Selection on floral characters mediated by diurnal and nocturnal pollinators of the orchid *Gymnadenia conopsea*

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Summary

Natural selection drives the evolution of adaptive diversity within and among natural populations. Pollinators have the ability to shape selection on floral characters and interactions with pollinators are thought to be one of the primary causes of plant diversification. The relative contribution of pollinators to plant reproduction varies as they differ in abundance and in efficiency as pollinators. I quantified the importance of pollination by diurnal and nocturnal flower visitors, quantified pollinator-mediated selection on floral traits through female function experimentally, and compared selection mediated by diurnal and nocturnal pollinators in the rewarding orchid *Gymnadenia conopsea*. Comparisons of selection gradients estimated for open-pollinated control plants and plants receiving supplemental hand-pollination indicated that pollinators contributed to selection for more flowers and were responsible for selection for longer nectar spurs in the study population. Observations of pollinia removal and pollen deposition indicated that the nocturnal pollinators became active later in the season than the diurnal pollinators did. However, day- and night-active pollinators appeared equally important for fruit production in the study population, and selection on floral traits did not differ significantly between the two categories. The results demonstrate that pollinators can mediate selection both on traits involved in pollinator attraction and on traits affecting pollination efficiency, and they suggest that interactions with diurnal and nocturnal pollinators result in similar selection on floral traits in this system.

Content

Introduction

A majority of the angiosperms are animal-pollinated and depend on pollinators for successful reproduction (Goulson 1999). Plants attract pollinators by visual cues, bright colors, nectar guides, scent and most species offer a reward. Some plant species are visited only during the day or only during the night, and others are visited during both. The relative contribution of different pollinators to plant reproduction varies as flower visitors differ in abundance and in effectiveness as pollinators (Fenster *et al.* 2004). This study compares diurnal and nocturnal pollinators in terms of their importance for fruit production and selection on floral characters in the nectar-producing orchid *Gymnadenia conopsea*.

Natural selection is the primary mechanism for the evolution of adaptive diversity within and among natural populations. The angiosperm flower is often used as a model for studying adaptive evolution (Johnston 1991) as the flower shape reflects adaptations to the morphology of pollinators (Johnson & Steiner 1997). Pollinators have the ability to shape selection on floral characters, affect plant population dynamics, and interactions with pollinators are thought to be one of the primary causes of plant diversification (Harder & Barrett 2006)**.** Variation in strength and direction of natural selection, within and between populations, is common (Johnston 1991). Differences between populations may contribute to the evolution of local adaptation, while temporal variation in direction of selection might contribute to the diversity of genetic variation within populations (Sletvold & Ågren 2010).

Pollinator efficiency
The interaction between plants and their visitors is a relationship full of compromises. Pollinators visit flowers with the intent of finding reward and not necessarily for transport of pollen, but the transport service is crucial for successful reproduction of most animal-pollinated species (Matsuki *et al.* 2008). The most frequent visitor is not necessarily the most effective pollinator (Huber *et al.* 2004; Matsuki *et al.* 2008). Reproductive success in plants depends on how well the plant succeeds in attracting pollinators and how efficient the visitors are as pollinators (Harder & Barrett 2006). Pollinator efficiency is determined by the amount of matching-pollen deposited and the precision of pollen deposition on the flower. Where the pollen ends up on the flower depends on pollinator morphology and pollinator behavior Corolla tube width and length and spur length are traits that have been shown to affect the mechanical fit in orchid flowers (Sletvold & Ågren 2010). The importance of a deep spur for effective pollination by hawkmoths has been documented in several studies (Nilsson 1988; Alexanderson & Johnson 2002; Boberg and Ågren 2009). Floral characters that are important for the mechanical fit between the flower and the pollinator often show less phenotypic variation than other floral characters (Cresswell 1998).

Reward or deceit
Nectar is the most common reward in animal-pollinated orchids *(*Neilan & Wilcock 1998) although about one-third of the species in the orchid family are rewardless (Hansen & Olesen 1999). In general, rewarding species of the orchid family have a higher visitation rate and higher fruit set and seed production than non-rewarding species. Why deceit is so common in

the orchid family is still under debate. Two main explanations are that reward is costly to produce even though it enhances reproductive success, and that geitonogamy is lower in nectarless plants as pollinators visit fewer flowers per inflorescences (Hansen & Olesen1999; Jersakova & Johnson 2005).

