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Evolutionary Ecology of Floral Traits in Fragrant Orchids

ELODIE CHAPURLAT

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2018

ISSN 1651-6214
ISBN 978-91-513-0425-0
urn:nbn:se:uu:diva-358249

Dissertation presented at Uppsala University to be publicly examined in Zootissalen, Villavägen 9, Uppsala, Friday, 12 October 2018 at 13:00 for the degree of Doctor of Philosophy. The examination will be conducted in English. Faculty examiner: Ass. Pr. Kathleen Kay (University of California Santa Cruz, Ecology and Evolutionary Biology Department).

Abstract

Chapurlat, E. 2018. Evolutionary Ecology of Floral Traits in Fragrant Orchids. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology* 1713. 64 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-0425-0.

Why are flowers so diverse? Much of floral evolution is thought to be driven by pollinator-mediated selection. However, the connection between macroevolutionary patterns of floral diversity and microevolutionary processes remains poorly understood. In this thesis, I have used the fragrant orchids *Gymnadenia conopsea* s.s. and *Gymnadenia densiflora* to investigate the role of pollinators as agents of selection on floral traits and to test whether they cause spatial variation in selection. I addressed the following questions (1) Is there divergent selection on flowering phenology and floral traits between these two closely related species? (2) What is the contribution of pollinators relative to other selective agents to selection on phenology, visual display, floral scent and spur length? (3) Do diurnal and nocturnal pollinators mediate different selection patterns? (4) Does spatial variation in pollinator communities cause spatial variation in selection?

A phenotypic selection study in *G. conopsea* s.s. and *G. densiflora* indicated that divergent selection on flowering time contributes to the maintenance of phenological differentiation between the two species. Hand-pollination experiments combined with selection analysis showed that while pollinators were the main selective agent on spur length, their contribution to selection on phenology, visual display and floral scent was more variable and sometimes opposed by non-pollinator mediated selection. Selection analyses combined with a selective exclusion experiment showed that diurnal and nocturnal pollinators exerted different selection patterns on floral traits. Hand-pollination experiments also demonstrated that variation in pollinator-mediated selection largely explained spatial variation in net selection on phenology, visual display and spur length among four populations. A study of floral scent emission of *G. conopsea* s.s. in the field coupled with a growth-chamber experiment revealed genetically-based variation in floral scent consistent with a scenario where spatial variation in relative importance of nocturnal and diurnal pollinators has resulted in the evolution of different scent emission rhythms.

Taken together, the results support the hypothesis that pollinators cause spatial variation in selection on floral traits. They also highlight the importance of experimentally identifying sources of selection to reveal conflicting and reinforcing selection by multiple agents and thus advance our understanding of the evolutionary ecology of floral traits.

Keywords: agents of selection, diurnal and nocturnal pollination, divergent selection, field experiment, floral evolution, floral scent, Gymnadenia conopsea, Gymnadenia densiflora, Orchidaceae, phenotypic selection, pollinator-mediated selection, spatial variation, scent rhythm

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ISSN 1651-6214
ISBN 978-91-513-0425-0
urn:nbn:se:uu:diva-358249 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-358249)

“Va revoir les roses. Tu comprendras que la tienne est unique au monde”

Antoine de Saint-Exupéry, Le Petit Prince

A ma famille

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Chapurlat, E., Le Roncé, I., Ågren, J., Sletvold, N. Divergent selection on flowering phenology but not on floral morphology between two closely related orchids. (Manuscript)

II Chapurlat, E., Ågren, J., Sletvold, N. (2015) Spatial variation in pollinator-mediated selection on phenology, floral display and spur length in the orchid *Gymnadenia conopsea*. *New Phytologist*, 208(4):1264–1275

III Chapurlat, E., Anderson, J., Ågren, J., Friberg, M., Sletvold, N. (2018) Diel pattern of floral scent emission matches the relative importance of diurnal and nocturnal pollinators in populations of *Gymnadenia conopsea*. *Annals of Botany*, 121(4):711–721

IV Chapurlat, E., Anderson, J., Ågren, J., Friberg, M., Sletvold, N. Conflicting selection on floral scent in the fragrant orchid *Gymnadenia conopsea* s.s.. (Submitted manuscript)

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Abbreviations

C	Control, open-pollinated plants
HP	Hand-pollinated plants
D	Diurnal pollination treatment
N	Nocturnal pollination treatment
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
EAD	Electroantennographic detection
GC	Gas chromatograph
MS	Mass spectrometer
NMDS	Non-metric multidimensional scaling
PERMANOVA	Nonparametric multivariate analysis of variance
SEM	Standardized emission rate
SIMPER	Similarity percentage analysis

Introduction

The diversity in shape, size, colour and scent of angiosperm flowers is tremendous (Harder and Barrett 2006) and understanding what causes and maintains this diversity remains a central theme in plant evolution. The large majority of angiosperms are pollinated by animals (Ollerton et al. 2011) and thus much of floral evolution is thought to have been driven by pollinator-mediated selection (Darwin 1862; Faegri and Van der Pijl 1966; Fenster et al. 2004; Harder and Johnson 2009; van der Niet and Johnson 2012). However, the connection between macroevolutionary patterns of diversity and microevolutionary processes remains poorly understood (Herrera et al. 2006; Harder and Johnson 2009). Experimental approaches combined with selection analyses can help to clarify the role of pollinators and advance our understanding of floral evolution.

Spatial variation in selection and adaptive divergence

Several processes, neutral or adaptive, can cause divergence of taxa and thus increase diversity. Adaptive divergence occurs when selection drives the evolution of traits towards different optima in different populations or species (*Figure 1*). Depending on the current trait distributions in relation to these respective optima, divergent selection can be linear in different directions (e.g. Hall & Willis, 2006; *Figure 1*A) or stabilizing with different optima (e.g. Benkman, 2003; *Figure 1*B). Several studies have detected divergent linear selection on flowering phenology (Nuismer and Cunningham 2005; Hall and Willis 2006; Sandring et al. 2007) and on floral morphology (Campbell 2003; Sandring et al. 2007; Gómez et al. 2009; Boberg 2010; Rymer et al. 2010). Although some of these studies suggest that variation in abiotic conditions, pollinators or herbivores could be involved, most of them have not clearly identified the causes of divergent selection, with the exception of Campbell (2003) who showed that, in some years, pollinators caused divergent selection on corolla width in *Ipomopsis*. Moreover, most of these studies provide examples of divergent selection within species, and only a few studies have tested whether floral differentiation between species is maintained by divergent selection (Johnston 1991; Campbell 2003), which should be expected if floral trait differences between closely related taxa are adaptive.

