

# Separating selection by diurnal and nocturnal pollinators on floral display and spur length in *Gymnadenia conopsea*

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**Abstract.** Most plants attract multiple flower visitors that may vary widely in their effectiveness as pollinators. Floral evolution is expected to reflect interactions with the most important pollinators, but few studies have quantified the contribution of different pollinators to current selection on floral traits. To compare selection mediated by diurnal and nocturnal pollinators on floral display and spur length in the rewarding orchid *Gymnadenia conopsea*, we manipulated the environment by conducting supplemental hand-pollinations and selective pollinator exclusions in two populations in central Norway. In both populations, the exclusion of diurnal pollinators significantly reduced seed production compared to open pollination, whereas the exclusion of nocturnal pollinators did not. There was significant selection on traits expected to influence pollinator attraction and pollination efficiency in both the diurnal and nocturnal pollination treatment. The relative strength of selection among plants exposed to diurnal and nocturnal visitors varied among traits and populations, but the direction of selection was consistent. The results suggest that diurnal pollinators are more important than nocturnal pollinators for seed production in the study populations, but that both categories contribute to selection on floral morphology. The study illustrates how experimental manipulations can link specific categories of pollinators to observed selection on floral traits, and thus improve our understanding of how species interactions shape patterns of selection.

**Key words:** floral evolution; generalized pollination; *Gymnadenia conopsea*; pollen limitation; pollination efficiency; pollinator attraction; pollinator importance; pollinator-mediated selection; spatial variation; temporal variation.

## INTRODUCTION

The evolution of floral traits is believed to be shaped by pollinator-mediated selection to a large extent (Darwin 1862, Fægri and van der Pijl 1979, Fenster et al. 2004). This view is supported by an increasing number of studies connecting floral diversity and pollination mode in a phylogenetic context (e.g., Graham and Barrett 2004, Kay et al. 2005, Wilson et al. 2006, Whittall and Hodges 2007), and experimentally demonstrating that pollinators mediate selection on floral traits (Galen 1996, Fishman and Willis 2008, Sandring and Ågren 2009, Caruso et al. 2010, Parachnowitsch and Kessler 2010, Sletvold and Ågren 2010, Sletvold et al. 2010). In highly specialized plant–pollinator interactions, it may be straightforward to infer the agents of selection on floral design (Peakall and Handel 1993, Muchhala and Thomson 2009). However, community surveys frequently demonstrate that the

majority of plants are visited by multiple potential pollinators (Waser et al. 1996, Armbruster et al. 2000, Ollerton et al. 2006), and their relative importance as selective agents is not necessarily obvious.

The importance of a given pollinator as selective agent on floral traits should depend both on its relative contribution to plant reproductive success through pollen transfer, and on the way its visitation rate and pollination efficiency varies with trait expression. The relative contribution to plant reproductive success may vary widely among pollinators (Schemske and Horvitz 1984, Herrera 1987, Wilson and Thomson 1991), and should be proportional to the product of visitation rate and pollen transfer efficiency per visit (Stebbins 1970). Pollinator importance estimates based on mean visitation rate and efficiency have been used to infer likely selective agents on floral traits (reviewed in Reynolds and Fenster 2008), but such estimates ignore potential differences among pollinators in how visitation rate and pollen transfer efficiency vary with plant trait expression. Instead, the importance as selective agent should depend both on the relative contribution to plant reproductive success and the strength and shape of selection resulting from a particular interaction. Only those pollinators whose visitation rate and/or efficiency of pollen transfer vary with trait expression will

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contribute to selection on floral traits, and the pollinator responsible for most pollen transfers need not be the most important selective agent (see also Aigner [2001] for a discussion of effects of interactions between pollinators). Pollinators may differ in the traits to which they respond (Schemske and Bradshaw 1999, Lunau et al. 2011), or favor different trait values (Medel et al. 2003, Aigner 2004, Muchhala 2007), and correlative approaches have been used to assess the relative importance of different pollinators as selective agents (e.g., Gómez et al. 2009). However, few studies have experimentally separated the contribution from different pollinators to selection on floral traits, and our current understanding of the correspondence between pollinator importance in terms of reproductive success and patterns of selection is limited.

The composition of the pollinator assembly may vary both spatially (Gómez et al. 2010) and temporally (Brunet 2009), which may result in associated variation in plant reproductive success and patterns of selection on floral traits. A particular kind of temporal variation in pollination is experienced by plants that expose their flowers to both diurnal and nocturnal visitors. In several systems, diurnal and nocturnal pollinators differ in pollination efficiency (Jennersten and Morse 1991, Young 2002) and relative importance for plant reproductive success (Fleming et al. 2001, Reynolds et al. 2009, Pérez-Barrales and Arroyo 2010) and gene flow (Barthelmess et al. 2006). Selection on floral traits mediated by diurnal and nocturnal pollinators is also likely to differ. In general, visual display traits should be more important for the attraction of diurnal visitors than for the attraction of nocturnal visitors, whereas the importance of traits affecting functional fit should not differ in a consistent manner between the two categories of visitors. For example, experimental manipulation of floral traits in the diurnally bee-pollinated orchid *Dactylorhiza lapponica* showed that both display size and spur length affected pollination success and female fitness (Sletvold and Ågren 2011a), whereas in the nocturnally hawk moth-pollinated orchid *Platanthera bifolia*, spur length but not corolla size influenced pollination success (Boberg and Ågren 2009). However, some studies have shown that visual cues affect nocturnal hawk moth behavior (Goyret et al. 2007), indicating that visual display can be important also in nocturnally pollinated plants.

Selection on floral traits can also result from abiotic factors (Totland 2001, Elle 2004) or interactions with antagonists (Gómez 2003, Sletvold and Grindeland 2008, Toräng et al. 2008, Kolb and Ehrlén 2010, Wise and Hébert 2010), and experimental manipulation of the pollination environment is therefore necessary to determine the importance of pollinators as agents of selection. A straightforward way to quantify pollinator-mediated selection is to compare selection gradients estimated for plants that are saturated with pollen experimentally (representing non-pollinator selection),

with those estimated for naturally pollinated plants (representing selection by all agents, including pollinators; Galen 1996, Fishman and Willis 2008, Sandring and Ågren 2009, Sletvold and Ågren 2010). For a given trait, the difference in selection gradient estimates the strength and direction of pollinator-mediated selection ( $\Delta\beta_{\text{poll}} = \beta_{\text{C}} - \beta_{\text{HP}}$ ; Sletvold and Ågren 2010, Sletvold et al. 2010). This approach pools the contribution of all pollinators, and to separate effects of specific groups, selective exclusion experiments are needed.

In the present study, we combine supplemental hand-pollinations with pollinator exclusions to examine the relative importance of diurnal and nocturnal pollinators for female fitness and pollinator-mediated selection on floral traits in two Norwegian populations of the rewarding orchid *Gymnadenia conopsea*. This species exhibits a semi-generalized pollination system, i.e., it receives visits from a large number of species (40 species listed in Claessen and Kleynen [2011]), but most of them are lepidopteran, and can be considered to belong to the same functional group (sensu Fenster et al. 2004). Both diurnal and nocturnal visitors are common (Darwin 1862, Huber et al. 2005, Sletvold and Ågren 2010). Variation in the strength of pollinator-mediated selection can explain much of the spatiotemporal variation in selection in the study populations (Sletvold and Ågren 2010), and here we examine whether this reflects variation in the importance of diurnal vs. nocturnal pollinators, as a first step toward linking specific pollinators to observed selection in this system. We specifically ask whether (1) the relative contribution of nocturnal and diurnal pollinators to seed production differs between populations, (2) diurnal pollinators mediate stronger selection on traits influencing floral display (plant height, number of flowers, flower size) than nocturnal pollinators do, (3) diurnal and nocturnal pollinators mediate similar selection on traits expected to affect pollination efficiency (spur length).

