POLLINATOR-MEDIATED SELECTION ON FLORAL DISPLAY AND SPUR LENGTH IN THE ORCHID *GYMNADENIA CONOPSEA*

Nina Sletvold[1],* and Jon Ågren[2],†

*Norwegian University of Science and Technology, Museum of Natural History and Archaeology, N-7491 Trondheim,
Norway; and †Plant Ecology, Department of Ecology and Genetics, Evolutionary Biology Centre,
Uppsala University, Norbyvägen 18 D, SE-752 36 Uppsala, Sweden

Floral diversification and specialization are thought to be driven largely by interactions with pollinators, but the extent to which current selection on floral traits is mediated by pollinators has rarely been determined experimentally. We documented selection through female function on floral traits in two populations of the rewarding orchid *Gymnadenia conopsea* in two years and quantified pollinator-mediated selection ($\Delta\beta_{poll}$) by subtracting estimates of selection gradients for plants receiving supplemental hand pollination from estimates obtained for open-pollinated control plants. There was directional selection for taller plants, more flowers, larger corollas, and longer spurs in the study populations. Pollinator-mediated selection ranged from weak to moderately strong ($\Delta\beta_{poll}$, range -0.01–0.21, median 0.08). All observed selection on spur length could be attributed to interactions with pollinators, while the proportion of observed selection on plant height (0%–77%), number of flowers (13%–42%), and corolla size (13%–97%) caused by pollinators varied among populations and years. Our results demonstrate that pollinators can mediate selection on both traits likely to be involved in pollinator attraction and traits affecting pollination efficiency. They further show that spatiotemporal variation in the strength of pollinator-mediated selection can contribute substantially to differences in selection between years and populations.

Keywords: female fitness, natural selection, plant-animal interactions, pollen limitation, selection gradients, spatiotemporal variation.

Introduction

Floral evolution is thought to be driven to a large extent by interactions with pollinators (Fenster et al. 2004), and it is well documented that variation in floral display traits may translate into variation in pollinator attraction (Grindeland et al. 2005) and pollination efficiency (Muchhala 2007). Phenotypic selection studies in natural populations have also demonstrated significant selection on a multitude of floral traits (reviewed in Harder and Johnson 2009), but the link between patterns of current selection and interactions with pollinators has been established conclusively in only a few cases (Sandring and Ågren 2009). Though it seems reasonable to assume that pollinators, at least to some degree, are responsible for the observed selection on flower morphology and phenology, studies that actually quantify the importance of pollinators as selective agents and how it varies in space and time are needed to fully understand the role of pollinator-mediated selection in floral evolution.

Pollen limitation of female reproductive success is common in many animal-pollinated plants (Ashman et al. 2004), indicating a considerable potential for pollinator-mediated selection on traits involved in pollinator attraction and pollination efficiency. Pollinator visitation rate is usually positively re-

[1] E-mail: nina.sletvold@vm.ntnu.no.
[2] Author for correspondence; e-mail: jon.agren@ebc.uu.se.

lated to visual display, and traits such as plant height (Lortie and Aarssen 1999), number of flowers (Mitchell et al. 2004), and flower size (Schemske and Ågren 1995; Armbruster et al. 2005) may influence pollinator attraction. Traits that are known to influence the functional fit between pollinator and plant include corolla tube width (Campbell et al. 1996), corolla tube length (Alexandersson and Johnson 2002; Bloch and Erhardt 2008; Muchhala and Thomson 2009), and spur length (Nilsson 1988; Johnson and Steiner 1997). However, other biotic (Gómez 2003; Toräng et al. 2008) and abiotic (Totland 2001; Caruso et al. 2003; Maad and Alexandersson 2004) agents may also exert selection on floral display and flower morphology, and the contribution from pollinators relative to other interactions is either inferred through the use of path analysis (for a recent example, see Rey et al. 2006) or, in most cases, unaddressed. This is likely to be particularly problematic for the interpretation of selection on morphological traits that are strongly correlated with plant size. Selection on traits such as number of flowers and flower size may reflect selection on plant resource acquisition rather than selection mediated by pollinators (Harder and Johnson 2009; Sandring and Ågren 2009), pointing to the importance of disentangling the effects of different selective agents to be able to determine the role of interactions with pollinators in the evolution of floral traits.

The strength and direction of phenotypic selection in natural populations may vary in both space and time. Among-population variation in the direction of selection is a prerequisite

for the evolution of local adaptation, while temporal variation in the direction of selection may contribute to the maintenance of genetic variation within populations (Bell 1997; Turelli et al. 2001). Spatiotemporal variation in selection on floral traits is frequent (Harder and Johnson 2009). However, since few studies have identified experimentally the selective agents in natural populations, little is known about the role of plant-pollinator interactions for spatiotemporal variation in selection on floral traits.