Many of the fundamental ideas of floral evolution assume that pollinators of a given kind prefer a similar combination of floral traits (Harder & Barrett 2006) and mediate similar selection on plants. Pollination syndromes organize plants into functional groups based on floral characters. The concept of pollination syndromes is fairly controversial, but is commonly used in literature (Waser *et al.* 1996; Ollerton *et al.* 2009).

Pollinator-mediated selection
Pollinator-mediated selection should be important for the evolution of floral characters. Strong selection on floral traits has been documented in a variety of plant species (Harder & Barrett 2006). Pollinators can mediate selection both on traits likely to be involved in pollinator attraction (corolla size, inflorescence height, number of flowers and corolla shape) and on traits affecting pollination efficiency (spur length) (Medel *et al.* 2003; Sletvold & Ågren 2010). The pollinator preference results in variation in fitness and phenotypic selection in the plant population (Medel *et al.* 2003). Pollinator visitation rate is usually positively related to visual attractiveness, and traits like plant height, number of flowers and flowers size have been demonstrated to influence pollinator attraction. Other factors that might, directly or indirectly, contribute to variation in pollination success, are weather conditions, resource availability and herbivory (Bennington & McGraw 1995; Wilson 1995; Caruzo 2000). Non-pollinator mediated selection is easier to detect when it is conflicting with pollinator-mediated selection (Harder & Barrett 2006). Herbivores and pollinators may mediate conflicting selection on floral traits (Harder & Barrett 2006). For example, it has been demonstrated that individuals with larger flowers receive more visits by pollinators, but also experience greater pressure from herbivores then individuals with smaller flowers (Strauss & Irwin 2004). Herbivory on flowers may reduce plant fitness directly by destroying reproductive structures, but also indirectly through effects on pollination as damaged flowers are less attractive to pollinators and by reducing nectar production. Conflicting selection usually creates floral traits with optima that differ from the optimum preferred by pollinators (Harder & Barrett 2006).

Purpose
In this study I quantified the relative importance of nocturnal and diurnal flower visitors as pollinators and as selective agents on floral characters in the animal-pollinated, nectar-producing orchid *Gymnadenia conopsea*. Previous studies of *G. conopsea* have demonstrated pollinator-mediated selection on several floral characters (Sletvold & Ågren 2010). By comparing fruit production in plants pollinated by nocturnal and diurnal flower visitors, I determined their relative importance as pollinators. I quantified pollinator-mediated selection on morphological traits likely to affect pollinator attraction (plant height, number of flowers and corolla size) and pollinator efficiency (spur length), by subtracting for each trait the estimated selection gradient for plants receiving supplemental hand-pollination (βHP) from

the estimate obtained for open-pollinated controls (βC), $\Delta\beta poll = \beta C - \beta HP$ (Sletvold & Ågren 2010). To determine whether selection mediated by diurnal and nocturnal flower visitors differed, I compared selection gradients among plants pollinated by diurnal and nocturnal visitors, respectively. Finally, I examined selection on floral characters caused by herbivores, to determine whether there is any conflicting selection on floral traits.

The main questions addressed were: (I) Are plant height, number of flowers, corolla size and spur length subject to pollinator-mediated selection in *Gymnadenia conopsea*? (II) Does selection on floral traits mediated by nocturnal and diurnal pollinators differ? (III) Are nocturnal and diurnal flower visitors equally important as pollinators of *G. conopsea*? (IV) Is the intensity of herbivory related to plant height, number of flowers, corolla size and spur length?