## MATERIALS AND METHODS

### *Study species and field sites*

*Gymnadenia conopsea* (L.) R. Br. s.l. is a terrestrial orchid, distributed across Eurasia (Hultén and Fries 1986). It occurs on calcareous soils in grazed meadows and margins of marshes and fens. The species is a tuberous, non-clonal, and long-lived perennial (Øien and Moen 2002). At the study sites, individuals emerge aboveground in late May to early June, and flowering individuals produce a single inflorescence with ~10–70 flowers 3–4 weeks later. The fragrant flowers vary in color from pale to bright pink, and rarely pure white. Flowers open sequentially from the bottom to the top. Individual flowers remain open for up to a week, while individual plants may flower for a month. A long, narrow spur contains nectar that is produced throughout anthesis (Stpiczynska and Matusiewicz 2001). Each flower contains two pollinaria, which are situated above the spur entrance. Plants are self-compatible, but depend

on pollinators for successful fruit set (N. Sletvold, unpublished data).

The study populations are located within two nature reserves in central Norway, Sølendet (62°40' N, 11°50' E) and Tågdalen (63°03' N, 9°05' E), separated by 145 km. The nature reserves are situated at the transition between the middle and north boreal vegetation zones (Moen 1999). The coastal Tågdalen population (450 m a.s.l., above sea level) has an oceanic climate (annual precipitation 1507 mm), while the inland Sølendet population (770 m a.s.l.) has a more continental climate (annual precipitation 670 mm). The number of flowering *G. conopsea* individuals varies among years from ~200 to 600 individuals in the Tågdalen population, and from 500 to 1000 individuals in the Sølendet population. The studied populations are found in open, wet grasslands, dominated by *Molinia caerulea* (L.) Moench, *Succisa pratensis* Moench, and *Thalictrum alpinum* L. The *G. conopsea* populations begin to flower in late June to early July, and flower for 3–4 weeks. In the Tågdalen population, the abundant hawk moth *Hemaris tityus* L. (Sphingidae) is the main diurnal pollinator of *G. conopsea* (Fig. 1a; >90% of observed diurnal visits), with occasional visits from butterflies of the genus *Boloria* Moore (Nymphalidae) (Fig. 1b) and *Aglais urticae* L. (Nymphalidae). Nocturnal pollinators observed include the hawk moth *Hyles gallii* Rott. (Sphingidae) and the noctuid *Autographa pulchrina* Haworth (Noctuidae). At Sølendet, the common fly *Empis tessellata* F. (Empididae) is the most frequent diurnal visitor (Fig. 1c; >50% of observed diurnal visits), and visits from butterflies of the genus *Boloria* Moore and *Pyrgus centaurea* Hübner (Hesperiidae) (Fig. 1d) have been observed. The few records of nocturnal activity are limited to the hawk moth *Hyles gallii* Rott and the noctuid *Papestra biren* Goeze (Noctuidae). The spur of *G. conopsea* is markedly shorter than the proboscises of all lepidopteran visitors, but considerably longer than the proboscis of the empidid fly (Fig. 1). High levels of fruit set are common in the study populations (60–80%), but fruit production and fruit mass are significantly pollen limited (Sletvold and Ågren 2010).

#### Field experiment

To quantify pollinator-mediated selection on floral display and spur length, and to separate the effects of diurnal and nocturnal pollinators, we experimentally manipulated the pollination environment in both study populations in 2010. In late June, a total of 480 plants with flower buds were haphazardly chosen and individually tagged in each of the two populations. We randomly assigned 120 plants to each of four treatments: supplemental hand-pollination (HP), natural pollination (C, control), only diurnal pollination (D), and only nocturnal pollination (N). Plants in the HP and C treatments were exposed to all visitors throughout the flowering period, while plants in the D treatment were caged during night (18:00–06:00 h), receiving only

diurnal visits, and plants in the N treatment were caged during day (06:00–18:00), receiving only nocturnal visits. Supplemental hand-pollinations were conducted as flowers opened, and all flowers were pollinated at least twice with a minimum of two pollen donors situated >5 m from the recipient plant. Cocktail sticks were used to collect pollinia, and pollinations were performed by rubbing one to two pollinia across each stigma, saturating the surface with pollen. Pollinia were primarily collected from other plants in the hand-pollination treatment, but also from individuals not included in the study. During its flowering period, each hand-pollinated plant received pollen from multiple donors.

In the Sølendet population, caging treatments continued until all flowers had wilted (14 August), while in the Tågdalen population, a few plants had open flowers when we terminated the experiment on 8 August (on average 1.5 flowers in 31 individuals, no difference between the D and N treatment, one-way ANOVA;  $F_{1,29} = 2.62$ ,  $P = 0.12$ ). At termination, we recorded pollinia removal and pollen deposition for all flowers that remained open. None of the flowers that were virgin at the termination of the experiment produced a fruit, indicating that no pollination occurred after we ended the caging treatments.

#### Measured traits

At the onset of flowering, we recorded the height of all plants included in the experiment (distance from ground to topmost flower). On one of the three lowermost flowers on each individual we measured spur length (distance from corolla to spur tip) and maximum corolla width and height to the nearest 0.1 mm with digital calipers. Corolla size was quantified as the product of width and height. The number of flowers was recorded at the end of the flowering period. To quantify female reproductive success, we recorded the number of fruits and collected three non-dehiscent capsules from each plant at maturation. Fruits were dried at room temperature for a month, and their dry mass was then determined to the nearest 0.01 mg. Fruit mass is positively related to number of seeds with embryos in *G. conopsea* (Sletvold and Ågren 2010). For each plant we estimated female fitness as the product of number of fruits and mean fruit mass. In both populations, we quantified pollen limitation as  $1 - (\text{mean female fitness of C plants} / \text{mean female fitness of HP plants})$ .

In the Sølendet population, 220 experimental plants were lost due to herbivory by larvae of the noctuid *Cerastis rubricosa* Denis and Schiffermüller (Noctuidae) during flowering (probability of herbivory did not vary among treatments, generalized linear model, GLM;  $\chi^2 = 3.28$ ,  $df = 3$ , 479,  $P = 0.35$ ).

#### Caging effects

In a separate experiment in the Sølendet population, we assessed whether caging per se affected female



FIG. 1. Diurnal insect visitors of the rewarding orchid *Gymnadenia conopsea* in central Norway: (a) *Hemaris tityus*, (b) *Boloria* sp., (c) *Empis tessellata*, and (d) *Pyrgus centaurea*. Photos by N. Sletvold.

reproductive success (e.g., through changing light and temperature conditions). We compared the female fitness of hand-pollinated plants with that of plants that were hand-pollinated but also caged either during the night or during the day. We randomly assigned 105 plants to three treatments with equal sample size: supplemental hand-pollination without caging (identical to the HP treatment in the main experiment), hand-pollination during daytime (06:00–18:00 h) combined with caging during night (18:00–06:00), and hand-pollination at nighttime (18:00–06:00) combined with caging during day (06:00–18:00). Hand-pollinations were conducted as in the main experiment. We recorded the total number of flowers at the end of the flowering period, and the number of fruits produced at fruit maturation. From each plant, we collected three fruits

and determined fruit mass, and estimated female fitness as the product of number of fruits and mean fruit mass.