Pollinator-mediated selection implies that the relationship between plant trait expression and relative fitness is at least partly the result of interactions with pollinators. Pollinator-mediated selection through female function can be detected by comparing the mode and intensity of selection in open-pollinated control plants and in plants receiving supplemental hand pollination (Galen 1996; Fishman and Willis 2008; Sandring and Ågren 2009). If supplemental hand pollination influences the slope or curvature of the relationship between plant trait expression and relative fitness, this shows that the magnitude of pollen limitation (quantified as the proportional increase in female fitness following hand pollination) varies with plant phenotype and thus that pollinators mediate selection on plant traits through female function. Here we use this approach to quantify pollinator-mediated selection on floral traits expected to affect attractiveness to pollinators (plant height, number of flowers, and flower size) and efficiency of pollination (spur length) in the rewarding orchid *Gymnadenia conopsea*. We estimated the strength of pollinator-mediated selection through female function ($\Delta\beta_{poll}$) by subtracting estimates of selection gradients for plants receiving supplemental hand pollination from estimates obtained for open-pollinated control plants. The study was conducted in 2 yr in two Norwegian populations found at sites that differ in pollinator fauna and vegetation height and in abiotic conditions such as temperature and precipitation. We specifically ask whether selection on floral morphology can be attributed to interactions with pollinators and, if so, whether the strength of pollinator-mediated selection varies in space and time.

Material 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 is found on calcareous soils in grazed meadows, traditional hay meadows, and margins of marshes and fens and is highly variable in terms of morphology and phenology (Marhold et al. 2005). In Norway, *G. conopsea* ssp. *conopsea* is found scattered throughout most of the country, from sea level up to altitudes beyond 1500 m a.s.l. The species is long-lived, tuberous, and nonclonal. Individuals produce a single inflorescence with ~10–70 flowers that open sequentially from the bottom to the top. The flowers vary in color from pale pink to cerise or lilac and rarely pure white. A long, narrow spur contains nectar that is produced throughout anthesis (Stpiczynska and Matusiewicz 2001). Each flower contains two pollinaria, situated above the spur entrance and composed of about a hundred massulae (tightly packed pollen masses; Nazarov and Gerlach 1997). The flowers are fra-

grant, emitting a spicy, clovelike scent and are visited by diurnal and nocturnal visitors (Huber et al. 2005; Meyer et al. 2007; N. Sletvold and J. Ågren, personal observation). High levels of fruit set are reported from British (Darwin 1862; Neiland and Wilcock 1998) and Swedish (Hansen and Olesen 1999; Lönn et al. 2006) populations. *Gymnadenia conopsea* is self-compatible but depends on pollinators for successful fruit set (N. Sletvold, unpublished data). Pollinaria are picked up singly or in pairs by visitors. The pollinium usually disintegrates, and massulae from a given pollinium can be deposited on multiple flowers. Fruits mature 4–6 wk after pollination, and the minute seeds are wind dispersed during autumn after capsule dehiscence.

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 boreal and the north boreal vegetation zones (Moen 1999) and experience a short growing season, usually lasting from late May to late August. The areas differ in local climate; the coastal Tågdalen population (450 m a.s.l.) 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 in 2008 and 2009 was approximately 200 and 400 in the Tågdalen population and 600 and 1000 in the Sølendet population, respectively. The studied populations are found in open, wet grasslands, dominated by *Molinia caerulea* (L.) Moench, *Succisa pratensis* Moench, and *Thalictrum alpinum* L. In the study areas, this vegetation type is typical for the transitional zone from fen margins to more herb-dominated birch woodlands. Vegetation is taller at the Tågdalen site than at the Sølendet site (N. Sletvold, personal observation). The *G. conopsea* populations begin to flower in late June and flower for 3–4 wk. In both populations, *G. conopsea* flowers are visited by diurnal butterflies of the genus *Boloria* Moore (Nymphalidae) and the nocturnal hawkmoth *Hyles gallii* Rott. (Sphingidae). In addition, the diurnal fly *Empis tessellata* F. (Empididae) is a common flower visitor at Sølendet, and the diurnal hawkmoth *Hemaris tityus* L. (Sphingidae) is common at Tågdalen (N. Sletvold, personal observation).

Field Experiment

Phenotypic selection on floral display and spur length was quantified in 2008 and 2009 at Sølendet and in 2009 at Tågdalen, while pollen limitation was quantified in both years in both populations. In mid-late June each year, plants with visible flower buds were haphazardly chosen and individually tagged. Sampling was independent between years. At Tågdalen, a total of 140 plants were marked in 2008, with 60 plants randomly assigned to the supplemental hand pollination treatment and 80 serving as open-pollinated controls. In 2009, we marked 250 plants, of which 85 received supplemental hand pollination and 165 served as open-pollinated controls. At Sølendet, a total of 340 plants were included both years, with 120 plants randomly assigned to the supplemental pollination treatment and 220 to the open-pollinated control. Sample size was lower in the hand pollination treat-

ment based on the expectation of reduced variation in female fitness following supplemental pollination. The study populations were visited one to three times per week throughout the flowering period, and on each visit, all open flowers on plants in the experimental treatment were pollinated by hand with cross-pollen from the local population. All flowers received supplemental pollination at least once. Cocktail sticks were used to collect pollinaria, and pollinations were performed by rubbing one or two pollinia across each stigma, saturating the surface with pollen. Pollinaria were collected primarily from other plants in the hand pollination treatment but, because of high natural rates of pollen removal, also from individuals not included in the study.

Measured Traits

At the onset of flowering, we recorded plant height (distance from ground to topmost flower) on every individual. 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 at maturation, and we collected three nondehisced capsules from each plant to determine mean fruit mass. Fruit mass is positively related to number of seeds with embryos in *G. conopsea* (linear regression, $b = 0.40$, $R^2 = 0.67$, $n = 44$ fruits, each fruit sampled from a separate individual in the Sølendet population, $P < 0.001$). For each plant, we estimated female fitness as the product of number of fruits and mean fruit mass. We quantified pollen limitation (PL) for each population and year as $1 -$ (mean female fitness of open-pollinated control plants/mean female fitness of hand-pollinated plants). In 2008, corolla size and spur length were measured only in the Sølendet population.