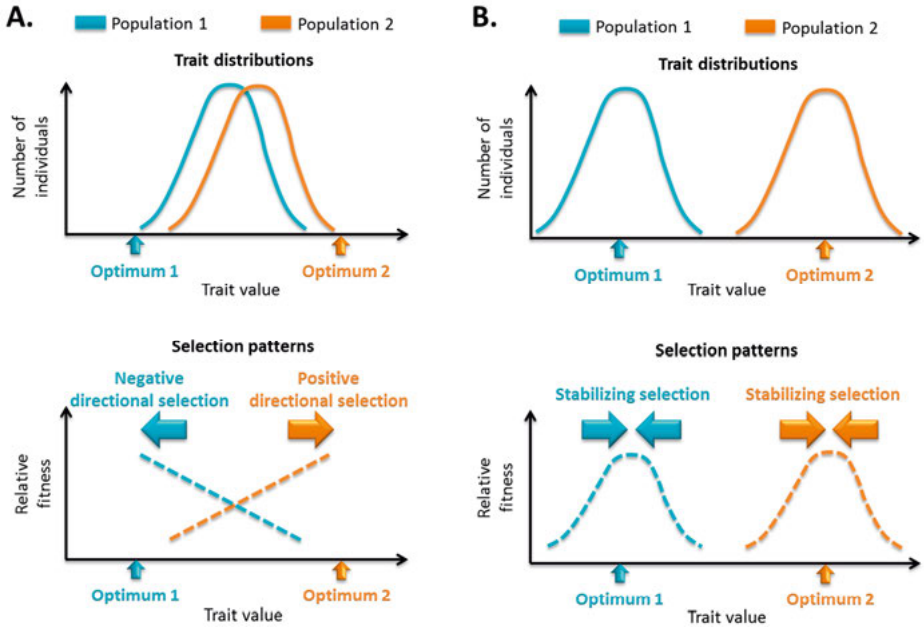


Figure 1. Patterns of divergent selection between two populations (represented by different colours) in function of the trait distributions in these populations relative to each population optimum. **A**. The distributions are intermediary and do not overlap with the optima, resulting in linear selection in different directions in each population. **B**. The population distributions span over the respective optima, resulting in stabilizing selection for the different optima in each population.

Interaction with pollinators is one aspect that can shape selective optima of floral traits. Because different pollinator species are likely to differ in their preferences and in their pollination efficiency, geographic variation in pollinator communities across the range of a given plant species is expected to cause spatial variation in pollinator-mediated selection. This is thought to be a central mechanism driving adaptive divergence of floral traits (Grant and Grant 1965; Stebbins 1970; Grant 1981). The quantification of pollinator-mediated selection in multiple populations is therefore an important step to understand the role of pollinators in plant adaptive differentiation and in driving patterns at the macroevolutionary level (Wilson and Thomson 1996; Herrera et al. 2006; Kay and Sargent 2009). Numerous studies have linked floral and pollinator traits across multiple populations (e.g. Anderson and Johnson 2008; Boberg et al. 2014; Newman et al. 2014), and one further linked among-population variation in selection on floral shape to variation in the composition of the local pollinator communities (Gómez et al. 2008, 2009). In contrast, very few studies have quantified current pollinator-mediated selection in multiple populations, and the present evidence for a geographic mosaic of pollinator-mediated selection remains predominantly correlative (but see Ågren et al. 2013; Weber and Kolb 2013).

Identifying the agents of selection on floral traits

Identifying the causes of geographical variation in natural selection is central for the understanding of adaptive differentiation and speciation (MacColl 2011). Selection on floral traits can be mediated by pollinators but also by antagonistic biotic agents (Gómez 2003; Parachnowitsch and Caruso 2008; Burkhardt et al. 2012; Ågren et al. 2013; Sletvold et al. 2015), and abiotic agents (Petit and Thompson 1998; Galen 2000; Totland 2001). In natural populations, plants interact with these agents simultaneously, and different agents can cause reinforcing or conflicting selection on the same trait (Strauss and Whittall 2006; Gómez 2008; Irwin and Brody 2011; Sletvold et al. 2015). It is thus necessary to experimentally unravel the contributions of different agents to net selection by manipulating the environment and comparing selection in the different treatments (Wade and Kalisz 1990; Caruso et al. 2017).

Supplemental hand-pollination can be used to separate the contribution from pollinators to net selection (Sandring and Ågren 2009; Sletvold and Ågren 2010). Indeed, hand-pollination leads to maximal pollination of all individuals and thus removes variation in relative fitness due to plant-pollinator interactions. Consequently, the selection gradients estimated for plants receiving supplemental hand-pollination represent non-pollinator mediated selection while gradients estimated for open-pollinated control plants represent net selection, i.e. the combined result of selection by all environmental factors. Pollinator-mediated selection can then be calculated by subtracting gradients obtained for hand-pollinated plants from gradients obtained for open-pollinated control plants.

The role of floral traits in plant-pollinator interactions

In Angiosperms, flowering time, flower morphology, colour and scent can critically influence pollen transfer. Timing of flowering determines which pollinators can visit the flowers, and can influence pollination success or competition for pollination (Kudo 2006; Elzinga et al. 2007). The shape, colour, scent and size of flowers or inflorescences are important traits for attracting pollinators and can thus be defined as display traits (Raguso 2008; Burger et al. 2010; Ida and Kudo 2010; Jersáková et al. 2012; Trunschke 2018). Flower morphology can be crucial for pollination efficiency by influencing the mechanical fit between the body of pollinators and the floral reproductive organs (Darwin 1862; Nilsson 1988; Conner et al. 1995; Alexandersson and Johnson 2002). In particular, the length of floral spurs, a tubular structure produced by some plant species, has been shown to positively influence pollination efficiency (Nilsson 1988; Boberg and Ågren 2009; Ellis and Johnson 2010). When one trait influences the rate of pollinator visitation

and a second trait influences pollination effectiveness per visit, correlational selection is expected on the pair of traits because pollination success will depend on the product of these two components (Sletvold and Ågren 2011b; Campbell et al. 2014). Correlational selection may also be expected for pairs of display traits if they influence pollinator visitation non-additively (Andersson 1996).

Floral scent: a complex and understudied trait

Whereas pollinator-driven adaptation of visual cues and floral morphologies provide some of the best examples of natural selection (e.g. Bradshaw and Schemske 2003; Whittall and Hodges 2007), the evolution and diversification of floral scent is less understood (Raguso 2008; Junker and Parachnowitsch 2015).

Floral scent is a complex trait as it can vary among plants not only in composition, but also in amount and timing of emission. Moreover, floral scent emissions of a single plant can show plasticity in response to environmental factors, including temperature (e.g. Hansted et al. 1994; Jakobsen and Olsen 1994; Farré-Armengol et al. 2014; Friberg et al. 2014a), humidity (Jakobsen and Olsen 1994; Friberg et al. 2014a), light (Jakobsen and Olsen 1994; Friberg et al. 2014a) and nutrient availability (Majetic et al. 2017). This makes it difficult to infer the importance of plant-pollinator interactions for the evolution of floral scent variation from field data alone (Majetic et al. 2009a). Thus, there is a need to disentangle innate and environmentally induced causes of floral scent variation among populations by collecting data on scent variation in a common environment.