#### *Statistical analyses*

We used one-way ANOVA to examine the effect of the caging treatment on female reproductive success, comparing the three hand-pollination treatments in the second experiment (open, caged during night, caged during day).

The effects of pollination treatment and population on plant traits and plant performance in the main experiment were examined with two-way ANOVA. We used planned contrasts to examine differences in fruit production, fruit mass, and female fitness between pollination treatments. First, we tested for pollen-limitation by comparing the HP and C treatments. Second, we tested for differences in female reproductive

TABLE 1. Trait means  $\pm$  SD for plants receiving supplemental hand-pollination (HP), open-pollinated control plants (C), plants with diurnal pollination (D), and plants with nocturnal pollination (N) in the *Gymnadenia conopsea* populations at Tågdalen and Sølendet, Norway in 2010.

Trait, by site	HP <i>n</i> = 108/68	C <i>n</i> = 118/61	D <i>n</i> = 117/73	N <i>n</i> = 117/70	Population	
					<i>F</i> <sub>1,728</sub>	<i>P</i>
Plant height (cm)					103	<b>&lt;0.0001</b>
Tågdalen	26.2 $\pm$ 4.0	25.4 $\pm$ 4.8	26.5 $\pm$ 4.5	25.8 $\pm$ 4.3		
Sølendet	22.5 $\pm$ 4.4	21.6 $\pm$ 3.8	22.6 $\pm$ 4.3	23.6 $\pm$ 4.7		
No. flowers					56.0	<b>&lt;0.0001</b>
Tågdalen	29.3 $\pm$ 7.7	28.8 $\pm$ 8.5	29.4 $\pm$ 9.1	28.4 $\pm$ 8.4		
Sølendet	24.7 $\pm$ 7.6	24.6 $\pm$ 9.8	23.4 $\pm$ 8.5	23.8 $\pm$ 7.8		
Corolla size (mm <sup>2</sup> )					0.98	0.32
Tågdalen	92.6 $\pm$ 15.6	92.4 $\pm$ 21.5	92.3 $\pm$ 17.3	92.6 $\pm$ 16.5		
Sølendet	90.0 $\pm$ 25.2	85.8 $\pm$ 21.4	91.5 $\pm$ 22.3	96.8 $\pm$ 26.6		
Spur length (mm)					112	<b>&lt;0.0001</b>
Tågdalen	12.3 $\pm$ 1.7	12.1 $\pm$ 2.0	12.3 $\pm$ 1.7	12.2 $\pm$ 1.5		
Sølendet	10.4 $\pm$ 1.4	10.8 $\pm$ 1.7	11.1 $\pm$ 1.5	11.1 $\pm$ 1.5		
No. fruits					95.4	<b>&lt;0.0001</b>
Tågdalen	28.7 $\pm$ 7.6	25.4 $\pm$ 9.8	25.3 $\pm$ 10.1	23.6 $\pm$ 9.4		
Sølendet	22.3 $\pm$ 9.0	19.5 $\pm$ 10.6	17.0 $\pm$ 9.7	15.7 $\pm$ 9.7		
Fruit mass (mg)					90.5	<b>&lt;0.0001</b>
Tågdalen	10.5 $\pm$ 2.36	9.49 $\pm$ 3.08	9.17 $\pm$ 2.87	7.02 $\pm$ 2.74		
Sølendet	8.25 $\pm$ 2.58	6.98 $\pm$ 2.08	6.70 $\pm$ 2.23	6.62 $\pm$ 1.96		
Female fitness					127.6	<b>&lt;0.0001</b>
Tågdalen	303 $\pm$ 111	253 $\pm$ 143	248 $\pm$ 148	178 $\pm$ 125		
Sølendet	197 $\pm$ 137	147 $\pm$ 106	127 $\pm$ 114	112 $\pm$ 88		

Notes: *F* and *P* values associated with the effects of population, pollination treatment, and their interaction in two-way ANOVA are shown; *P* < 0.05 is in bold. The first *n* is for Tågdalen and the second *n* is for Sølendet.

success between plants exposed to diurnal and nocturnal pollinators, respectively (D vs. N treatment). Finally, we compared both the D and N treatment with the C treatment, to test whether removal of one pollinator type reduced plant reproductive success. In cases with a significant population  $\times$  treatment interaction, contrasts were calculated separately for each population.

Selection was estimated following Lande and Arnold (1983), using multiple regression analyses with relative fitness (individual fitness divided by mean fitness) as the response variable and standardized trait values (with a mean of 0 and a variance of 1) as explanatory variables. Fitness was quantified as number of fruits  $\times$  mean fruit mass, and relative fitness and standardized trait values were calculated separately for each treatment and population. In the Tågdalen population, we initially included quadratic ( $\gamma_{ii}$ ) and cross-product ( $\gamma_{ij}$ ) terms in regression models to quantify nonlinear and correlational selection, but none of the quadratic or correlational selection gradients were statistically significant. In the Sølendet population, reduced sample size following herbivory prevented the inclusion of nonlinear terms in the model. We therefore chose to analyze an identical model including only linear gradients for both populations.

We used ANCOVA to determine whether selection differed between pollination treatments and populations, comparing the C vs. HP treatment in one analysis,

and the D vs. N treatment in a second analysis. The model included relative fitness as the dependent variable and the four standardized traits (plant height, number of flowers, corolla size, and spur length), pollination treatment (C vs. HP, or D vs. N), population, and trait  $\times$  pollination treatment, trait  $\times$  population, and trait  $\times$  pollination treatment  $\times$  population interactions as independent variables. Because statistically significant three-way interactions were detected when comparing the D and N treatments, we further tested the effect of pollination treatment on selection gradient estimates separately for each population (contrasting C vs. HP and D vs. N). To quantify pollinator-mediated selection, we subtracted for each trait the estimated selection gradient for plants receiving supplemental hand-pollination ( $\beta_{HP}$ ) from the estimate obtained for open-pollinated controls ( $\beta_C$ ),  $\Delta\beta_{poll} = \beta_C - \beta_{HP}$  (cf. Sletvold and Ågren 2010).

To examine how herbivory influenced patterns of selection in the Sølendet population, we first used logistic regression to determine whether the probability of herbivory was related to floral traits. The probability of herbivory increased with number of flowers ( $\chi^2 = 4.95$ , *df* = 1, 479, *P* = 0.026) and tended to increase with plant height ( $\chi^2 = 2.84$ , *df* = 1, 479, *P* = 0.092), but was not significantly related to flower size ( $\chi^2 = 1.93$ , *df* = 1, 479, *P* = 0.16) or spur length ( $\chi^2 = 1.44$ , *df* = 1, 479, *P* = 0.23). Second, we compared selection models including

TABLE 1. Extended.

Pollination		Population × pollination	
$F_{3,728}$	$P$	$F_{3,728}$	$P$
2.30	0.076	1.38	0.25
0.31	0.82	0.39	0.76
2.14	0.093	2.01	0.11
1.63	0.18	1.96	0.12
11.6	<b>&lt;0.0001</b>	0.67	0.57
27.5	<b>&lt;0.0001</b>	6.45	<b>0.0003</b>
25.4	<b>&lt;0.0001</b>	1.18	0.32

and excluding plants subject to herbivory. Because the two selection models yielded similar results, we report only results from the latter. All analyses were conducted with SAS 9.2 (SAS Institute, Cary, North Carolina, USA).