Statistical Analyses

The effects of pollination treatment, population, and year on plant traits and plant performance were examined with three-way ANOVA, except for corolla size and spur length, for which data were not collected at Tågdalen in 2008. For the latter two traits, we used two-way ANOVA to determine the effects of population and pollination treatment (data from 2009) and year and pollination treatment (data from the Sølendet 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 × mean fruit mass, and relative fitness and standardized trait values were calculated separately for each treatment and population. We initially included quadratic (γ_{ii}) and cross-product (γ_{ij}) terms in regression models to quantify nonlinear and correlational selection. However, none of the quadratic or correlational gradients were statistically significant, and they improved model fit as evaluated by R^2 only

marginally. We therefore report only linear gradients. Multi-collinearity was assessed by inspection of variance inflation factors, which in no case exceeded 2, indicating that the level of collinearity was not problematic (Quinn and Keough 2002).

We used ANCOVA to determine whether linear selection gradients varied among pollination treatments, populations, and years. Since we lacked estimates of selection gradients in Tågdalen in 2008, we used two different models, examining variation between populations and years separately. Both models included relative fitness as the dependent variable and the four standardized traits (plant height, number of flowers, corolla size, and spur length) as independent variables. The first model, applied to data from the Sølendet population, also included pollination treatment, year, and trait × pollination treatment, trait × year, and trait × pollination treatment × year interactions as independent variables. The second model, applied to data from both populations in 2009, included pollination treatment, population, and trait × pollination treatment, trait × population, and trait × pollination treatment × population interactions as independent variables. Because statistically significant three-way interactions were detected, we further tested the effect of pollination treatment on selection gradient estimates separately for each population and year. To quantify pollinator-mediated selection, we subtracted for each trait the estimated selection gradient for plants receiving supplemental hand pollination (β_{HP}) from the estimate obtained for open-pollinated controls (β_C), $\Delta\beta_{poll} = \beta_C - \beta_{HP}$. Selection gradients were illustrated with added-variable plots, in which the residuals from a linear regression model of relative fitness on all traits except the focal trait were plotted against the residuals from a regression model of the focal trait on the other traits.

Results

Floral Traits

Plant height, number of flowers, and corolla size varied among populations and years, while spur length varied only among populations (table 1). Plants in the Tågdalen population were taller and produced more flowers compared to plants in the Sølendet population in both years. Flower production was higher in 2009 than in 2008 in both populations. The difference in flower production between years was larger at Tågdalen than at Sølendet, resulting in a significant population × year interaction for number of flowers. Corolla size was larger in the Sølendet population than in the Tågdalen population, whereas the opposite was true for spur length. Corolla size was smaller in 2009 than in 2008 (table 1).

Floral traits were moderately positively correlated in both years and populations (table 2). Tall plants tended to produce many flowers with large corollas and long spurs.

Pollen Limitation

Fruit production and fruit mass were pollen limited in both populations in both years (table 1). Plants produced more and larger fruits in the Tågdalen population than in the Sølendet population in both years and more and larger fruits in 2009

Table 1

Trait Means ± SD for Open-Pollinated Control Plants (C) and Plants Receiving Supplemental Hand Pollination (HP) in the *Gymnadenia conopsea* Populations at Tågdalen (Tåg) and Sølendet (Søl) in 2008 and 2009

Trait	C Tåg ($n_{2008} = 71$, $n_{2009} = 163$)	HP Tåg ($n_{2008} = 40$, $n_{2009} = 83$)	C Søl ($n_{2008} = 197$, $n_{2009} = 215$)	HP Søl ($n_{2008} = 115$, $n_{2009} = 114$)	P poll	P yr	P pop	P poll × yr	P poll × pop	P pop × yr	P poll × pop × yr
Plant height (cm)	26.4 ± 4.9, 28.4 ± 4.4	25.8 ± 4.5, 27.5 ± 4.2	23.6 ± 3.8, 23.3 ± 4.0	23.1 ± 3.7, 22.8 ± 3.6	.15	.0052	<.0001	.61	.60	.069	.59
No. flowers	24.5 ± 9.4, 30.8 ± 8.9	24.5 ± 7.2, 31.5 ± 10.4	19.9 ± 7.1, 23.8 ± 7.7	20.0 ± 6.1, 23.8 ± 7.0	.70	<.0001	<.0001	.76	.82	.016	.72
Corolla size (mm[2])	NA, 91.2 ± 18.9	NA, 91.4 ± 16.2	112.1 ± 26.4, 104.6 ± 28.9	108.3 ± 24.5, 104.5 ± 21.1	.11[a]/.94[b]	<.0001[a]	<.0001[b]	.42[a]	.17[b]	NA	NA
Spur length (mm)	NA, 12.8 ± 1.6	NA, 13.1 ± 1.5	11.8 ± 1.8, 12.1 ± 1.5	11.7 ± 1.8, 11.9 ± 1.4	.90[a]/.58[b]	.10[a]	<.0001[b]	.95[a]	.22[b]	NA	NA
No. fruits	18.3 ± 9.4, 26.2 ± 10.3	22.0 ± 7.4, 30.9 ± 10.1	14.5 ± 7.6, 15.8 ± 10.2	19.2 ± 6.1, 23.2 ± 6.7	<.0001	<.0001	<.0001	.15	.16	<.0001	.48
Fruit mass (mg)	7.06 ± 1.78, 9.96 ± 3.10	8.25 ± 1.86, 11.00 ± 2.40	6.94 ± 1.78, 7.86 ± 2.81	7.73 ± 1.62, 8.56 ± 2.41	<.0001	<.0001	<.0001	.67	.38	<.0001	.83
No. fruits × fruit mass	138 ± 105, 276 ± 182	187 ± 85, 342 ± 153	110 ± 78, 138 ± 118	152 ± 73, 201 ± 96	<.0001	<.0001	<.0001	.37	.93	<.0001	.77

Note. P values associated with the effects of pollination treatment (poll), year (yr), population (pop), and their interactions in ANOVA are indicated. NA = data not available.