Material & Methods

Gymnadenia conopsea (L.) Br.R. (Orchidaceae) is a perennial, terrestrial orchid, with a distribution across Europe and parts of Asia (Lönn *et al.* 2006; Hansen and Olesen 1999). It grows preferably on calcareous soil in grazed meadows, often close to marshes and fens. *G. conopsea* is rare but represented almost all over Norway, growing from sea level up to altitudes beyond 1500 m a.s.l (Sletvold & Ågren 2010). It is a non-clonal long-lived tuberous orchid. Since reproduction is costly it seldom flowers two years in a row. *G. conopsea* has one single inflorescence containing 10-70 flowers. The color varies from pink to white, although light pink is most common. The flower lacks nectar guides (Hansen and Olesen 1999). The flowers open from the bottom to the top of the inflorescence. The flowering time in the Norwegian populations usually stretches from the end of June to the beginning of August, and peaks in July. Each plant flowers for 3-4 weeks. Each flower has a nectar containing spur (mean 10.8 mm) and emits a spicy, sweet scent that attracts both diurnal and nocturnal flower visitors (Huber *et al.* 2004; Meyer *et al.* 2007; Sletvold & Ågren 2010). *Gymnadenia conopsea* is self-compatible but depends on pollinators for successful pollination and fruit set (Meyer *et al.* 2007). It is hermaphroditic, the reproductive organ consists of male and female parts (Hansen and Olesen 1999). Two pollinia are situated above the spur entrance. The pollinium contains pollen packed in numerous massulae (tightly packed pollen-masses). As the pollinator searches for nectar and inserts its proboscis into the spur the pollinia attach, singly or in a pair, to the proboscis. When successful pollination occurs the pollinator visits a second flower, the pollinia rubs onto the stigma and pollen is deposited. The pollinia have a bending mechanism to prevent self-pollination if pollinators visit several flowers on the same inflorescences. The massulae breaks off easily and pollen from a single pollinium can be deposited on several flowers (Sletvold and Ågren 2010). High levels of fruit set have been reported in both British and Swedish populations (Darwin 1862; Neiland and Wilcock 1998; Hansen and Olesen 1999; Lönn *et al.* 2006). Successfully pollinated flowers start to wilt soon after pollination. The fruits mature 4-6 weeks after pollination. Once the fruits mature, they burst and the dust-like seeds are dispersed by wind in the autumn (Sletvold & Ågren 2010).

The study was conducted in a population of 600 to 1000 flowering *Gymnadenia conopsea* individuals within the nature reserve Sølendet in central Norway (62°40'N, 11°50'E).

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Sølendet has a relatively short growing season, lasting from late May to late August and is located at the transition between middle and north boreal vegetative zones (Moen, 1999). Sølendet experience continental climate with an annual precipitation of 670 mm. The mean temperature in July 2010 was 12.5 degrees. The Sølendet population grows in open, wet grassland vegetation dominated by *Molinia caerulea* (L.), *Succisa pratensis* Moench, and *Thalictrum alpinum* (L).. The vegetation type is typical for the transition zone between the middle boreal and north boreal vegetation zones in Norway, from fen margins to more herb-dominated birch woodlands. The *G. conopsea* populations in Sølendet are mainly visited by diurnal butterflies of the genus *Boloria* Moore (Nymphalidae), the diurnal fly *Empis tessellata* F. (Empididae) and the nocturnal hawkmoth *Hyles gallii* Rott. (Sphingidae) (Sletvold & Ågren 2010).

Field experiment

In early July 2010, a total of 480 *G. conopsea* that had not yet begun to flower were haphazardly chosen and individually tagged. The plants were divided into four groups: 120 for hand-pollination treatment, 120 open for pollination by diurnal pollinators only, 120 open for pollination by nocturnal pollinators only, and 120 open for pollination by both diurnal and nocturnal flower visitors (control). The pollinators were excluded with 35*16 cm net cages (Figure 1.). The 120 plants pollinated by diurnal flower visitors were covered with cages between 6 PM and 6 AM and exposed to flower visitors between 6 AM and 6 PM. The plants pollinated by nocturnal pollinators were covered with cages between 6 AM and 6 PM and exposed to flower visitors between 6 PM and 6 AM. The plants were caged just before the first flower was about to open and experimental caging was continued until all flowers had wilted. Each flower on plants in the hand-pollination treatment was pollinated by hand at least once with pollen from two donors before wilting. Pollinia were haphazardly collected from other plants in the hand-pollination treatment. Towards the end of flowering, pollinia also from individuals outside of the experiment were collected and used in the hand-pollination treatment. The distance to pollen donors was never less than a few meters. Pollinaria were collected with a cocktail-stick, and pollinia were rubbed across the stigma of the receiving flower, making sure that the stigma was saturated with pollen.

Figure 1. The picture to the left shows *Gymnadenia conopsea*. In the picture to the right, the plant is covered with a cage.