Studies documenting phenotypic selection on scent are few and recent (Schiestl et al. 2010; Ehrlén et al. 2012; Parachnowitsch et al. 2012; Gross et al. 2016), and although floral scent is predicted to be under pollinator-mediated selection, the selective agents are still largely unidentified. Quantifying selection on emission rates of individual scent compounds is difficult because floral scent bouquets can be comprised of tens of compounds (e.g. Schiestl et al. 2010; Friberg et al. 2013; Gross et al. 2016) and the biological function of each compound is often unknown. Previous studies have used either total scent emission rate (Majetic et al. 2009b; Parachnowitsch et al. 2012) or statistical methods such as principal component analysis (PCA, Schiestl et al. 2010; Gross et al. 2016) and model selection criteria (Ehrlén et al. 2012; Parachnowitsch et al. 2012) to reduce the number of variables included in phenotypic selection analysis. Such approaches reduce comparability among studies, and using PCA or total scent makes it difficult to link selection to specific scent compounds. When pollination is the main agent of interest, an alternative approach is to use electroantennography to identify which compounds are detected by the pollinators and thus potential targets of pollinator-mediated selection. Surprisingly, electroantennography is rarely

used in combination with studies of intraspecific scent variation (Delle-Vedove et al. 2017) and has not been used as a criterion to identify and reduce the number of potential targets of pollinator-mediated selection in previous studies of selection on floral scent.

Aims of the thesis

In this thesis, I have used the fragrant orchids *Gymnadenia conopsea* s.s. and *Gymnadenia densiflora* to investigate the **role of pollinators as agents of selection on floral traits and to test whether they cause spatial variation in selection among populations**. To do so, I used a combination of **descriptive and experimental approaches** in a microevolutionary perspective.

More specifically, I addressed the following questions:

1. Do we observe divergent selection on flowering phenology and floral traits between two closely related and morphologically similar orchid species (I)?

2. What is the contribution of pollinators relative to other selective agents to phenotypic selection on floral traits, including flowering phenology, visual display traits, floral scent and a trait influencing pollination efficiency, spur length (II, IV)?

3. What is the relative contribution of diurnal and nocturnal pollinators to reproductive success of *Gymnadenia conopsea* s.s. in southern Sweden (II, IV)? Do these two categories of pollinators mediate different patterns of selection (II, III, IV)?

4. Does spatial variation in pollinator communities, including variation in the relative importance of diurnal and nocturnal pollinators, cause spatial variation in selection (II, III)?

Material and Methods

This thesis is based on four papers, using a combination of descriptive and experimental approaches.

In **paper I**, I document net phenotypic selection in several populations of *G. conopsea* s.s. and *G. densiflora* to test for divergent selection between the two species.

In **paper II**, I experimentally quantify the contribution of pollinators to net selection in a subset of the populations included in **paper I**.

In **papers III** and **IV**, I focus on a complex and understudied trait, **floral scent**. In **paper III**, I characterize spatiotemporal variation in floral scent emission of *G. conopsea* s.s. in multiple populations in the field and in controlled conditions. In **paper IV**, I experimentally quantify pollinator-mediated selection on scent compounds in one of the populations included in **paper III**.

The fragrant orchids *Gymnadenia conopsea* s.s. and *Gymnadenia densiflora*

Gymnadenia conopsea (L.) s.l. is a terrestrial orchid distributed across Eurasia (Hultén and Fries 1986). The tuberous, non-clonal and long-lived perennial plants occur on calcareous soils in grazed meadows and margins of marshes and fens (Øien and Moen 2002). The *Gymnadenia* complex is highly variable with regards to morphology, flower colour, scent production, flowering phenology and habitat (Soliva and Widmer 1999; Gustafsson and Lönn 2003; Jersáková et al. 2010; Stark et al. 2011).

Phylogeny and taxonomy

The most recent classification recognizes two taxa within *Gymnadenia conopsea* s.l.: *G. conopsea* (L.) R.Br. s.s. and *G. densiflora* A. Dietr (Bateman et al. 2003; Stark et al. 2011). These two taxa do not have a sister-species relationship as phylogenetic analyses of the genus have shown that *G. odoratissima* is the sister species of *G. conopsea* s.s. (Bateman et al. 2003; Sun et al. 2015). *G. conopsea* s.s. exhibits ploidy level variation, with presence of

diploids and tetraploids reported in several European locations (Travnicek et al. 2010, 2012; Stark et al. 2011).

Floral morphology and phenology: similarities and differences between the two species

Both species produce fragrant pink flowers that are similar in morphology and colour (Jersáková et al. 2010; *Figure 2*). The plants produce a single inflorescence of ca 10-100 flowers. Flowers open sequentially from the bottom to the top of the inflorescence. 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. *Gymnadenia conopsea* s.s. plants are self-compatible, but depend on pollinators for successful fruit set (Sletvold et al. 2012a).

The available literature indicates that diploid *G. conopsea* s.s. flowers earlier than tetraploid *G. conopsea* s.s. and *G. densiflora* (*Figure 2*C, Jersáková *et al.*, 2010), and produces shorter inflorescences with less flowers than *G. densiflora* (Stark et al. 2011). The two species also differ in floral scent (Jersáková et al. 2010). In contrast, there is no consistent difference in spur length, as diploid *G. conopsea* s.s. had shorter spurs than *G. densiflora* in a study conducted in the Czech Republic (Jersáková et al. 2010), while the opposite has been reported in Germany (Stark et al. 2011).

Species identification by flow cytometry

Large within-species variation in flowering phenology and floral morphology, as well as variation in ploidy levels within *G. conopsea* s.s. (Jersáková et al. 2010; Stark et al. 2011; Travnicek et al. 2012) makes it difficult to identify taxa reliably in the field without genetic identification (Stark et al. 2011). This is why I used flow cytometry to reliably identify species for each of my study populations on the island of Öland, southern Sweden (*Figure 3*B), where both species co-occur. Leaf samples from *Gymnadenia* were collected in 2014 and analysed by the Plant Cytometry Services company in the Netherlands following the protocol of Travnicek et al. 2010.