## RESULTS

### *Effects of caging*

Fruit production ( $F_{2,102} = 0.16$ ,  $P = 0.86$ ), fruit mass ( $F_{2,102} = 0.17$ ,  $P = 0.84$ ), and female fitness ( $F_{2,102} = 0.01$ ,  $P = 0.99$ ) did not differ significantly among hand-pollinated plants that were caged during day, night, or not at all, indicating that the caging treatment per se did not affect female reproductive success.

### *Floral traits*

Plant height, number of flowers, and spur length varied among populations, but not among pollination treatments (Table 1). Plants in the Tågdalen population were taller and produced more flowers with longer spurs compared to plants in the Sølendet population. Floral traits tended to be moderately positively correlated in both populations (Appendix: Table A1).

### *Pollen limitation*

Fruit production and fruit mass were pollen limited in both populations. Plants produced more and larger fruits in the Tågdalen population than in the Sølendet population (Table 1, Fig. 2a–d). Supplemental hand-pollination increased the number of fruits by 13% at

Tågdalen and by 14% at Sølendet, and the contrast between the HP and C treatment was statistically significant ( $P = 0.0039$ ; Fig. 2a, b). The overall effect of pollination treatment on fruit mass differed between populations (significant population × treatment interaction; Table 1), but the effect of hand-pollination was similar (Fig. 2c, d). Fruit mass increased by 11% at Tågdalen and by 18% at Sølendet, and the increase was significant in both populations (Tågdalen  $P = 0.0085$ , Sølendet  $P = 0.00045$ ). The female fitness of hand-pollinated plants was significantly higher than that of open-pollinated controls ( $P < 0.0001$ ; Fig. 2e, f). Pollen limitation (1 – mean female fitness of C plants divided by mean female fitness of HP plants) was 0.16 in the Tågdalen population and 0.25 in the Sølendet population, and did not differ significantly between populations (no significant population × pollination treatment interaction in analysis of female fitness; Table 1).

### *Female reproductive success in the diurnal and nocturnal pollination treatments*

Female reproductive success was higher among plants exposed to diurnal visitors than among plants exposed to nocturnal visitors (D vs. N). In both populations, plants exposed to nocturnal pollinators produced fewer fruits than plants exposed to pollinators during both day and night (contrast C vs. N,  $P = 0.0075$ ), while fruit production in plants with diurnal pollination was intermediate, and did not differ significantly from either group (C vs. D,  $P = 0.23$ ; D vs. N,  $P = 0.12$ ; Fig. 2a, b). The effect of diurnal vs. nocturnal pollinator exclusion on fruit mass differed between populations (significant population × treatment interaction; Table 1). At Tågdalen, nocturnally pollinated plants produced significantly smaller fruits than diurnally or open-pollinated plants ( $P < 0.0001$  for both contrasts; Fig. 2c), whereas at Sølendet, no significant differences in fruit mass were detected between the N, D, and C treatments (all  $P > 0.28$ ; Fig. 2d). In both populations, female fitness of plants with nocturnal pollination was significantly lower than that of plants with diurnal pollination ( $P = 0.0010$ ), whereas the difference between diurnal and open pollination was not statistically significant ( $P = 0.23$ ; Fig. 2e, f).

### *Pollinator-mediated selection*

We detected significant directional selection for taller plants, more flowers and longer spurs in both populations, and pollinators contributed to selection on both plant height and spur length. In the Tågdalen population, linear selection gradients for spur length differed significantly between the control and supplemental hand-pollination treatment (significant trait × pollination treatment interaction in ANCOVA;  $F_{9,216} = 24.4$ ,  $P < 0.0001$ ). The strength of pollinator-mediated selection ( $\Delta\beta_{\text{poll}}$ ) was 0.18, and all selection observed on spur length among open-pollinated control plants was due to interactions with pollinators (Fig. 3a; Appendix: Fig.

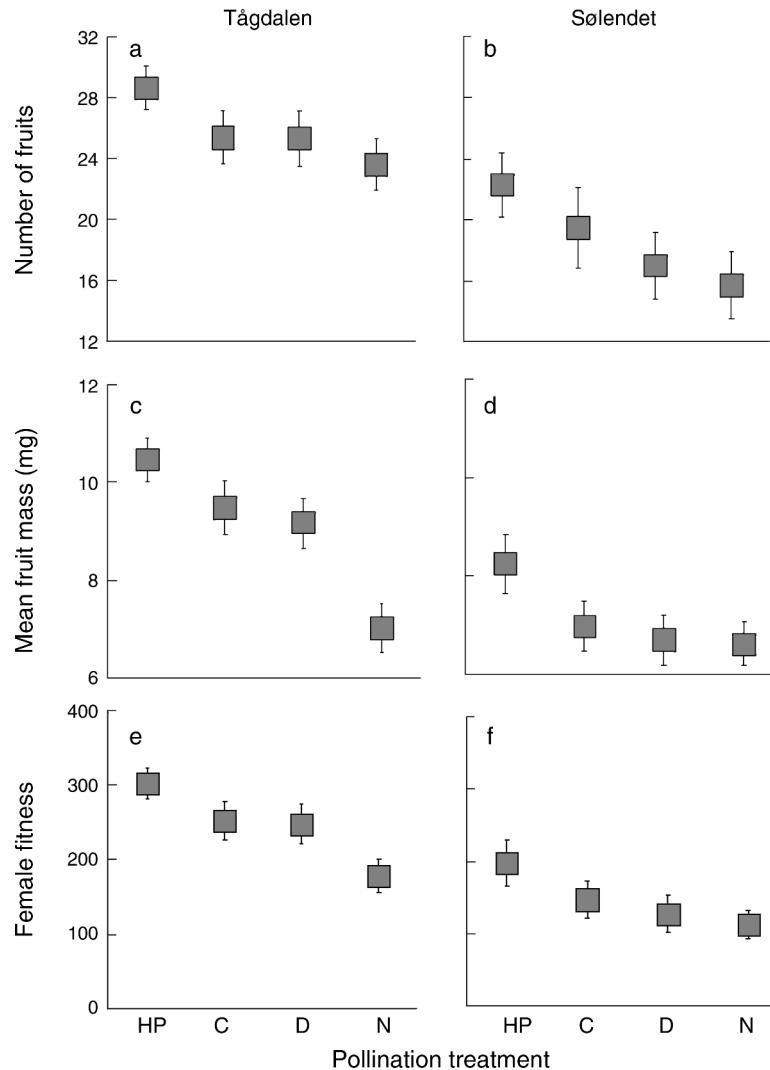


FIG. 2. The effect of pollination treatment on number of fruits, mean fruit mass, and the combined estimate of female fitness (number of fruits  $\times$  fruit mass) in the *Gymnadenia conopsea* populations at (a, c, e) Tågdalen and (b, d, f) Sølendet, Norway. Symbols are means per individual  $\pm$  1.96 SE. Treatments are: HP, hand-pollination; C, control, open pollination; D, diurnal pollination; N, nocturnal pollination.

A1a). Pollinators also accounted for 77% of the observed selection on plant height ( $\Delta\beta_{\text{poll}} = 0.10$ ;  $F_{9,216} = 5.82$ ,  $P = 0.017$ ; Fig. 3a; Appendix: Fig. A2). The selection documented on number of flowers could not be attributed to pollinators; estimates of selection gradients were identical in both pollination treatments ( $F_{9,224} = 0.02$ ,  $P = 0.89$ ; Fig. 3a).