Herbivory by lepidopteran larvae (unknown spp.) on flowers, fruits and even entire plant was common during the experiment. Every day the number of eaten flowers was recorded and early on in the experiment eaten plants were replaced. Because of the massive pressure from herbivores, the final sample size in each treatment was much reduced to 79 plants in the hand-pollination treatment, 79 plants pollinated by diurnal flower visitors only, 78 plants pollinated by nocturnal flower visitors only, and 65 plants in the control treatment.

The pollination intensity was investigated on three occasions. The number of massulae deposited (MD) and the number of pollinia removed (PR) were recorded on the 17th of July, the 29th of July and the 5th of August. On the 17th of July, a total of 195 plants were scored, 63 control plants, 64 day-pollinated plants and 68 night-pollinated plants. On the 29th of July, a total of 87 plants were scored, 28 control plants, 28 day-pollinated plants and 31 night-pollinated plants. On the 5th of August, all plants in the control, the day-pollinated and night-pollinated treatments were scored. I also recorded pollen deposition and removal on 15 hand-pollinated plants to estimate my efficiency as pollinator. Number of massulae received per flower (Prop MD) was estimated as the number of massulae deposited per plant divided with the number of flower scored. Proportion of pollinia removed (Prop PR) was estimated as (the number of pollinia removed per plant / (2 * the number of flower scored)).

Measured traits

Flowering start was recorded on all plant and height were measured to the nearest mm (length from the ground to the top of the inflorescences). On one of the three lowest flower of each plant, spur length (length from the corolla base to the spur tip) and corolla height and length were measured to the nearest 0.1 mm with a digital caliper. Corolla size was calculated as the product of corolla height and length (Figure 2.). The number of flowers was recorded at the end of the flowering period. Number of flowers and number of intact fruits (number of initiated fruits minus number of fruits consumed by herbivores) were counted on each plant at the end of the flowering period. Three intact fruit capsules were harvested from each plant. Fruit mass is positively correlated with number of seeds with embryos in *G. conopsea* (Sletvold & Ågren 2010). The fruits were weighed individually to determine mean fruit mass.

Figure 2. Plant height was measured from the ground to the top of the inflorescences (see picture to the left). The upper right picture shows nectar-containing spurs, measured from the corolla base to the tip of the spur. The lower right picture shows a corolla and two pollinia.

For each plant, female fitness was estimated as the product of number of fruits and mean fruit mass. Relative fitness was calculated as female fitness / mean female fitness of a given treatment. Fruit set was calculated as number of intact fruits / number of flowers not consumed by herbivores. Pollen limitation (PL) in the population was estimated as 1 – (mean female fitness of open-pollinated control plants/mean female fitness of hand-pollinated plants).

Statistical analyses

Statistical analyses were performed in Minitab 15. The effect of treatment on floral traits, fruit production and pollination success was analyzed with one-way ANOVAs. Post hoc tests (Tukey HSD) were performed for variables showing statistically significant effect of treatment in ANOVA.

I estimated phenotypic selection on floral traits following Lande and Arnold (1983), using multiple regression analysis with relative fitness (individual fitness / mean fitness) as the response variable and standardized trait values as explanatory variables. Fitness was quantified for each individual as number of fruits × mean fruit mass. Relative fitness and standardized trait values were calculated separately for each treatment. Differences in selection gradients between open-pollinated control plants and hand-pollinated plants and between day- and night-pollinated plants were analyzed with ANCOVA. By using ANCOVA it is possible to compare linear selection on floral traits in different treatments. Plants completely consumed by herbivores were excluded from the analysis of trait differences among treatments and the selection analysis.

The ANCOVA model:

Relative fitness = trait 1 + trait 2 + trait 3 + trait 4 + treatment + trait 1 * treatment + trait 2 * treatment + trait 3 * treatment + trait 4 * treatment

The strength of pollinator-mediated selection ($\Delta\beta poll$) was estimated by subtracting for each trait the estimated selection gradient for plants receiving supplemental hand-pollination (βHP) from the estimate obtained for open-pollinated controls (βC), $\Delta\beta poll = \beta C – \beta HP$.

I used logistic regression to ask whether the risk of being completely consumed by herbivores was related to any of the floral traits documented. This analysis included also replaced plants whose phenotype had been recorded before damage (N = 457).

Results

Floral traits
No statistically significant differences in number of flowers, corolla size or spur length were detected between treatments (one-way ANOVA; Table 1). There were statistical significant differences in plant height between treatments. Post-hoc test (Tukey HSD) indicated that plants were shorter in the open-pollinated control group (Table 1).

