finding and recognizing host-plants (Burger *et al*., 2010; Milet-Pinheiro *et al*., 2012). Pink petals are attractive to bees (Menzel and Shmida, 1993; Reynolds *et al*., 2009), so they can be a visual cue of *S. colorata* early in the morning, when they are still open, and even when they are completely rolled up in the afternoon. Although we previously found that *S. colorata* did not emit flower VOCs at mid-day (Prieto-Benítez *et al*., 2015), the flowers still emit a small amount of scent before complete petal closure, which differs in composition from the nocturnal scent. Among the compounds released during the day, *p*-benzoquinone, benzyl acetate, benzyl alcohol and 2-phenylethanol have the potential to attract bees and flies (Knudsen and Mori, 1996; Dobson,

2006; Dötterl and Vereecken, 2010; Burger *et al*., 2012). It is also interesting to note that three unknown compounds are emitted in significant amounts only in the morning. These compounds may act as olfactory cues for diurnal attraction of pollinators or as deterrents of herbivores. Small bees and hoverflies visited the flowers of *S. colorata* to collect pollen, and bombilids to drink nectar. Both rewards are less abundant but still available during the day, especially in the early morning, if they have not been consumed the previous night. Moreover, our hand pollination experiment showed that one pollen grain has the same probability to develop a seed when it is deposited in a flower at night as in the early morning, but pollination success decreases by 2-fold in the afternoon. This suggests that phenotypic specialization to night pollination in *S. colorata* does not cause a strong fitness trade-off to early morning pollination, so the functional diversity of pollinators can be maintained (Aigner, 2001, 2004; Armbruster, 2014). However, in the afternoon, the attractiveness and fertility of flowers reduce drastically, and this may result in a large decrease in fertilization success.

Our pollinator censuses showed that the abundance of day and night visitors varies between the three populations (Fig. 5), and moths were the most frequent visitors only in one population. The number of seeds per fruit was positively correlated with night, afternoon and total visit rates. The number of seeds was not correlated with the morning visit rate, despite the fact that our hand pollination experiment found that morning pollination can produce high amounts of seeds. This apparent contradiction could be due to a low efficiency (pollen removed and deposited per single visit) of the morning pollinators. We did not collect efficiency data, but Reynolds *et al.* (2009) reported that pollinator importance (visitation frequency × pollen deposition) was higher for nocturnal moths than for diurnal bees in *S. stellata* (another species with nocturnal moth syndrome) in two of three studied years. In summary, the results of hand pollinations and field censuses together suggest that when nocturnal pollinators are scarce, the combined effect of diurnal and nocturnal pollination may ensure the plant's reproductive success. Complementarity of diurnal and nocturnal pollination has been described before (Miyake and Yahara, 1999; Wolff *et al*., 2003; Reynolds *et al*., 2009; Amorim *et al*., 2013). We believe that our observations on the flower circadian rhythm and receptivity of *S. colorata* may be generalized to other *Silene* species with presumed nocturnal syndrome. Many of them have their flowers open in the early morning (pers. obs.), and diurnal pollinators are also frequent and have a substantial role in their reproductive success (Giménez-Benavides *et al*., 2007; van Putten *et al*., 2007; Reynolds *et al*., 2009; Martinell *et al*., 2010; Buide *et al*., 2015). These results suggest functional reasons to support the general agreement that traits associated with classical pollination syndromes can vary and directly impact the pollinators observed in the field (Waser *et al*., 1996; Ollerton *et al*., 2007, 2015; Armbruster, 2014).

The presence of the nursery pollinator *Hadena sancta* in the nocturnal pollinator guild of *S. colorata*, which is for the first time described as a host in the present work, may explain why there was no positive effect of the visitation rate on the fruit set. *Hadena sancta* pollinates the plant but also rears its offspring in the flowers and developing fruits. Although the pollinator service provided by *Hadena* species may be prominent (Reynolds *et al*., 2009, 2012; Labouche and Bernasconi, 2010), the net effect of the interaction may be negative when the cost of fruit predation is taken into account (Kephart *et al*., 2006; Reynolds *et al*., 2012; Kula *et al*., 2014). Unfortunately, we could not accurately distinguish *Hadena* from other moths in the night video recordings to estimate its visitation frequency. However, the overall nocturnal visitation rate was positively correlated with fruit predation by *Hadena* larvae in two out of three populations (2 Paso and Mostoles) (Fig. 5), suggesting that loss by fruit predation is proportional to pollination service in these two populations. If the net outcome of the *S. colorata*–*H. sancta* interaction shifts towards parasitism, the complementary pollination provided by diurnal pollinators may help to compensate the high cost of fruit predation. The outcome of nursery pollination has been studied in other *Silene*–*Hadena* systems (Petersson, 1991; Reynolds *et al*., 2012; Kula *et al*., 2014). These works have shown that the frequency of the nursery pollinators and co-pollinators contributes to shifts between mutualism and parasitism with the host plant, and this outcome also varies in space and time and with host plant density. Selective pressures exerted by pollinators and predators may also vary in space and time (Thompson, 1994, 1999) and in trait combinations. For instance, Fenster *et al*. (2015) demonstrated complex selection by hummingbirds in artificial combinations of flower traits from three contrasting *Silene* species. Therefore, studies that involve several populations and various years are needed to clarify the constancy or lability of selective pressures acting on floral traits of *S. colorata*.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: localities from where the seed came and the data of the flower visitor census. Video S1: petal nyctinasty, anther dehiscence and style elongation of *Silene colorata*.

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