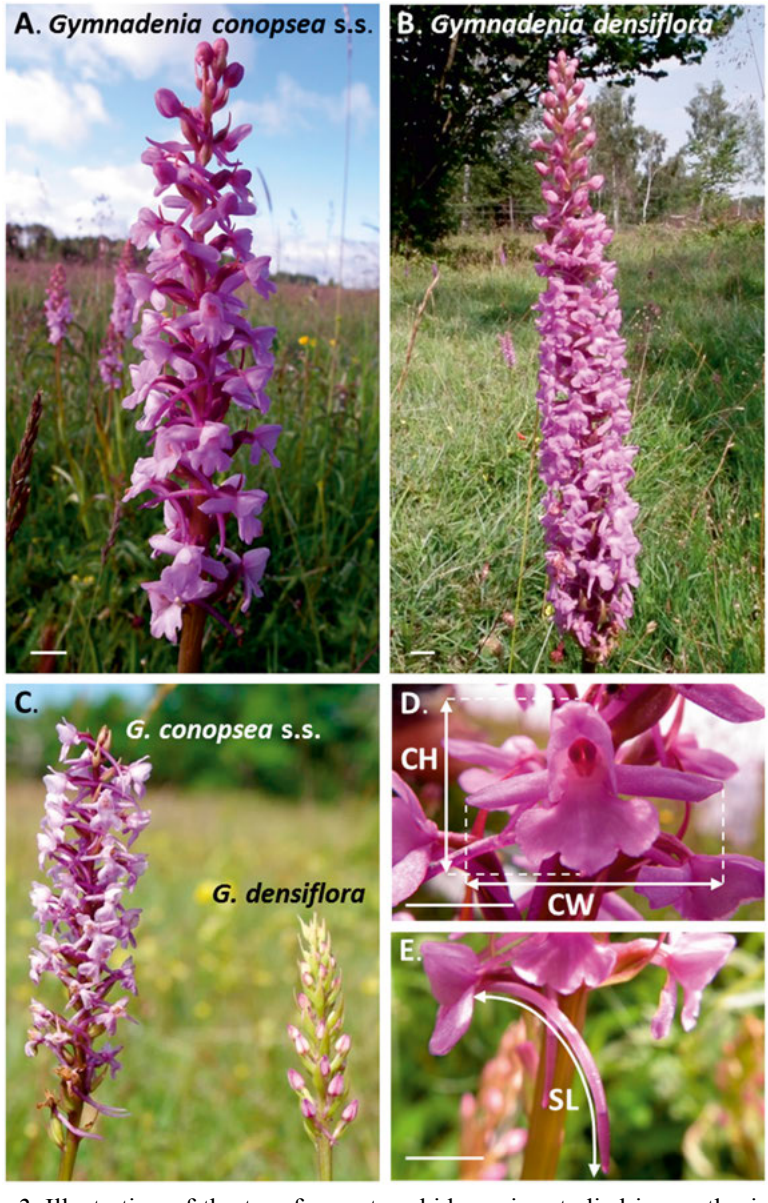


Figure 2. Illustration of the two fragrant orchid species studied in my thesis, *Gymnadenia conopsea* s.s. (**A**) and *Gymnadenia densiflora* (**B**) which differ in flowering time (**C**). The flowers of the two species are morphologically similar, and I measured their corolla area as the product of corolla height (CH) and corolla width (CW) (**D**) and their spur length (SL) (**E**). The horizontal white bars indicate 0.5 cm scale.

Pollinators

Gymnadenia conopsea s.s. and *G. densiflora* have a semi-generalized pollination system: they are visited by numerous species but most of them are lepidopterans (Claessens and Kleynen 2011) and thus belong to similar functional groups (*sensu* Fenster et al. 2004). Both species are pollinated by diurnal and nocturnal lepidopteran species. *Gymnadenia conopsea* s.s. can also be visited by long-tongued diptera of the *Empis* genus in some populations (Sletvold et al. 2012b, E. Chapurlat, pers. obs.). The observations I collected show that on Öland, where I conducted most of my fieldwork, the two species share several nocturnal pollinators, namely *Autographa gamma*, *Deilephila porcellus* and *Hyles gallii*, but *G. conopsea* s.s. is also pollinated by additional nocturnal lepidopterans. In contrast, diurnal pollinators differ for the two species.

Study populations

In **paper I**, I quantified net phenotypic selection in **six populations of *G. conopsea*** s.s. and **four populations of *G. densiflora*** located in the central part of Öland, an island in the Baltic sea, off the coast of southern Sweden (*Table 1, Figure 3B*). Both species grow in sympatry at five of the nine study sites, but except for Gråborg, I estimated selection in only one of the species at each site.

In **paper II**, I quantified pollinator-mediated selection in **four populations that are a subset** of the ten populations included in **paper I** (*Table 1*).

In **paper III**, I quantified diurnal and nocturnal floral scent emission in *G. conopsea* s.s. in **four populations on Öland** and **two populations in central Norway** (*Table 1, Figure 3*). I chose to include the two Norwegian populations because previous studies had characterized pollinator communities and shown that diurnal pollinators contributed more to reproductive success than nocturnal pollinators in these populations (Sletvold and Ågren 2010; Sletvold et al. 2012b).

In **paper IV**, I quantified pollinator-mediated selection on floral scent in **one of the Öland populations from paper III** (*Table 1, Figure 3B*).

Table 1. Summary of data included in each paper of this thesis. The name of each population is abbreviated as follows (for locations, see maps in *Figure 3*): F = Folkeslunda, G = Gråborg, Ig = Igelmossen, Is = Ismantorp, Ka = Kalkstad, Kv = Kvinneby, L = Långlöt, Me = Melösa, Mö = Mörbylånga, Ö = Österskog, S = Sølendet, T = Tågdalen. Pollination treatments are abbreviated as follows: C = open-pollinated control, HP = hand-pollination, D/N = diurnal and nocturnal exclusion experiment.

Paper	Year	Species	Populations	C	HP	D/N	Scent
I	2012	*G. conopsea* s.s.	G, Ka, Kv, L, Me, Mö	×			
		G. densiflora	G, Ig, Is, Ö				
II	2012	*G. conopsea* s.s.	Kv, L, Me	×	×	×	
		G. densiflora	G	(data of I)		(L, Me)	
III	2013	*G. conopsea* s.s.	F, Kv, L, Me, T, S				×
IV	2016	*G. conopsea* s.s.	F	×	×	×	×