In the Sølendet population, interactions with pollinators contributed to selection on spur length. Linear selection gradients for spur length were significantly stronger among open-pollinated control plants compared to hand-pollinated plants ( $F_{9,126} = 7.86$ ,  $P = 0.0059$ ), and all selection was mediated by pollinators ( $\Delta\beta_{\text{poll}} = 0.21$ ; Fig. 3b; Appendix: Fig. A1b). We also documented significant selection on plant height and

number of flowers, but selection gradients for these two traits were not affected by supplemental hand-pollination (both  $P > 0.90$ ; Fig. 3b). Selection for more flowers was stronger at Sølendet than at Tågdalen (significant trait  $\times$  population interaction in ANCOVA,  $P < 0.0001$ ), but the contribution from pollinators to selection did not differ significantly between the two populations for any trait (no significant trait  $\times$  pollination treatment  $\times$  population interaction in ANCOVA, all  $P > 0.13$ ).

#### Selection in the diurnal and nocturnal pollination treatments

There was significant selection on both floral display and spur length among plants exposed exclusively to

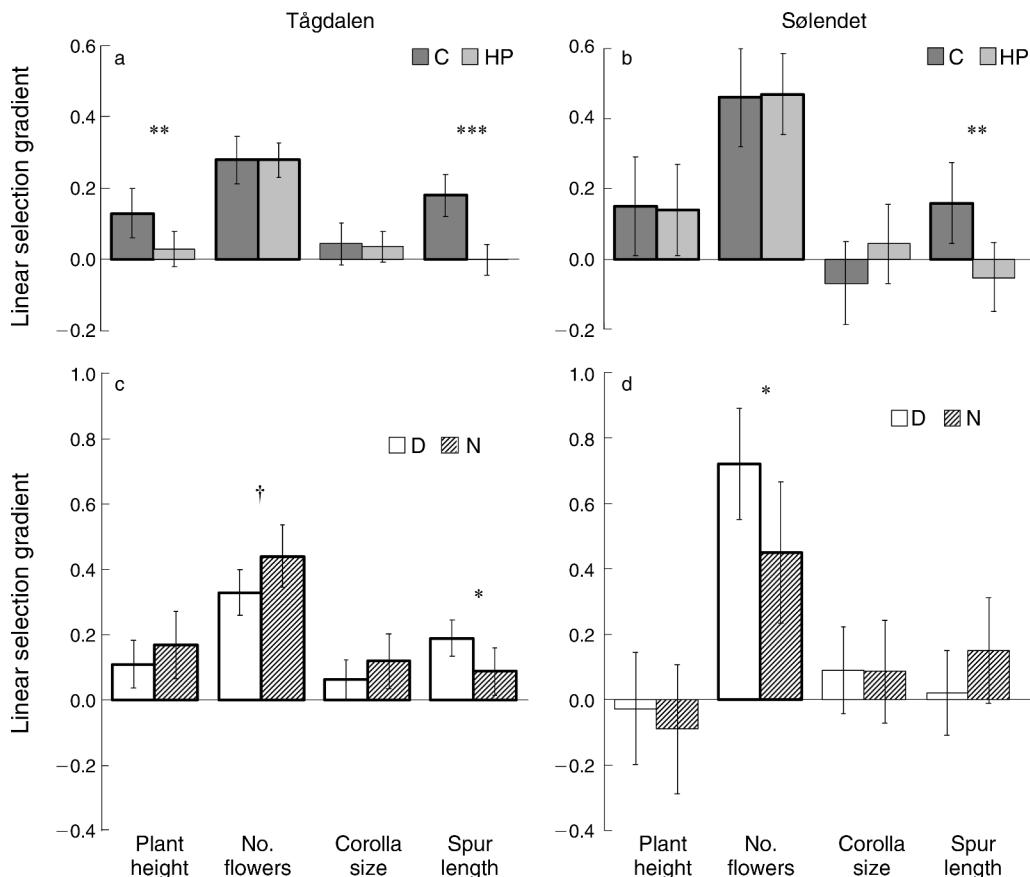


FIG. 3. Phenotypic linear selection gradients estimated with multiple regression ( $\pm 1.96$  SE) for plant height, number of flowers, corolla size, and spur length among plants (a, b) receiving supplemental hand-pollination (HP) and open-pollinated control plants (C), and plants (c, d) exposed exclusively to diurnal pollinators (D) or nocturnal pollinators (N) in the (a, c) Tågdaalen and (b, d) Sølendet populations of *Gymnadenia conopsea*. Histogram bars drawn with a bold line indicate significant gradients at the  $P < 0.05$  level. Traits for which selection gradients differed between pollination treatments are indicated with asterisks above bars (significant trait  $\times$  pollination treatment in ANCOVA).

\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; †  $P = 0.066$ .

either diurnal or nocturnal pollinators, and the direction of selection was consistent in the two treatments. In the Tågdaalen population, there was significant selection on all floral traits in both the diurnal and nocturnal pollination treatment (Fig. 3c). Selection on spur length was significantly stronger among diurnally pollinated plants compared to nocturnally pollinated plants ( $\beta_D = 0.19$  vs.  $\beta_N = 0.088$ ;  $F_{9,224} = 4.69$ ,  $P = 0.031$ ; Appendix: Fig. A1c). In contrast, selection gradients for number of flowers tended to be stronger among plants exposed to nocturnal pollinators compared to those exposed to diurnal pollinators ( $\beta_D = 0.33$  vs.  $\beta_N = 0.44$ ;  $F_{9,224} = 3.42$ ,  $P = 0.066$ ). Selection gradients for plant height and corolla size did not differ between the diurnal and nocturnal pollination treatment (both  $P > 0.30$ ).

In the Sølendet population, there was selection for more flowers in both the diurnal and nocturnal pollination treatment, and selection for longer spurs was close to significant in the nocturnal treatment ( $P = 0.077$ ; Fig. 3d; Appendix: Fig. A1d). Selection on

number of flowers was significantly stronger among plants visited by diurnal pollinators compared to those visited by nocturnal pollinators ( $\beta_D = 0.72$  vs.  $\beta_N = 0.45$ ;  $F_{9,133} = 3.94$ ,  $P = 0.049$ ). The relative strength of selection in the diurnal vs. nocturnal pollination treatment differed between the two populations for both spur length and number of flowers (significant trait  $\times$  pollination treatment  $\times$  population interactions in ANCOVA; spur length  $F_{19,357} = 4.80$ ,  $P = 0.029$ ; number of flowers  $F_{19,357} = 8.54$ ,  $P = 0.0037$ ; Fig. 3c, d).

#### DISCUSSION

The rewarding orchid *Gymnadenia conopsea* exhibits floral traits indicating generalization toward both diurnal and nocturnal pollination; it has a conspicuous floral display and emits scent both during the day and at night. We documented selection on floral display and spur length among plants exposed exclusively to either diurnal or nocturnal visitors, suggesting that both categories of flower visitors contribute to selection on

floral morphology in this system. The strength of selection from diurnal and nocturnal pollinators varied among traits and populations, but the direction was consistent. The results indicate that fluctuations in relative abundance of the two categories of pollinators is unlikely to cause major shifts in the pattern of selection on floral morphology.