[a] Only data from Sølendet.

[b] Only data from 2009.

Table 2

Phenotypic Correlations among Traits in the *Gymnadenia conopsea* Populations at Tågdalen (above Diagonal) and Sølendet (below Diagonal) in 2008 and 2009, Based on Open-Pollinated Control Plants

Trait	Plant height (2008, 2009)	No. flowers (2008, 2009)	Corolla size (2008, 2009)	Spur length (2008, 2009)
Plant height		.66***, .52***	NA, .36***	NA, .31***
No. flowers	.48***, .56***		NA, .36***	NA, .49***
Corolla size	.37***, .41***	.17*, .33***		NA, .44***
Spur length	.15*, .21**	.27***, .27***	.15*, .34***	

Note. $n_{\text{Tåg08}} = 71$, $n_{\text{Tåg09}} = 163$, $n_{\text{Søl08}} = 197$, $n_{\text{Søl09}} = 215$. NA = data not available.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

than in 2008 in both populations. The difference between years was larger in the Tågdalen population, resulting in significant population × year interactions for both fitness components, as well as for the combined fitness estimate (number of fruits × fruit mass). The effect of supplemental hand pollination on female fitness did not differ significantly between years or populations (table 1). Pollen limitation (PL = 1 − (mean female fitness of open-pollinated control plants/mean female fitness of hand-pollinated plants)) was 0.26 in 2008 and 0.19 in 2009 in the Tågdalen population and 0.28 and 0.31, respectively, in the Sølendet population.

Phenotypic Selection

Significant selection on all four traits examined was detected in both populations. In the Sølendet population, there was directional selection for more flowers, larger corollas, and longer spurs in both years; in 2008 there was also significant

selection for taller plants (open-pollinated controls; table 3). In the Tågdalen population, there was directional selection for taller plants, more flowers, larger corollas, and longer spurs in 2009 (table 3).

Pollinator-Mediated Selection

Pollinators contributed to selection on both floral display and spur length. In the Sølendet population, all traits except plant height were subject to pollinator-mediated selection in at least one year. In both years, linear selection gradients for spur length differed significantly between the two pollination treatments, and all selection observed on spur length among open-pollinated control plants could be attributed to interactions with pollinators (figs. 1, 2e, 3d; table 3). Pollinator-mediated selection on spur length ($\Delta\beta_{\text{poll}}$) was 0.077 in 2008 and 0.16 in 2009 (table 3). Among hand-pollinated plants, estimates of selection gradients for spur length were low

Table 3

Phenotypic Linear Selection Gradients ($\pm$SE) for Open-Pollinated Control Plants (β_C) and for Plants Receiving Supplemental Hand Pollination (β_{HP}) in the Sølendet Population in 2008 and 2009 and in the Tågdalen Population in 2009

| Trait	β_C	β_{HP}	$\Delta\beta_{\text{poll}}$	P
Sølendet 2008:				
Plant height	.11 ± .042***	.12 ± .033***	−.010	.30
No. flowers	.38 ± .043***	.30 ± .032***	.080	.37
Corolla size	.070 ± .039*	.061 ± .025**	.0090	.77
Spur length	.067 ± .038*	−.010 ± .025	.077	.035
Sølendet 2009:				
Plant height	.034 ± .045	.044 ± .042	−.010	.88
No. flowers	.50 ± .044***	.29 ± .044***	.21	.0031
Corolla size	.20 ± .041**	.054 ± .037	.15	.020
Spur length	.16 ± .038**	−.0022 ± .035	.16	.0053
Tågdalen 2009:				
Plant height	.16 ± .033***	.037 ± .029	.12	.019
No. flowers	.39 ± .035***	.34 ± .027***	.050	.33
Corolla size	.088 ± .032**	.0023 ± .028	.086	.050
Spur length	.093 ± .033**	−.011 ± .027	.10	.033

Note. $\Delta\beta_{\text{poll}}$ is the strength of pollinator-mediated selection ($\Delta\beta_{\text{poll}} = \beta_C - \beta_{HP}$). P values associated with the effect of the trait × pollination treatment interaction in ANCOVA are indicated.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.
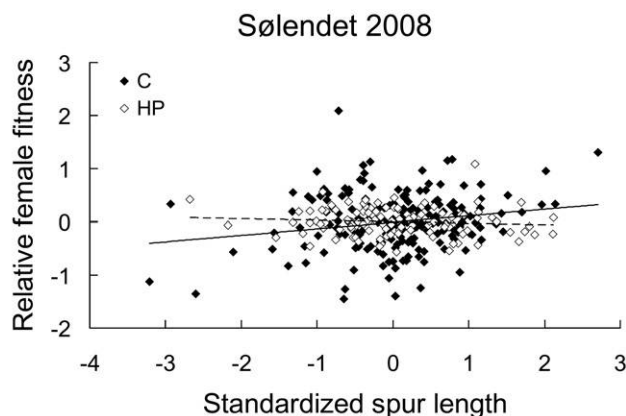
Fig. 1 Standardized linear phenotypic selection gradient for spur length in open-pollinated control plants (*C*; filled symbols, solid line) and in plants receiving supplemental hand pollination (*HP*; open symbols, dashed line) in the Sølendet population in 2008. The selection gradient is illustrated with an added-variable plot, in which the residuals from a linear regression model of relative fitness on all traits except the focal trait are plotted against the residuals from a regression model of the focal trait on the other traits.