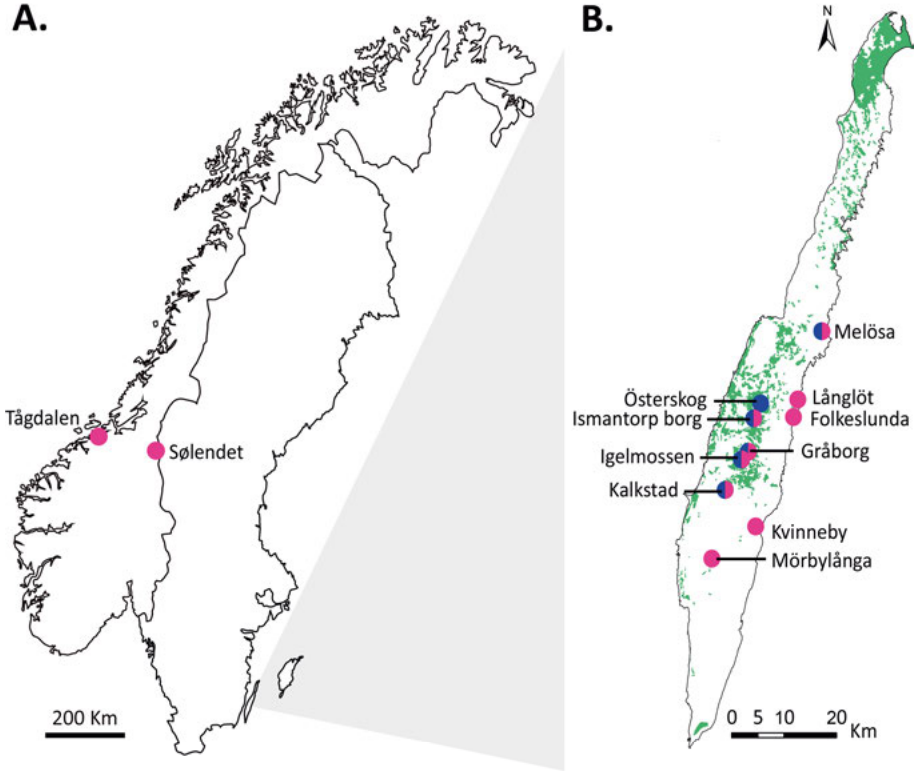


Figure 3. **A**. Locations of the two Norwegian *Gymnadenia conopsea* s.s. populations included in paper III. **B**. Map of all the sites studied on the island Öland in south-eastern Sweden in papers I to IV. Pink = *Gymnadenia conopsea* s.s., blue = *Gymnadenia densiflora*, while symbols with mixed colours correspond to sites where both species occur in sympatry.

Pollinator exclusion experiment (II and IV)

To **quantify the respective contribution of diurnal and nocturnal pollinators to reproductive success**, I conducted a pollinator exclusion experiment in three of my study populations on Öland (*Table 1*): Melösa and Långlöt in 2012 (**paper II**) and Folkeslunda in 2016 (**paper IV**). In each population, 240 (**paper II**) or 60 (**paper IV**) plants were tagged and randomly assigned to each of two treatments: diurnal pollination (D) or nocturnal pollination (N). Plants in the D treatment were caged during night (18:00 h – 06:00 h), receiving only diurnal visits, and plants in the N treatment were caged during day (06:00 h – 18:00 h), receiving only nocturnal visits. Caging continued until all flowers had wilted. The cages were made of a white mosquito net wrapped around a metallic wire cylinder.

I used one-way ANOVA to examine the effects of selective pollinator exclusion (diurnal *vs.* nocturnal pollination) on reproductive performance. Number of flowers and fruits were analyzed with generalized linear models with a quasi-poisson error distribution, because of overdispersion.

Floral scent sampling and analyses (III and IV)

Sampling design

As *G. conopsea* s.s. is pollinated both at day and at night, I decided to study floral scent emissions during both periods. For each plant, I **always sampled volatiles for one hour during the day and one hour during the night**, at the time corresponding to peak activity of the pollinators.

In **paper III**, my goal was to test whether scent emission rhythms during day and night differed between populations in southern Sweden and in central Norway, where nocturnal pollinators are less important for reproductive success. In 2013, I **sampled plants *in situ* in four populations in southern Sweden and in the two Norwegian populations**. In 2015, to test whether patterns described in the field were due to plastic responses to environmental factors, I **transferred plants from two of the southern populations and the two Norwegian populations to a growth chamber** where all plants were exposed to temperature and photoperiod conditions intermediate to field conditions in Norway and Sweden.

In **paper IV**, I sampled all plants included in the selection analysis *in situ* in the natural population.

Dynamic headspace scent sampling and sample preparation

I used a method called **dynamic headspace sampling (*Figure 4*), which allows the calculation of a standardized emission rate** of each scent compound. Inflorescences were enclosed in oven bags together with a scent trap (*Figure 4*B). Air was extracted from the bags through a small hole at the top of the bag by a pump maintaining a steady flow of 200 mL/min monitored by flow meters (*Figure 4*A). At each sampling occasion, a control sample of ambient air was collected to identify background contamination (*Figure 4*C). After sampling, adsorbed volatiles were eluted from the traps with hexane, stored at -20°C before being concentrated to 50µL. An internal standard of 5µL of 0.03% toluene solution (1300 ng) was added to each sample.

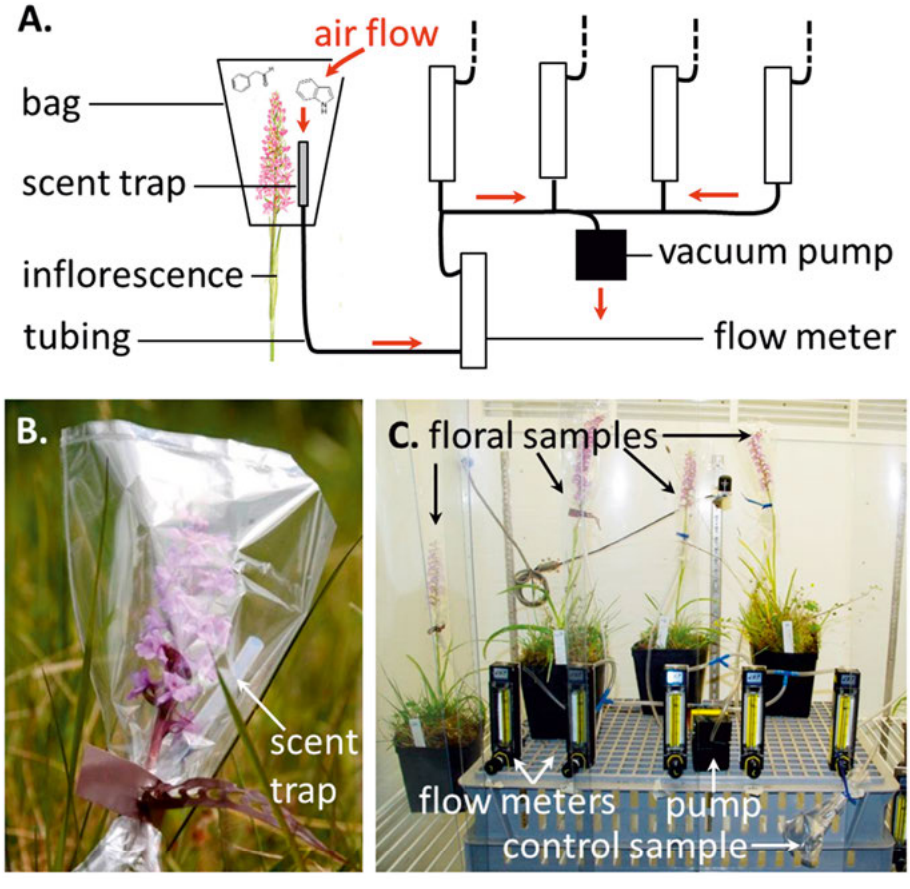


Figure 4. **A**. Diagram illustrating the set-up used for the dynamic headspace sampling of floral scent **B**. Detail of a *Gymnadenia* inflorescence enclosed in a bag together with a scent trap (Photo: Nina Sletvold) **C**. Set-up used in the growth chamber (**paper III**) showing the simultaneous sampling of several inflorescences and of one air control (empty bag).