Diurnal and nocturnal pollinators have been found to differ in their contribution to plant reproductive success in many systems. Commonly, this reflects a qualitative shift in pollinator type, from bats or hawk moths at night, to birds or bees at daytime (Fleming et al. 2001, Wolff et al. 2002, Muchhala 2003, Reynolds et al. 2009). The present results document that variation in relative abundance and efficiency of pollinators belonging to similar functional groups also can affect reproductive success. In both study populations, seed production of plants exposed only to diurnal visitors was higher than that of plants exposed only to nocturnal visitors (87–98% vs. 70–76% relative to that of open-pollinated controls). This suggests that the visitation rate by nocturnal pollinators is lower or that they deposit fewer pollen per visit than diurnal pollinators do. In several systems, per-visit efficiency of nocturnal pollinators is higher than that of diurnal pollinators, but because diurnal insects are more abundant, they make an equal or larger overall contribution to seed production (Jennersten and Morse 1991, Wolff et al. 2002, Young 2002). At present, we lack information on per-visit efficiency for the different pollinators of *G. conopsea*, but observations in both study populations support a higher visitation rate from diurnal pollinators (N. Sletvold, unpublished data). Our results contrast with findings in more southern *G. conopsea* populations, where nocturnal visitors seem to be more abundant or efficient than diurnal visitors. In Germany, the exclusion of nocturnal pollinators reduced fruit and seed set compared to open-pollinated plants, whereas the exclusion of diurnal pollinators had no effect (Meyer et al. 2007), and in southern Sweden, pollinia removal and deposition were considerably higher during night than during day (S. D. Johnson, S.-L. Steenhuisen, C. Fortunel, and J. Ågren, unpublished data). This could reflect a decreasing relative contribution from nocturnal pollinators with increasing latitude, as low nocturnal temperatures may limit insect activity, and light conditions during night and day become increasingly similar. The daily onset of flight activity in nocturnal moths often coincides with the termination of twilight (Dreisig 1980), and it has been shown that illumination in the subarctic is continuously at levels that normally inhibit the activity of moths (Dreisig 1981). Experimental manipulations of the pollination environment along a latitudinal gradient could reveal whether the relative contribution from diurnal and nocturnal visitors varies with latitude, or in a more mosaic pattern in response to variation in the composition of the pollinator community within different geographic regions.

The overall pattern of selection on floral display was similar among plants exposed to diurnal and nocturnal pollinators. We expected stronger selection on floral display among plants exposed only to diurnal visitors, but both categories of pollinators selected for taller plants with more flowers, suggesting that they respond similarly to visual cues. Visual signals may intuitively be expected to be more important for the attraction of diurnal visitors, but nocturnal moths can discriminate among colors at very low light intensities (Kelber et al. 2003), and experiments with artificial flowers have shown that hawk moths can exhibit stronger innate preferences for visual targets than scent signals (Goyret et al. 2007). In the Tågdalen population, the relationships between floral display traits and female fitness actually tended to be stronger in the nocturnal treatment than in the diurnal treatment (Fig. 3c). One explanation could be that the degree of pollen limitation was stronger in the nocturnal treatment, causing more intense competition for pollination. However, in the Sølendet population, selection on flower production was stronger in the diurnal treatment, despite weaker pollen limitation. It is likely that such reversals in the relative importance of the two pollinator groups reflect among-site variation in species composition of the diurnal and nocturnal community.

The strength of selection on spur length was similar in the two *G. conopsea* populations, and as previously documented, all selection on spur length was mediated by pollinators (Sletvold and Ågren 2010). The consistent selection for longer spurs probably reflects improved pollination efficiency in long-spurred plants, as experimentally shown in several systems (cf. Nilsson 1988, Johnson and Steiner 1997, Boberg and Ågren 2009, Ellis and Johnson 2010, Sletvold and Ågren 2011a). Both diurnal and nocturnal visitors mediated selection on spur length, but the results suggest that the relative importance of the two pollinator categories for selection on spur length may differ between populations. Selection was stronger among plants with diurnal pollination in the Tågdalen population, and tended to be stronger among plants with nocturnal pollination in the Sølendet population. This may reflect between-population differences in the proboscis length of the two pollinator categories. In the Sølendet population, the most frequently observed diurnal visitor is the empidid fly *Empis tessellata* (Fig. 1c). Because the proboscis of *E. tessellata* is markedly shorter than the spur of *G. conopsea*, *E. tessellata* is not expected to exert selection for longer nectar spurs. By contrast, nocturnal pollinators at Sølendet and both diurnal and nocturnal pollinators at Tågdalen are lepidopteran species with a markedly longer proboscis than the spur of *G. conopsea*. In these species, pollinaria attach along the length of the proboscis, suggesting that pollinaria from long-spurred plants may fail to contact the stigma in short-spurred plants, which should reduce female reproductive success (Sletvold and Ågren 2010). Moreover, a positive

correlation between spur length and nectar volume may make long-spurred plants more attractive to flower visitors (Sletvold and Ågren 2010). Selection for longer spurs mediated by the lepidopteran pollinators of *G. conopsea* may thus reflect effects of spur length on efficiency of pollination as well as on rates of visitation.

Pollinator-mediated selection may be due to trait-induced variation in both the quantity and quality of pollen deposited, and the experimental design of the present study does not distinguish between these possibilities. Plants receiving supplemental hand-pollination received more pollen, but may also have received pollen of higher quality. Specifically, open-pollinated plants may have received a mixture of self- and cross-pollen, while supplemental hand-pollinations were conducted using cross-pollen from plants located at least 5 m from the focal plant, which should have reduced the risk of mating between closely related individuals. *Gymnadenia conopsea* is self-compatible, but pollination with self-pollen results in fruits that are ~35% smaller than fruits produced after cross pollination, and staining of pollinia indicate that a substantial proportion of natural pollinations in the Sølendet population represents geitonogamous self-pollination (Sletvold et al. 2012). Additional studies are required to determine the extent to which pollinator-mediated selection on floral traits in *G. conopsea* is due to trait-related variation in the quantity and quality of pollen received, respectively.

Our approach to estimate selection shares a problem with other experiments studying natural phenotypic variation. Without manipulating trait expression, we cannot distinguish the effects of focal traits from those of potentially correlated traits not included in the analyses (Mitchell-Olds and Shaw 1987, Wade and Kalisz 1990). For example, if scent production is strongly correlated with display size, differences in selection exerted by diurnal and nocturnal pollinators may reflect variation in response to scent as well as visual display. Experimental manipulation will be required to determine conclusively the relative importance of scent and floral display for pollination success in this system (cf. Sletvold and Ågren 2011a).

In the present experiments, we used diurnal and nocturnal caging treatments of equal length (12 h). At the latitude of the study populations (62° N), summers are characterized by long days, with 17–20 sunhours during the *G. conopsea* flowering period. To separate accurately the effects of diurnal and nocturnal pollinators, the caging periods should agree with activity time for each pollinator category, and one alternative could be to delimit each treatment based on light intensity measurements. We did not observe any activity by diurnal pollinators outside the 06:00–18:00 h window, but we cannot completely exclude the possibility that there was some contribution by day-flying pollinators to the nocturnal pollination treatment.

*Gymnadenia conopsea* is a long-lived perennial that shows significant short-term costs of reproduction, and costs differ between the two studied populations in terms of magnitude and fitness components affected (Sletvold and Ågren 2011b). Life history differences across environments can affect trait selection (Kolb and Ehrlén 2010), and estimates of selection based on fitness components may or may not correlate well with those based on lifetime fitness (Gómez 2008, Kolb and Ehrlén 2010). To compare selection gradients among different populations based on overall fitness, we are collecting demographic data that will link effects on components of fitness throughout the life cycle.