Fruit set, fruit size, and female fitness were higher in the hand-pollinated group than in the other treatments (Table 1), indicating pollen-limited female reproductive success. The pollen limitation (PL = 1 - (mean female fitness of open-pollinated control plants/mean female fitness of hand pollinated plants)) was 0.11, which is fairly low. No statistically significant difference was found in one-way ANOVA comparing fruit set, fruit size and female fitness of day-pollinated, night-pollinated and control plants (Table 1).

Pollination intensity

Pollen removal and pollen deposition varied among treatments on 29 July, but not on 5 August; pollen removal and deposition on 17th July was too low to be statistically analyzed. On 29 July, pollen removal and deposition was lower among plants exposed to nocturnal flower visitors only than among plants exposed to diurnal flower visitors, or to both (control; Table 3).

Phenotypic selection

Significant selection on several floral traits was detected. There was directional selection for more flowers in all four treatments (Table 2). In the open-pollinated control group marginally significant selection for longer spurs was detected (P = 0.06) and in the hand-pollinated group selection for shorter spurs (P < 0.05) and taller plants (P < 0.01) was detected (Table 2).

Pollinator-mediated selection

Pollinators contributed to selection both on number of flowers and on spur length. Pollinator-mediated selection (Δβpoll) was 0.192 on spur length and 0.175 on number of flowers (Table 2).

Day- and night comparisons

There was no statistically significant difference in selection on floral characters between plants pollinated only by day- and night-active flower visitors, respectively (Table 2).

Herbivory

Logistic regression indicated that the risk of complete herbivory increased with increasing number of flowers (chi-square = 3.9, df = 1, P = 0.047), but was not significantly related to plant height (chi-square = 2.5, P = 0.11), flower size (chi-square = 0.01, P = 0.93), or spur length (chi-square = 0.72, P = 0.39).

Discussion

This study has documented pollinator-mediated selection for more flowers and longer nectar spurs in the nectar-producing orchid *G. conopsea*. The results indicate that the phenology of day- and night-active pollinators differs, but that their importance as pollinators is similar. Moreover, no statistically significant difference in selection on floral traits mediated by day- and night-active flower visitors was detected.

Plants in the hand-pollination treatment produced more and larger fruits and had higher female fitness than plants in the other treatments. This indicates pollen limitation of female reproductive success in the population, which is common in animal-pollinated species (Ashman et al. 2004). The estimate of pollen limitation (0.11) was lower than estimates obtained in the same population in two previous years, 0.28 in 2008 and 0.31 in 2009. Variation in pollen limitation among years is not unexpected and may be related to temporal variation in abundance of pollinators and the composition of the pollinator assembly.

Pollinators contributed to selection for more flowers. Statistically significant selection for more flowers was found in all treatments. This is expected when fitness is quantified as seed production as the number of flowers sets a limit to the number of seeds that can be produced. However, experimental hand-pollination showed that part of the selection for more flowers was due to higher pollination success among many-flowered plants. Significant pollinator-mediated selection for more flowers has previously been demonstrated in *G. conopsea* (Sletvold & Ågren 2010), but also in other species including the rewardless orchid *Dactylorhiza lapponica* (Sletvold et al. 2010) and the fly-pollinated crucifer *Arabidopsis lyrata* (Sandring & Ågren 2009). Pollinator-mediated selection for more flowers can be expected when floral display is important for pollinator attraction (Harder & Barrett 2006).

A large floral display may attract more pollinators and enhance the chance of pollination, but may also increase the risk of being damaged by herbivores (Goméz 2003). In the present study, the risk of herbivores consuming all flowers increased with increasing number of flowers produced, indicating that herbivores counteract pollinator-mediated selection on flower production in this population.