Gas chromatography-Mass spectrometry (GC-MS) analysis

To identify the scent compounds present in the *G. conopsea* s.s. floral bouquet, I analysed the floral scent samples with a gas chromatograph connected to a mass spectrometer. The gas chromatograph separates the volatile compounds present in the sample by a slow increase in temperature: the most volatile compounds exit the chromatograph first while heavier, less volatile compounds necessitate higher temperatures. The mass spectrometer then fragments the volatile compounds that exit the chromatograph and detects the nature and quantity of ions produced by this fragmentation, which allows compound identification and quantification.

Compound scoring and estimation of emission rates

Most compounds were identified by verification of MS library suggestion using Kovats retention index values obtained from the literature, and some using authentic standards rerun on the GC-MS. The remaining compounds were denoted as unknowns. In **paper III**, I manually integrated the floral volatile peaks, while in **paper IV**, I developed an automatic scoring method using the MS manufacturer's software.

I **calculated standardized emission rates per inflorescence** (SEM) of each compound as follows (from Svensson et al., 2005, amount of standard was 1300 ng per sample):

$$SEM_{compound\ i}(ng.h^{-1}) = \frac{peak\ area_{compound\ i}}{peak\ area_{standard}} \times standard\ amount\ in\ ng$$

In **paper III**, I added the emission rates of all compounds to obtain total scent emission rate per inflorescence.

Statistical analyses on floral scent

In **paper III**, to test whether total floral scent emission rates varied among populations and differed between day and night, I analysed the standardized emission rate per inflorescence and per flower with a **repeated measures ANOVA** including sampling period (day or night) as a within-subject factor, population as a between-subject factor, and their interaction. In this analysis, a significant interaction term indicates that the difference between diurnal and nocturnal emission varies among populations. I used **planned contrasts** to test if the effect of period (day *vs.* night) on scent emission differed between the two Norwegian populations mainly pollinated by diurnal and the four Swedish populations mainly pollinated by nocturnal pollinators. **Pairwise t-tests** were used to determine in which populations the difference between day and night was statistically significant. I analysed field and growth chamber data in separate analyses.

To examine **qualitative variation in the floral scent bouquets**, I conducted **multivariate analyses** based on the proportion of each compound with the *vegan* package (Oksanen et al. 2015). I explored differences in scent composition between groups of samples (populations (III) or pollination treatment (IV) and period of sampling) graphically by **nonmetric multidimensional scaling** (NMDS) with different symbols for each group. I also examined variation among groups with **nonparametric multivariate analysis of variance** (perMANOVA, function *adonis*, 10 000 permutations). In **paper III**, I also conducted a **similarity percentage analysis** (SIMPER, function *simper*) on proportions to determine which compounds contributed the most to the difference between diurnal and nocturnal samples.

Gas chromatography coupled to electroantennographic detection (GC-EAD) (IV)

To **determine which floral scent compounds can be detected by the different pollinator** species observed in the study population of **paper IV**, I used GC-EAD (*Figure 5*). As for the GC-MS analysis, the gas chromatograph separates the compounds present in the floral scent sample. In GC-EAD, the gas effluent that exits the chromatograph and contains the floral volatile compounds is split to reach simultaneously a flame ionization detector (FID) on one side and the antennae of the insect on the other side (*Figure 5*A). The FID records when the volatile compounds exit the chromatograph, while electrodes record the antennal responses, and the two simultaneous traces can then be compared to identify which compounds elicit antennal responses (*Figure 5*B). To confirm the identity of the compounds detected by the FID, the floral scent used for the GC-EAD runs was analyzed by GC-MS with the same settings as for the GC-EAD analysis.

I **performed GC-EAD with two important local pollinators**: a nocturnal pollinator, *Deiliphila porcellus* (Sphingidae, 8 runs) and a diurnal pollinator, *Aglais urticae* (Nymphalidae, 6 runs). To complete this data, I **also searched the literature for GC-EAD data** for the different pollinator species observed in the study population (i.e. *Aglais urticae*, *Autographa gamma*, *Cucullia umbratica* and *Deilephila porcellus*). I did not find any data for *C. umbratica*.

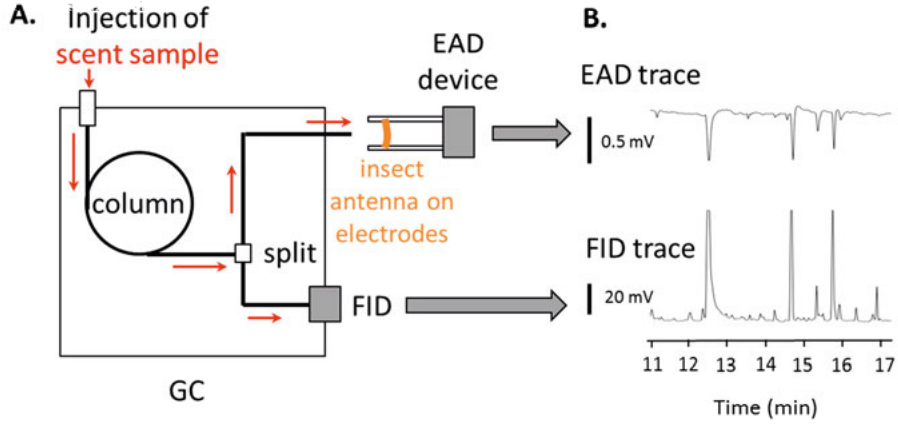


Figure 5. **A**. Diagram of GC-EAD set-up. The red arrows show the gas flow carrying the scent sample through the column of the gas chromatograph (GC), where the different scent compounds are separated, to the antenna connected to the electroantennographic detection (EAD) device and to the flame ionization detector (FID). **B**. Examples of EAD and FID traces obtained for an antenna of *Deilephila porcellus* exposed to the floral scent sample of *Gymnadenia conopsea* s.s.