and not significantly different from 0 (table 3). In 2009, selection gradients for number of flowers and corolla size also differed significantly between pollination treatments (figs. 2*a*, 2*c*, 3*b*, 3*c*; table 3). Pollinator-mediated selection on number of flowers was stronger than on corolla size ($\Delta\beta_{poll}$, 0.21 vs. 0.15; table 3) but represented a smaller proportion of the selection documented among open-pollinated plants (42% vs. 73%). In 2008, pollinator-mediated selection on number of flowers and corolla size was weaker, and selection gradients did not differ significantly between pollination treatments (fig. 3; table 3). The selection documented on plant height in 2008 could not be attributed to pollinators (fig. 3*a*; table 3).

In the Tågdalen population, interactions with pollinators contributed to selection on all traits except number of flowers. Linear selection gradients for plant height, corolla size, and spur length all differed between plants that received supplemental hand pollination and open-pollinated control plants (figs. 2, 3; table 3). All observed selection on spur length among open-pollinated plants was mediated by pollinators ($\Delta\beta_{poll} = 0.10$). As in the Sølendet population, no statistically significant selection on spur length was detected among hand-pollinated plants (figs. 2*f*, 3*d*; table 3). Most of the selection on plant height and corolla size could also be attributed to interactions with pollinators; selection gradients in the hand pollination treatment were weak and not statistically significant (figs. 2*b*, 2*d*, 3*a*, 3*c*; table 3). The strength of pollinator-mediated selection on plant height was somewhat higher than on corolla size (0.12 vs. 0.086; table 3) but represented a smaller proportion of the selection observed among open-pollinated control plants (77% vs. 97%). Pollinators contributed relatively little to selection on number of flowers ($\Delta\beta_{poll} = 0.05$), and selection gradients did not differ significantly between treatments (fig. 3*b*; table 3).

The higher frequency of statistically nonsignificant selection gradients in the hand pollination treatment compared to the control was apparently not simply a function of lower sample size in the former treatment. In both treatments, all estimates of selection gradients larger than 0.06 and none lower than 0.06 were statistically significant (table 3).

Spatiotemporal Variation in Selection

Selection on plant height, corolla size, and spur length differed significantly between years in the Sølendet population (fig. 3; table 4). Temporal variation in selection on corolla size was due to significantly stronger pollinator-mediated selection in 2009 than in 2008 (0.15 vs. 0.009; significant trait × pollination treatment × year interaction in ANCOVA; table 4; fig. 3*c*). Pollinator-mediated selection on spur length was also more than twice as strong in 2009 compared to 2008 (0.16 vs. 0.077), but no significant three-way interaction was detected (table 4; fig. 3*d*). Among both control plants and hand-pollinated plants, selection on plant height was weaker in 2009 than in 2008 (trait × year interaction in ANCOVA; table 4), and temporal variation in selection on plant height could not be explained by variation in plant-pollinator interactions (fig. 3*a*).

Selection on plant height, number of flowers, and corolla size differed between populations in 2009 (fig. 3; table 4). Spatial variation in selection on plant height and number of flowers could be explained by variation in plant-pollinator interactions. Pollinator-mediated selection on plant height was stronger in the Tågdalen population than in the Sølendet population (0.12 vs. −0.01; significant trait × pollination treatment × population interactions in ANCOVA; table 4; fig. 3*a*), while the reverse was true for pollinator-mediated selection on number of flowers (0.05 vs. 0.21; marginally significant interaction; table 4; fig. 3*b*). Among both control plants and hand-pollinated plants, selection on corolla size was stronger in the Sølendet population than in the Tågdalen population (trait × population interaction in ANCOVA; table 4), and spatial variation in selection on corolla size could not be explained by variation in pollinator-mediated selection (fig. 3*c*).

Discussion

This study has experimentally demonstrated that interactions with pollinators contribute to selection on both traits expected to influence pollinator attraction and traits likely to affect pollination efficiency in the rewarding orchid *Gymnadenia conopsea*. Female reproductive success was pollen limited in both study populations, and there was evidence of pollinator-mediated selection on all investigated floral traits in at least one of the populations. Our study is among the first to determine experimentally the importance of variation in plant-pollinator interactions for differences in selection on floral traits between natural populations and between years.