Whether pollination systems should be considered generalized or specialized has been vigorously debated over the last decades (Waser et al. 1996, Fenster et al. 2004), but comparatively few attempts have been made to quantify the contribution of specific pollinators to selection on floral traits. As illustrated by the present study, manipulation of the pollination environment makes it possible to move beyond the recognition that pollinators vary in visitation frequency and efficiency, and provides quantitative estimates of selection exerted by different categories of pollinators. This represents an important step toward linking observed selection on floral traits to interactions with specific categories of pollinators.

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#### LITERATURE CITED

- Aigner, P. A. 2001. Optimality modeling and fitness trade-offs: when should plants become pollinator specialists? *Oikos* 95:177–184.
- Aigner, P. A. 2004. Floral specialization without trade-offs: optimal corolla flare in contrasting pollination environments. *Ecology* 85:2560–2569.
- Armbruster, W. S., C. B. Fenster, and M. R. Dudash. 2000. Pollination “principles” revisited: specialization, pollination syndromes, and the evolution of flowers. *Det Norske Videnskaps-akademi I. Matematisk Naturvidenskapelige Klasse Skrifter, Ny Serie* 39:179–200.
- Barthelmess, E. L., C. M. Richards, and D. E. McCauley. 2006. Relative effects of nocturnal vs diurnal pollinators and distance on gene flow in small *Silene alba* populations. *New Phytologist* 169:689–698.
- Boberg, E., and J. Ågren. 2009. Despite their apparent integration, spur length but not perianth size affects reproductive success in the moth-pollinated orchid *Platanthera bifolia*. *Functional Ecology* 23:1022–1028.
- Brunet, J. 2009. Pollinators of the Rocky Mountain columbine: temporal variation, functional groups and associations with floral traits. *Annals of Botany* 103:1567–1578.
- Caruso, C. M., S. L. Scott, J. C. Wray, and C. A. Walsh. 2010. Pollinators, herbivores, and the maintenance of flower color variation: a case study with *Lobelia siphilitica*. *International Journal of Plant Sciences* 171:1020–1028.

- Claessen, J., and J. Kleynen. 2011. The flower of the European orchid. Form and function. Schrijen-Lipperz, Stein, The Netherlands.
- Darwin, C. R. 1862. On the various contrivances by which British and foreign orchids are fertilised by insects. Murray, London, UK.
- Dreisig, H. 1980. The importance of illumination level in the daily onset of flight activity in nocturnal moths. *Physiological Entomology* 5:327–342.
- Dreisig, H. 1981. Daily flight activity of moths in the continuous daylight of the arctic summer. *Holarctic Ecology* 4:36–42.
- Elle, E. 2004. Floral adaptations and biotic and abiotic selection pressures. Pages 111–118 in Q. C. B. Cronk, J. Whitton, R. H. Ree, and I. E. P. Taylor, editors. *Plant adaptation: molecular genetics and ecology*. NRS Research Press, Ottawa, Ontario, Canada.
- Ellis, A. G., and S. D. Johnson. 2010. Gender differences in the effects of floral spur length manipulation on fitness in a hermaphrodite orchid. *International Journal of Plant Sciences* 171:1010–1019.
- Fægri, K., and L. van der Pijl. 1979. *The principles of pollination ecology*. Pergamon, Oxford, UK.
- Fenster, C. B., W. S. Armbruster, P. Wilson, M. R. Dudash, and J. D. Thomson. 2004. Pollination syndromes and floral specialization. *Annual Review of Ecology, Evolution and Systematics* 35:375–403.
- Fishman, L., and J. H. Willis. 2008. Pollen limitation and natural selection on floral characters in the yellow monkey-flower, *Mimulus guttatus*. *New Phytologist* 177:802–810.
- Fleming, T. H., C. T. Sahley, J. N. Holland, J. D. Nason, and J. L. Hamrick. 2001. Sonoran Desert columnar cacti and the evolution of generalized pollination systems. *Ecological Monographs* 71:511–530.
- Galen, C. 1996. Rates of floral evolution: adaptation to bumblebee pollination in an alpine wildflower, *Polemonium viscosum*. *Evolution* 50:120–125.
- Gómez, J. M. 2003. Herbivory reduces the strength of pollinator-mediated selection in the Mediterranean herb *Erysimum mediohispanicum*: consequences for plant specialization. *American Naturalist* 162:242–256.
- Gómez, J. M. 2008. Sequential conflicting selection due to multispecific interactions triggers evolutionary trade-offs in a monocarpic herb. *Evolution* 62:668–679.
- Gómez, J. M., M. Abdelaziz, J. Lorite, A. J. Munoz-Pajares, and F. Perfectti. 2010. Changes in pollinator fauna cause spatial variation in pollen limitation. *Journal of Ecology* 98:1243–1252.
- Gómez, J. M., F. Perfectti, J. Bosch, and P. M. Camacho. 2009. A geographic selection mosaic in a generalized plant–pollinator–herbivore system. *Ecological Monographs* 79:245–263.
- Goyret, J., P. M. Markwell, and R. A. Raguso. 2007. The effect of decoupling olfactory and visual stimuli on the foraging behavior of *Manduca sexta*. *Journal of Experimental Biology* 210:1398–1405.
- Graham, S. W., and S. C. H. Barrett. 2004. Phylogenetic reconstruction of the evolution of stylar polymorphisms in *Narcissus* (Amaryllidaceae). *American Journal of Botany* 91:1007–1021.
- Herrera, C. M. 1987. Components of pollinator “quality”: comparative analysis of a diverse insect assemblage. *Oikos* 50:79–90.
- Huber, F. K., R. Kaiser, W. Sauter, and F. P. Schiestl. 2005. Floral scent emission and pollinator attraction in two species of *Gymnadenia* (Orchidaceae). *Oecologia* 142:564–575.
- Hultén, E., and M. Fries. 1986. *Atlas of north European vascular plants north of the Tropic of Cancer*. Volumes 1–3. Koeltz Scientific Books, Königstein, Germany.
- Jennersten, O., and D. H. Morse. 1991. The quality of pollination by diurnal and nocturnal insects visiting common milkweed, *Asclepias syriaca*. *American Midland Naturalist* 125:18–28.
- Johnson, S. D., and K. E. Steiner. 1997. Long-tongued fly pollination and evolution of floral spur length in the *Disa draconis* complex (Orchidaceae). *Evolution* 51:45–53.
- Kay, K. M., P. A. Reeves, R. G. Olmstead, and D. W. Schemske. 2005. Rapid speciation and the evolution of hummingbird pollination in neotropical *Costus* subgenus *Costus* (Costaceae): evidence from nrDNA ITS and ETS sequences. *American Journal of Botany* 92:1899–1910.
- Kelber, A., A. Balkenius, and E. J. Warrant. 2003. Colour vision in diurnal and nocturnal hawkmoths. *Integrative and Comparative Biology* 43:571–579.
- Kolb, A., and J. Ehrlén. 2010. Environmental context drives seed predator-mediated selection on a floral display trait. *Evolutionary Ecology* 24:433–445.
- Lande, R., and S. J. Arnold. 1983. The measurement of selection on correlated characters. *Evolution* 37:1210–1226.
- Lunau, K., S. Papiorek, T. Eitz, and M. Sazima. 2011. Avoidance of achromatic colours by bees provides a private niche for hummingbirds. *Journal of Experimental Biology* 214:1607–1612.
- Medel, R., C. Botto-Mahan, and M. Kalin-Arroyo. 2003. Pollinator-mediated selection on the nectar guide phenotype in the Andean monkey flower, *Mimulus luteus*. *Ecology* 84:1721–1732.
- Meyer, B., J. Kroger, and I. Steffan-Dewenter. 2007. Contribution of diurnal and nocturnal pollinators to the reproductive success of the orchid species *Gymnadenia conopsea*. *Entomologia Generalis* 30:299–300.
- Mitchell-Olds, T., and R. G. Shaw. 1987. Regression analysis of natural selection: statistical inference and biological interpretation. *Evolution* 41:1149–1161.
- Moen, A. 1999. *National atlas of Norway: vegetation*. Norwegian Mapping Authority, Hønefoss, Norway.
- Muchhala, N. 2003. Exploring the boundary between pollination syndromes: bats and hummingbirds as pollinators of *Burmeistera cyclostigmata* and *B. tenuiflora* (Campanulaceae). *Oecologia* 134:373–380.
- Muchhala, N. 2007. Adaptive trade-off in floral morphology mediates specialization for flowers pollinated by bats and hummingbirds. *American Naturalist* 169:494–504.
- Muchhala, N., and J. D. Thomson. 2009. Going to great lengths: selection for long corolla tubes in an extremely specialized bat-flower mutualism. *Proceedings of the Royal Society B* 276:2147–2152.
- Nilsson, A. 1988. The evolution of flowers with deep corolla tubes. *Nature* 334:147–149.
- Øien, D.-I., and A. Moen. 2002. Flowering and survival of *Dactylorhiza lapponica* and *Gymnadenia conopsea* in the Sølendet Nature Reserve, Central Norway. Pages 3–22 in P. Kindlmann, J. H. Willems, and D. F. Whigham, editors. *Trends and fluctuations and underlying mechanisms in terrestrial orchid populations*. Backhyus, Leiden, The Netherlands.
- Ollerton, J., S. D. Johnson, and A. B. Hingston. 2006. Geographical variation in diversity and specification of pollination systems. Pages 283–308 in N. M. Waser and J. Ollerton, editors. *Plant–pollinator interactions: from specialization to generalization*. University of Chicago Press, Chicago, Illinois, USA.
- Parachnowitsch, A. L., and A. Kessler. 2010. Pollinators exert natural selection on flower size and floral display in *Penstemon digitalis*. *New Phytologist* 188:393–402.
- Peakall, R., and S. N. Handel. 1993. Pollinators discriminate among floral heights of a sexually deceptive orchid: implications for selection. *Evolution* 47:1681–1687.
- Pérez-Barrales, R., and J. Arroyo. 2010. Pollinator shifts and the loss of style polymorphism in *Narcissus papyraceus* (Amaryllidaceae). *Journal of Evolutionary Biology* 23:1117–1128.