Phenotypic selection analyses (I, II and IV)

Floral traits included in the analyses

In **papers I, II and IV**, I quantified phenotypic selection through female fitness on five traits: **flowering start, plant height, number of flowers, corolla area** (quantified as the product of corolla width and corolla height, *Figure 2*D) and **spur length** (*Figure 2*E).

In **paper IV**, I **also included 14 floral scent traits** in the selection analysis. There was a large number of potential scent traits, and I used, prior to analysis, the following criteria to reduce the set of scent variables to include in the selection model:

(1) I selected compounds for which we have GC-EAD evidence of response in at least one of the pollinator species;
(2) I used only compounds present in at least 20% of the scent samples collected at day (for compounds detected by *A. gamma* or *A. urticae*) or at night (for compounds detected by *D. porcellus* or *A. gamma*);
(3) I removed three variables because they had a correlation greater than 0.60 with another volatile emission rate.

Furthermore, the amount of scent emitted varied across the different days of sampling. To remove this effect, I used as scent variable for compound *i* the residuals of a linear model including scent emission rate of compound *i* as

response variable and sampling date (treated as a categorical variable) as explanatory variable.

Female fitness

For each plant, I estimated female fitness as the **product of number of fruits and mean fruit mass**. I recorded the number of fruits and harvested three mature capsules spread across the inflorescence to estimate fruit mass.

Estimation and analysis of selection gradients

I estimated **directional** (I, II and IV)**, quadratic** (I and II) **and correlational** (II) **selection gradients** using **multiple regression analyses** with relative female 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, following Lande & Arnold (1983). In **paper I**, I hypothesized that divergent selection between *G. conopsea* s.s. and *G. densiflora* could be driven by linear selection in different directions or by stabilizing selection for different optima, and I thus report only directional and quadratic selection gradients. In **paper II**, I estimated and reported all gradients except for the Kvinneby population, which had too low sample size to allow for estimation of quadratic and correlational gradients. In **paper IV**, due to the high number of variables in the selection model, I only estimated directional selection gradients.

To visualize non-linear (quadratic and correlational) gradients, I used **added-variable plots**. To produce these plots, the residuals from a linear regression of relative fitness on all floral traits except the focal trait(s) are plotted against the set(s) of residuals obtained when regressing the focal trait(s) on the remaining floral traits. To visualize correlational selection gradients, I used three-dimensional plots (Cook and Weisberg 1989), where the surfaces were estimated and plotted using the *gam* and *vis.gam* functions of the *mgcv* package (Wood 2006).

Net selection (I, II, and IV)
I estimated selection gradients among open-pollinated control plants (C), which represent **net selection** gradients. Statistical significance of net gradients was assessed by the multiple regression models.

Test of divergent selection between G. conopsea *s.s. and* G. densiflora (I)
To test for divergent linear selection, I conducted for each floral trait a **one-sided Welch t-test** on the linear selection gradients, with the alternative hypothesis being that selection gradients are greater in the species with the largest mean trait value. I examined whether there was stabilizing selection (presence of an intermediate optima) with the **added-variable plots**.

Pollinator-mediated selection (II and IV)
To separate the contribution from pollinators to net selection, I used a **hand-pollination experiment**. The selection gradients estimated for plants receiving supplemental hand-pollination (HP) represent **non-pollinator mediated selection**. Statistical significance of non-pollinator mediated gradients was assessed by the multiple regression models for HP plants.

I then estimated **pollinator-mediated selection** gradients by **subtracting gradients obtained for HP plants from gradients obtained for C plants**. **Significance** of pollinator-mediated selection was tested using an **ANCOVA** model including relative fitness as the response variable and the standardized floral traits and trait × pollination treatment interactions as explanatory variables.

Selection by diurnal and nocturnal pollinators (II)
To test whether diurnal and nocturnal pollinators mediated different patterns of selection, I used the **pollinator exclusion experiment** and estimated selection gradients among plants exposed only to diurnal pollinators (D) or to nocturnal pollinators (N). To determine **whether linear selection gradients differed between the D and N treatments**, I used a similar **ANCOVA** model as for testing pollinator-mediated selection with pollination treatment now being D and N.

Spatial variation in selection and correspondence between net and pollinator-mediated selection (II)
To determine **whether net directional selection varied among populations**, I analysed data from the control treatment with an **ANCOVA** including relative fitness as the response variable and the five standardized traits (flowering start, plant height, number of flowers, corolla size and spur length), population and the trait × population interactions as explanatory variables. I also tested **whether pollinator-mediated directional selection varied among populations** using an **ANCOVA** model including both the C and HP treatments in the four populations. The model included relative fitness as the response variable and the five standardized traits (flowering start, plant height, number of flowers, corolla size and spur length), pollination treatment (C *vs.* HP), population, and trait × pollination treatment, trait × population, and trait × pollination treatment × population interactions as explanatory variables. To estimate the **extent to which variation in pollinator-mediated selection explained variation in net selection**, I **regressed** net selection on pollinator-mediated selection separately for each floral trait.

Results and Discussion

Divergent selection on flowering time but not on floral morphology between two closely related species (I)

Partially consistent with my hypotheses, there was **indication of divergent linear selection on flowering time in the expected direction**, with selection for earlier flowering in two of the early-flowering *G. conopsea* s.s. populations, and for later flowering in two of the late-flowering *G. densiflora* populations (*Figure 6*A). In contrast, there was **no evidence of divergent selection on morphological traits** (*Figure 6*B-E), in spite of significant differences for most of these traits between the two *Gymnadenia* species**.** No significant stabilizing selection was detected.

Phenological isolation between two plant taxa is the earliest premating barrier possible and has the greatest potential for reproductive isolation (Widmer et al. 2009). My results suggest that **divergent natural selection contributes to the marked phenological differentiation** between *Gymnadenia conopsea* s.s. and *Gymnadenia densiflora* and **should reinforce** this barrier in this system. **Interspecific pollen deposition during the overlapping flowering period may be costly** and could potentially cause such divergent selection on flowering time (Nuismer and Cunningham 2005). The quantification of interspecific pollen transfers together with experimental crosses between the two *Gymnadenia* species would be necessary to test this hypothesis. **Abiotic agents or temporal variation in pollinators could also cause the observed selection on phenology** (Pilson 2000; Elzinga et al. 2007; Sandring and Ågren 2009; Sletvold et al. 2010, 2015).

In contrast, my results indicate that current selection patterns do not explain morphological floral divergence between the two species. This suggests that **non-adaptive processes may play a role in the floral trait differentiation** between these two species, or that **selection has driven it historically but is not strong any longer** (Harder and Johnson 2009). Further investigations are needed to fully understand to what extent floral differentiation between *G. conopsea* s.s. and *G. densiflora* is adaptive.