There is considerable evidence indicating that pollinators are the main selective agents behind the evolution of nectar spur length. In this study, there was directional selection for longer spurs in both populations, and all selection on spur length could be attributed to interactions with pollinators.
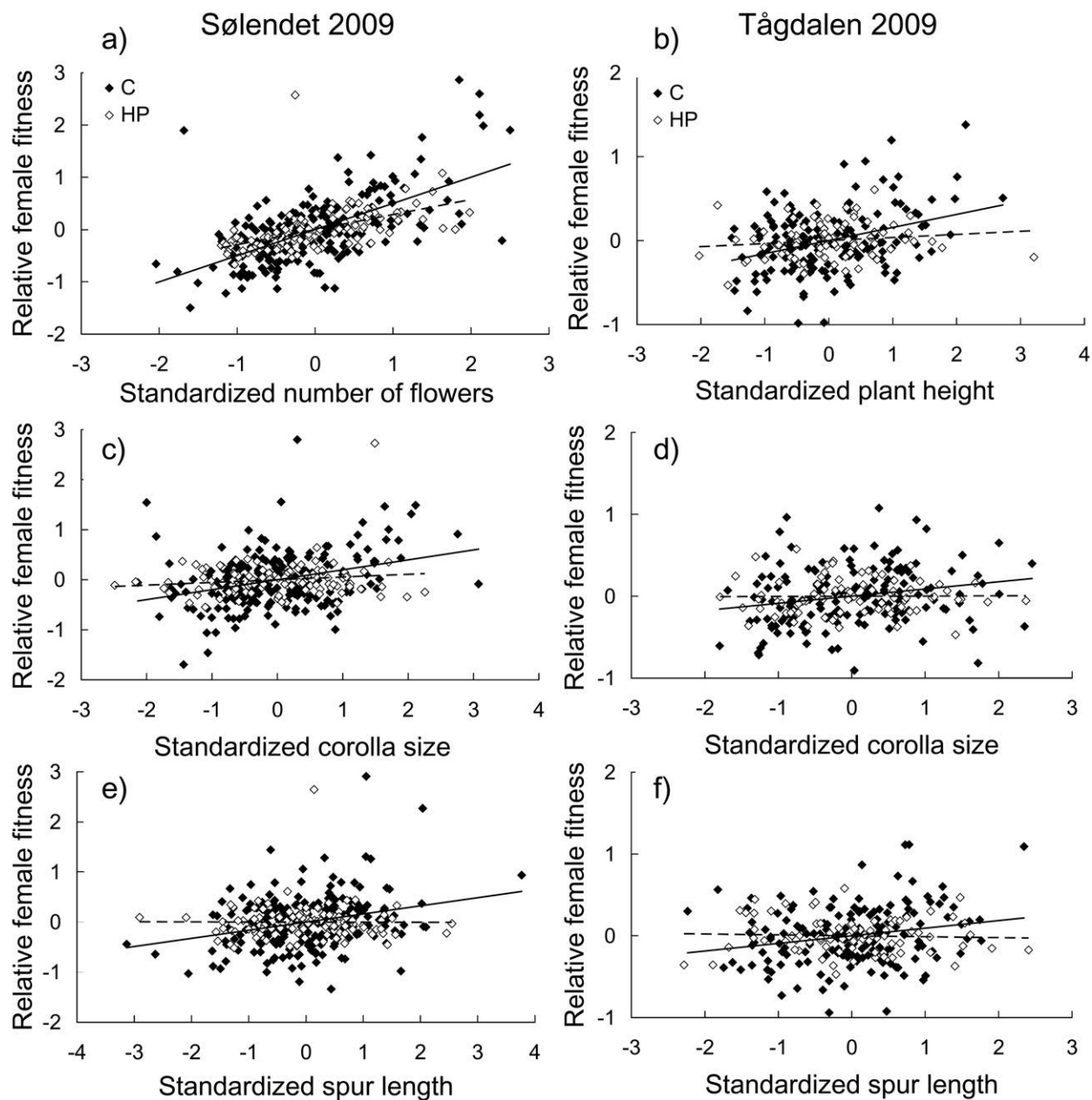
Fig. 2 Standardized linear phenotypic selection gradients (added-variable plots; see fig. 1) for plant height, number of flowers, corolla size, and spur length in open-pollinated control plants (*C*; filled symbols, solid lines) and in plants receiving supplemental hand pollination (*HP*; open symbols, dashed lines) in 2009 in the Sølendet population (*a*, *c*, *e*) and in the Tågdalen population (*b*, *d*, *f*).

Similarly, interactions with pollinators could explain all selection on spur length in a population of the deceptive, bumblebee-pollinated orchid *Dactylorhiza lapponica* ($\Delta\beta_{poll} = 0.32$; Sletvold et al. 2010). The consistent pollinator-mediated selection for longer spurs could be due to improved mechanical fit between the flower and flower visitors in long-spurred plants, resulting in higher pollination efficiency. All observed lepidopteran visitors have a proboscis longer than the spur of *G. conopsea*, and the pollinaria are consequently placed along the length of the proboscis, suggesting that pollinaria from long-spurred plants may fail to contact the stigma in