There was evidence of pollinator-mediated selection for longer nectar spurs in the study population. Marginally significant selection for longer spurs was found in the control group and selection for shorter spurs was detected in the hand-pollinated group. Spur length is

closely connected to pollination efficiency, as the length of the spur influences the fit between pollinator and flower morphology. Selection for longer spurs in the control treatment may be an indication that pollination efficiency increases with spur length or that pollinators find plants with long spurs more attractive. Longer spur and nectar content is often positively related which could explain pollinator preference (Whittall & Hodges 2007). The selection for shorter spurs in the hand-pollinated group is interesting as it might indicate that there is a cost associated with the production of long spurs, a cost that is not balanced by any benefits in the hand-pollinated group as all flowers were pollinated regardless of spur length. The cost could be related to the cost of producing the spur itself or to costs associated with an increased nectar production. Earlier studies of the population at Sølendet also demonstrated pollinator-mediated selection on floral display and spur length (Sletvold & Ågren 2010). The pollinator-mediated selection on spur length ($\Delta\beta poll$) was 0.077 in 2008, 0.16 in 2009 and 0.192 in 2010. This indicates strong consistent pollinator-mediated selection for longer spurs in the Sølendet population.

In the hand-pollination treatment, there was statistically significant selection for taller plants. This suggests that plant height is correlated to resource availability. Access to sunlight should increase with increasing plant height and this may be important in the relatively tall vegetation at Sølendet. Significant selection for taller plants is consistent with observations made in the same population in 2008 and 2009 (Sletvold & Ågren 2010).

Observations of pollinia removal and pollen deposition indicated that the nocturnal pollinators became active later in the season than the diurnal pollinators did, but there was no difference in fruit set or fruit size between plants pollinated by day- and night-active visitors, respectively. On 29th of July, the proportion of pollen deposition and pollinia removal were significantly lower among night-pollinated than among day-pollinated and control plants (Table 3). On 5th of August, there were no statistically significant differences in either pollinia deposition/removal or number of massulae received per flower between the groups. The differences in pollen deposition were not correlated with differences in fruit set or fruit size suggesting that either the low pollen deposition observed in night-pollinated plants was sufficient for fruit production or that the low pollen deposition early in the season in the night-pollinated treatment was compensated by higher deposition later in the season.

There was no significant difference in selection mediated by nocturnal and diurnal pollinators in the present study. This is surprising as I expected the floral display to be more important for attraction of diurnal pollinators that for attraction of nocturnal pollinators. I would expect scent to be a more important guide for attracting nocturnal visitors. Scent has been shown to play an important role in attraction of nocturnal pollinators over both long and short distances (Van der Pijl and Dodson 1966; Dodson et al 1969; Knudsen 2002). The results suggest that the diurnal and nocturnal pollinators mediate similar selection on the floral traits examined in the present study.

Fruit production and fruit size in 2010 partly differed from those recorded in the study population in previous years. The mean fruit mass of control plants and hand-pollinated plants was similar to that observed in 2008 (mean ± SE, control 7.06 ± 1.78, hand-pollinated plants

8.25 ± 1.86), but smaller than that recorded in 2009 (control 9.96 ± 3.10, hand-pollinated plants 11.00 ± 2.40). The mean number of fruits was lower in 2010 both among control plants and hand-pollinated plants compared to 2008 (control 18.3 ± 9.4, hand-pollinated plants 22.0 ± 7.4) and 2009 (control 26.2 ± 10.3, hand-pollinated plants 30.9 ± 10.1). One possible explanation is that the populations experienced stronger pollen limitation as a result of low pollinator activity in 2010. However, pollen limitation was stronger in 2008 and 2009 indicating that factors such as resource availability or herbivory were limiting in 2010.

Since *G. conopsea* is a hermaphroditic, perennial plant, one limitation of this study is that I studied selection only through female fitness and only estimated fitness in one season. Fruit production is the most frequently used measure of female reproductive success (Proctor and Harder 1994), although the survival and reproduction in the next generation might not be proportional to seed production (Harder & Barrett 2006). Correct estimation of male reproductive success is difficult to conduct. Estimating male reproductive success based on pollen removal may often be misleading as it does not consider the proportion of the removed pollen that ends up on receptive stigmas (Hansen & Olesen 1999; Harder & Barrett 2006). In orchids, a high reproductive success in one year can reduce reproductive success in subsequent seasons (Neiland & Wilcock 1998). Therefore, it is important to quantify selection over more than one season (Harder & Barrett 2006).

To summarize, the results of the present study demonstrate that pollinators can mediate selection both on traits involved in pollinator attraction and on traits affecting pollination efficiency, and they suggest that interactions with diurnal and nocturnal pollinators result in similar selection on floral traits in this system.