- Reynolds, R. J., and C. B. Fenster. 2008. Point and interval estimation of pollinator importance: a study using pollination data of *Silene caroliniana*. *Oecologia* 156:325–332.
- Reynolds, R. J., M. J. Westbrook, A. S. Rohde, J. M. Cridland, C. B. Fenster, and M. R. Dudash. 2009. Pollinator specialization and pollination syndromes of three related North American *Silene*. *Ecology* 90:2077–2087.
- Sandring, S., and J. Ågren. 2009. Pollinator-mediated selection on floral display and flowering time in the perennial herb *Arabidopsis lyrata*. *Evolution* 63:1292–1300.
- Schemske, D. W., and H. D. Bradshaw. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proceedings of the National Academy of Sciences USA* 96:11910–11915.
- Schemske, D. W., and C. C. Horvitz. 1984. Variation among floral visitors in pollination ability: a precondition for mutualism specialization. *Science* 255:519–521.
- Sletvold, N., and J. Ågren. 2010. Pollinator-mediated selection on floral display and spur length in the orchid *Gymnadenia conopsea*. *International Journal of Plant Sciences* 171:999–1009.
- Sletvold, N., and J. Ågren. 2011a. Nonadditive effects of floral display and spur length on reproductive success in a deceptive orchid. *Ecology* 92:2167–2174.
- Sletvold, N., and J. Ågren. 2011b. Among-population variation in costs of reproduction in the long-lived orchid *Gymnadenia conopsea*: an experimental study. *Oecologia* 167:461–468.
- Sletvold, N., and J. M. Grindeland. 2008. Floral herbivory increases with inflorescence size and local plant density in the facultative biennial *Digitalis purpurea*. *Acta Oecologica* 34:21–25.
- Sletvold, N., J. M. Grindeland, and J. Ågren. 2010. Pollinator-mediated selection on floral traits and flowering phenology in the deceptive orchid *Dactylorhiza lapponica*. *New Phytologist* 188:385–392.
- Sletvold, N., J. M. Grindeland, P. Zu, and J. Ågren. 2012. Strong inbreeding depression and local outbreeding depression in the rewarding orchid *Gymnadenia conopsea*. *Conservation Genetics*, *in press*.
- Stebbins, G. L. 1970. Adaptive radiation of reproductive characteristics in angiosperms. I. Pollination mechanisms. *Annual Review of Ecology and Systematics* 1:307–326.
- Stpiczynska, M., and J. Matusiewicz. 2001. Anatomy and ultrastructure of spur nectary of *Gymnadenia conopsea* (L.) Orchidaceae. *Acta Societatis Botanicorum Poloniae* 70:267–272.
- Torång, P., J. Ehrlén, and J. Ågren. 2008. Mutualists and antagonists mediate frequency-dependent selection on floral display. *Ecology* 89:1564–1572.
- Totland, Ø. 2001. Environment-dependent pollen limitation and selection on floral traits in an alpine species. *Ecology* 82:2233–2244.
- Wade, M. J., and S. Kalisz. 1990. The causes of natural selection. *Evolution* 44:1947–1955.
- Waser, N. M., L. Chittka, M. V. Price, N. M. Williams, and J. Ollerton. 1996. Generalization in pollination systems, and why it matters. *Ecology* 77:1043–1060.
- Whittall, J. B., and S. A. Hodges. 2007. Pollinator shifts drive increasingly long nectar spurs in columbine flowers. *Nature* 447:706–710.
- Wilson, P., M. C. Castellanos, A. Wolfe, and J. D. Thomson. 2006. Shifts between bee- and bird-pollination among penstemons. Pages 47–68 in N. M. Waser and J. Ollerton, editors. *Plant–pollinator interactions: from specialization to generalization*. University of Chicago Press, Chicago, Illinois, USA.
- Wilson, P., and J. D. Thomson. 1991. Heterogeneity among floral visitors leads to discordance between removal and deposition of pollen. *Ecology* 72:1503–1507.
- Wise, M. J., and J. B. Hébert. 2010. Herbivores affect natural selection for floral-sex ratio in a field population of horse-nettle, *Solanum carolinense*. *Ecology* 91:937–943.
- Wolff, D., M. Braun, and S. Liede. 2002. Nocturnal versus diurnal pollination success in *Isertia laevis* (Rubiaceae): a sphingophilous plant visited by hummingbirds. *Plant Biology* 5:71–78.
- Young, H. J. 2002. Diurnal and nocturnal pollination of *Silene alba* (Caryophyllaceae). *American Journal of Botany* 89:433–440.

## SUPPLEMENTAL MATERIAL

### Appendix

Table of phenotypic correlations among floral traits and added variable plots of selection gradients for floral traits (*Ecological Archives* E093-166-A1).