short-spurred plants, resulting in reduced female fitness (Johnson and Steiner 1997). In addition, long-spurred plants may be more attractive to pollinators due to a positive correlation between nectar volume and spur length (data from Sølendet in 2008; $r = 0.58$, $n = 312$; N. Sletvold, unpublished data). The evolution of floral nectar spurs is considered a key innovation in several plant lineages (Hodges 1997), allowing diversification as a result of pollinator-mediated selection. Consistent with this hypothesis, phylogenetic trait mapping in *Aquilegia* has indicated that an evolutionary trend for longer spurs is associated with shifts to pollinators with lon-
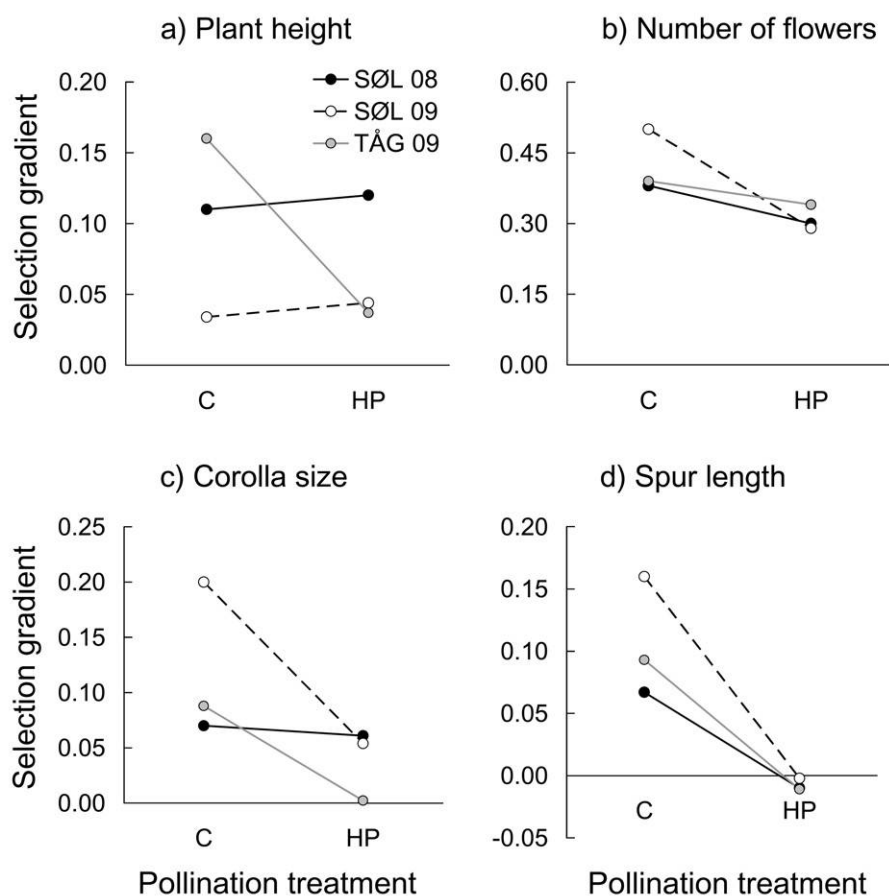
Fig. 3 Standardized linear phenotypic selection gradients in relation to pollination treatment (*C*, open-pollinated control plants; *HP*, hand-pollinated plants) for (*a*) plant height, (*b*) number of flowers, (*c*) corolla size, and (*d*) spur length in the Sølendet population in 2008 (black circles, solid lines) and 2009 (white circles, dashed lines) and in the Tågdalen population in 2009 (gray circles, gray lines).

ger tongues (Whittall and Hodges 2007), and population mean spur length has been found to be positively correlated with the tongue lengths of local pollinators in several plant taxa (Miller 1981; Robertson and Wyatt 1990; Johnson and Steiner 1997; Anderson and Johnson 2008; Anderson et al. 2010). Moreover, experimental shortening of spurs has been found to reduce estimates of male and/or female fitness in orchids (Nilsson 1988; Johnson and Steiner 1997; Boberg and Ågren 2009), indicating that spur length is a target of selection. The results of this study provide additional sup-

port for the view that pollinators can drive spur length evolution.

Pollinators contributed significantly to selection for more flowers in the Sølendet population but not in the Tågdalen population. Directional selection for more flowers is expected in studies using seed production as an estimate of fitness because the number of flowers sets an upper limit to seed production but also because the number of open flowers can affect attractiveness to pollinators (Mitchell et al. 2004; Grindeland et al. 2005). In the present study, pollinator-mediated

Table 4

P Values from ANCOVAs Testing for Differences in Pollinator-Mediated Selection between Years in the Sølendet Population and between Populations in 2009

Trait	Sølendet 2008 + 2009 (*n* = 641)			Tågdalen + Sølendet 2009 (*n* = 575)		
	P trait × poll	*P* trait × yr	*P* trait × poll × yr	*P* trait × poll	*P* trait × pop	*P* trait × poll × pop
Plant height	.62	.046	.78	.023	.21	.045
No. flowers	.00066	.17	.34	.0040	.52	.051
Corolla size	.043	.78	.050	.0063	.049	.48
Spur length	.0029	.041	.24	.0010	.34	.46

selection ($\Delta\beta_{poll}$) on number of flowers varied from 0.05 to 0.21 and was statistically significant in the Sølendet population in 2009. In this year, pollinator-mediated selection corresponded to 42% of the documented selection on number of flowers among open-pollinated plants, demonstrating that flower production may strongly influence plant female fitness through its effects on pollination success. Pollinator-mediated selection on number of flowers has also been demonstrated in the self-incompatible, fly-pollinated herb *Arabidopsis lyrata* ($\Delta\beta_{poll}$, range 0.21–0.81; Sandring and Ågren 2009) and in the bumblebee-pollinated orchid *D. lapponica* ($\Delta\beta_{poll} = 0.30$; Sletvold et al. 2010). Spatial and temporal variation in the strength of pollinator-mediated selection on number of flowers is likely to be common in many systems, reflecting shifts in the relative importance of resource and pollen limitation and in the effects of number of flowers on pollination success.