Acknowledgement

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Tables

Table 1. Trait means ± SD for open-pollinated control plants (C), plants receiving supplemental hand-pollination (HP), plants pollinated by day-active pollinators only (Day), and plants pollinated by night-active pollinators only (Night). P-values associated with the pollination treatment in ANOVA are indicated. P-values < 0.05 are in bold.

Trait	C (N = 63)	HP (N = 76)	Day (N = 75)	Night (N = 75)	P
Plant height (cm)	21.5 ± 3.7	22.1 ± 4.0	22.4 ± 4.2	23.4 ± 4.5	**0.047**
No. flowers	24.1 ± 8.7	23.6 ± 7.0	22.7 ± 6.1	23.6 ± 7.6	0.542
Corolla size (mm2)	83.4 ± 20.1	88.9 ± 23.5	89.1 ± 18.3	93.9 ± 25.5	0.073
Spur length (mm)	10.8 ± 1.7	10.4 ± 1.6	11.1 ± 1.6	11.0 ± 1.5	0.078
Number of fruits	18.5 ± 10.7	21.5 ± 8.7	15.3 ± 8.1	14.8 ± 9.1	**0.000**
Fruit mass (mg)	0.69 ± 0.22	0.78 ± 0.25	0.66 ± 0.23	0.63 ± 0.21	**0.000**
Female fitness	0.14 ± 0.10	0.17 ± 0.01	0.11 ± 0.09	0.10 ± 0.07	**0.000**
Fruit set %	77 ± 3	93 ± 2	72 ± 3	66 ± 3	**0.000**

Table 2. Phenotypic linear selection gradients (± SE) for open-pollinated control plants (C), plants receiving supplemental hand-pollination (HP), plants pollinated by day-active pollinators only (Day), and plants pollinated by night-active pollinators only (Night). Δβpoll is quantifies pollinator-mediated selection (βpoll = βC – βHP). P-values associated with the effect of the trait × pollination treatment interaction in ANCOVA are indicated. P-values < 0.05 are in bold.

Trait	β ± SE				Δβpoll	P	
	C (N = 63)	HP (N = 76)	Day (N = 75)	Night (N = 75)		C vs. HP	Day vs. Night
Plant height	0.122 ± 0.075	0.115 ± 0.043**	0.029 ± 0.068	-0.095 ± 0.081	0.007	0.9350	0.2411
No. flowers	0.581 ± 0.079***	0.406 ± 0.039***	0.498 ± 0.084***	0.515 ± 0.088***	0.175	**0.0368**	0.8898
Corolla size	0.013 ± 0.064	0.010 ± 0.038	0.034 ± 0.053	0.014 ± 0.062	0.003	0.9665	0.8079
Spur length	0.112 ± 0.059§	-0.080 ± 0.033*	0.054 ± 0.051	-0.035 ± 0.060	0.192	**0.0033**	0.2604

§ P = 0.06, * P < 0.05, ** P < 0.01, *** P < 0.001

Table 3. Pollen removal and deposition on 29th of July and 5th of August for open-pollinated control plants (C), plants pollinated by day-active flower visitors only (Day) and plants pollinated by night-active flower visitors only (Night). Number of pollinia removed (PR), number of massulae deposited (MD) and the proportion of pollinia removed and deposited were estimated. P-values associated with the pollination treatment in ANOVA are indicated, P-values < 0.05 are in bold.

Treatment	C	Day	Night	P
29th of July	(N = 28)	(N = 29)	(N = 30)	
#Flowers recorded	17 ± 7	14 ± 6	16 ± 7	0.167
PR	17 ± 14	13 ± 13	10 ± 12	0.13
MD	10 ± 7	8 ± 4	5 ± 6	**0.004**
PropPR	0.48 ± 0.3	0.44 ± 0,3	0.29 ± 0.3	**0.050**
PropMD	0.62 ± 0.3	0.59 ± 0.3	0.34 ± 0.3	**0.000**
5th of August	(N = 65)	(N = 76)	(N = 73)	
#Flowers recorded	9 ± 7	8 ± 6	8 ± 6	0.831
PR	5 ± 5	4 ± 4	5 ± 4	0.266
MD	5 ± 5	4 ± 4	4 ± 4	0.09
PropPR	0.54 ± 0.3	0.50 ± 0.3	0.48 ± 0.3	0.427
PropMD	0.32 ± 0.1	0.29 ± 0.2	0.30 ± 0.2	0.536