Spatial but not temporal variation in selection on plant height could be attributed to interactions with pollinators. At Sølendet, there was significant selection for taller plants among both open-pollinated controls and plants receiving supplemental pollination in 2008, whereas in 2009, no significant selection on plant height was documented. Temporal variation in selection on height in the Sølendet population was thus mediated by agents other than pollinators. In contrast, 77% of the selection for taller plants documented in the Tågdalen population could be attributed to interactions with pollinators. Vegetation is taller at Tågdalen than at Sølendet, which may explain why plant height is more critical for pollination success at the former site. Pollinator visitation has been found to be positively related to plant height in several animal-pollinated species (Peakall and Handel 1993; O'Connell and Johnston 1998; Lortie and Aarssen 1999), and field experiments suggest that pollinator-mediated selection on plant stature is stronger in tall than in low vegetation in the rosette herb *Primula farinosa* (Ehrlén et al. 2002; Ågren et al. 2006). Available evidence thus suggests that pollinators may contribute to selection on plant height in several species and that the strength of this selection may vary with vegetation context.

There was significant directional selection for larger corollas in both populations, but the importance of pollinator-mediated selection varied. In the Sølendet population in 2008, there was no significant difference in selection strength on corolla size between the two pollination treatments, while in 2009 most of the selection was mediated by pollinators in both populations (73%–97%). These findings indicate that large flowers increase pollination success in *G. conopsea* but also that other factors may contribute to selection for larger flowers. Selection for larger corollas in the Sølendet population could partly be the result of a positive correlation between corolla size and number of ovules (Fenster and Carr 1997). The documented selection for larger corollas was probably not caused by underlying variation in resource status. Corolla size was only weakly correlated with number of flowers, which is a trait likely to reflect plant vigor, and selection on corolla size was statistically significant also when controlling for variation in the number of flowers produced.

Spatiotemporal variation in pollinator-mediated selection on floral traits in *G. conopsea* may reflect variation in the importance of diurnal versus nocturnal pollinators. Intuitively, visual display should be more important for the attraction of

diurnal visitors than for the attraction of nocturnal visitors, while scent should play a major role for the attraction of pollinators at night. Boberg and Ågren (2009) recently showed that experimental reduction of corolla size did not affect fruit production in the nocturnally hawkmoth-pollinated orchid *Platanthera bifolia*. However, other experiments have shown that floral display traits may affect visitation patterns from nocturnal hawkmoths (White et al. 1994; Raguso and Willis 2002), suggesting that visual display may influence pollination success also during night. In ongoing work, we examine how trait variation is related to visitation and pollen transfer efficiency of diurnal and nocturnal pollinators and how this translates into selection on floral traits in *G. conopsea*.

The strength of pollinator-mediated selection through female function is expected to increase with increasing pollen limitation (Ashman and Morgan 2004). The results of this study cannot be used to evaluate this prediction because the range of pollen limitation observed was rather limited (PL = 0.19–0.31). However, even within this rather narrow range, both overall phenotypic selection on floral traits through female fertility and the component of this selection that could be attributed to pollinators varied considerably in *G. conopsea*.

Patterns of selection may differ between sex functions in hermaphroditic plants (Hodgins and Barrett 2008 and references therein), raising the question whether the documented relationships between floral traits and female fitness also hold for male fitness. Studies that used pollen removal as an estimate of male reproductive success in orchids have produced variable results. Some documented similar selection through both sex functions (O'Connell and Johnston 1998), while others detected sex-dependent selection (Maad 2000; Benitez-Vieyra et al. 2006). In *G. conopsea*, the proportion of pollen removed varied little in the Tågdalen population in 2008 (median 97%, range 91%–100%, $n = 68$), suggesting limited opportunities for selection via differential pollen removal. In the Sølendet population in 2008, pollen removal varied more (median 88%, range 42%–100%, $n = 84$) but was, on the other hand, highly correlated with fruit production ($r = 0.82$, $n = 84$; N. Sletvold, unpublished data), suggesting that selection estimates based on pollen removal would parallel those via female function. However, pollen removal may provide biased estimates of pollen export to other plants (Johnson et al. 2005) and siring success (Snow and Lewis 1993), and direct estimates of male fitness through paternity analyses would be needed to compare reliably selection patterns through the two sex functions.

Spatial and temporal variation in phenotypic selection on floral traits has been documented in several systems and has been related to variation in, for example, interactions with pollinators (Gómez et al. 2008) and antagonists (Toräng et al. 2008) and water availability (Caruso et al. 2003; Maad and Alexandersson 2004). However, the causes of variation in selection have seldom been determined experimentally. The results of this study show that pollinators may exert strong selection on floral traits, that spatiotemporal variation in interactions with pollinators contributes to among-year and among-population variation in selection on floral traits, but also that display traits such as plant height and corolla size may be subject to selection mediated by selective agents other than pollinators. A full understanding of the factors govern-

ing variation in selection on floral traits in this and other systems will thus require experimental manipulation of both the biotic environment and the abiotic environment. Because *G. conopsea* and closely related taxa display considerable variation in floral morphology, phenology, and scent (Huber et al. 2005; Marhold et al. 2005; Jersáková et al. 2010), they represent an attractive system for further exploring the importance of pollinator-mediated selection for the maintenance of a within- and among-population variation in floral traits using comparative and experimental approaches.

Acknowledgments

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