In paper I, I showed divergent net selection on flowering phenology between two closely related species. However, an experimental approach is necessary to identify the sources of this selection and better understand what may have contributed to floral divergence between these two species.

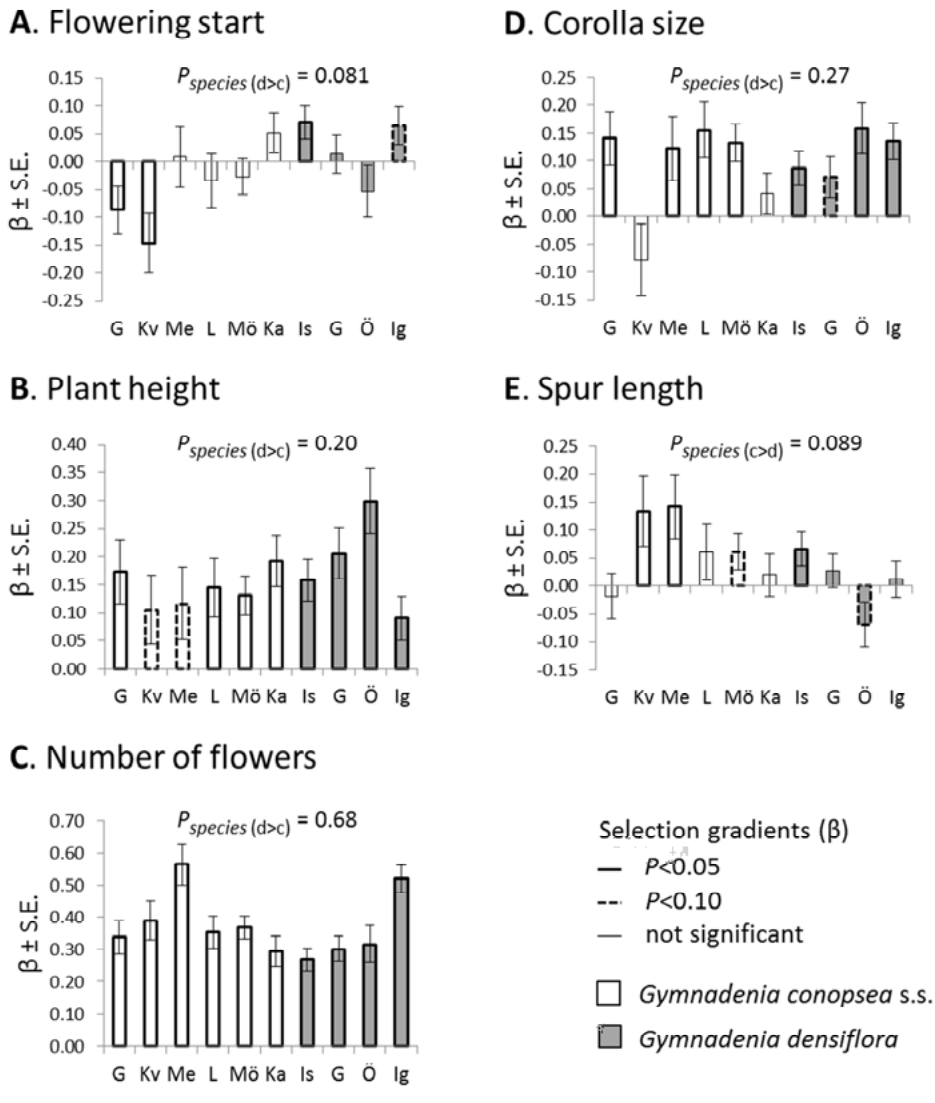


Figure 6. Linear selection gradients for five floral traits (panels A to E) in the six populations of *Gymnadenia conopsea* s.s. (white bars) and four populations of *Gymnadenia densiflora* (grey bars) in 2012 (**paper I**). The name of each population is abbreviated on the x axis as follows: G = Gråborg, Ig = Igelmossen, Is = Ismantorp, Ka = Kalkstad, Kv = Kvinneby, L = Långlöt, Me = Melösa, Mö = Mörbylånga, Ö = Österskog. Populations are ordered by mean flowering start. The P-value associated with the one-sided Welch t-test testing for differences in selection gradients between the two species is indicated above each bar-plot, with the tested alternative hypothesis indicated between parenthesis (c = *conopsea* s.s., d = *densiflora*, > = "greater than")

Pollinators contribute to selection on floral traits, notably to floral scent, in complex ways (II, IV)

In the two studies where I experimentally quantified pollinator-mediated selection (**papers II** and **IV**), I document significant pollinator-mediated selection on number of flowers, corolla size, spur length and floral scent. However, the strength and direction of pollinator-mediated selection varied among floral traits.

In **paper II**, **pollinators contributed significantly to the strong selection for more flowers** observed in one of the four study populations, consistent with the well-known role of display size in pollinator attraction (see e.g. Grindeland et al. 2005; Makino et al. 2007 and references therein). **More surprisingly, pollinators mediated selection for smaller corollas** in another population, opposing non-pollinator-mediated selection. In *G. conopsea* s.s., smaller flowers have narrower spurs (correlation between corolla size and spur diameter, $r = 0.28$, $p < 0.0001$, $n = 197$, N. Sletvold, unpublished data), which may increase pollination efficiency by facilitating pollinium transfer to the proboscis of the pollinator. Lepidopteran pollinators select for narrower floral tubes in other systems too (Campbell et al. 1997; Kulbaba and Worley 2012). Finally, **all observed selection for longer spurs was pollinator-mediated**, paralleling findings from Norwegian *G. conopsea* s.s. populations (Sletvold and Ågren 2014).

In **paper IV**, I document **significant pollinator mediated-selection on several floral scent compounds** (*Figure 7*). Given that floral scent attracts pollinators (e.g. Huber et al. 2004; Theis 2006; Andrews et al. 2007; Byers et al. 2014; Friberg et al. 2014b; Bischoff et al. 2015), pollinator-mediated selection for increased scent emissions is expected in natural populations (Schiestl 2015). I indeed found that **pollinators mediated selection for higher emission rates** of methyl eugenol and benzyl alcohol (*Figure 7*A-C) **but I also detected equally or even stronger pollinator-mediated selection for reduced emission** of indole and p-cresol (*Figure 7*D-E). All four compounds are present in the odor of many plant species, suggesting broad functional importance (Knudsen et al. 2006; Tan and Nishida 2012; Cna'ani et al. 2018). However, it appears that the function of each specific compound is highly context-dependent (Kite et al. 1998; Ômura et al. 1999; Andrews et al. 2007; Mishra and Sihag 2009; Tan and Nishida 2012; Bischoff et al. 2015; Cna'ani et al. 2018), and behavioral tests would be needed to identify the roles of these different compounds in this study system.

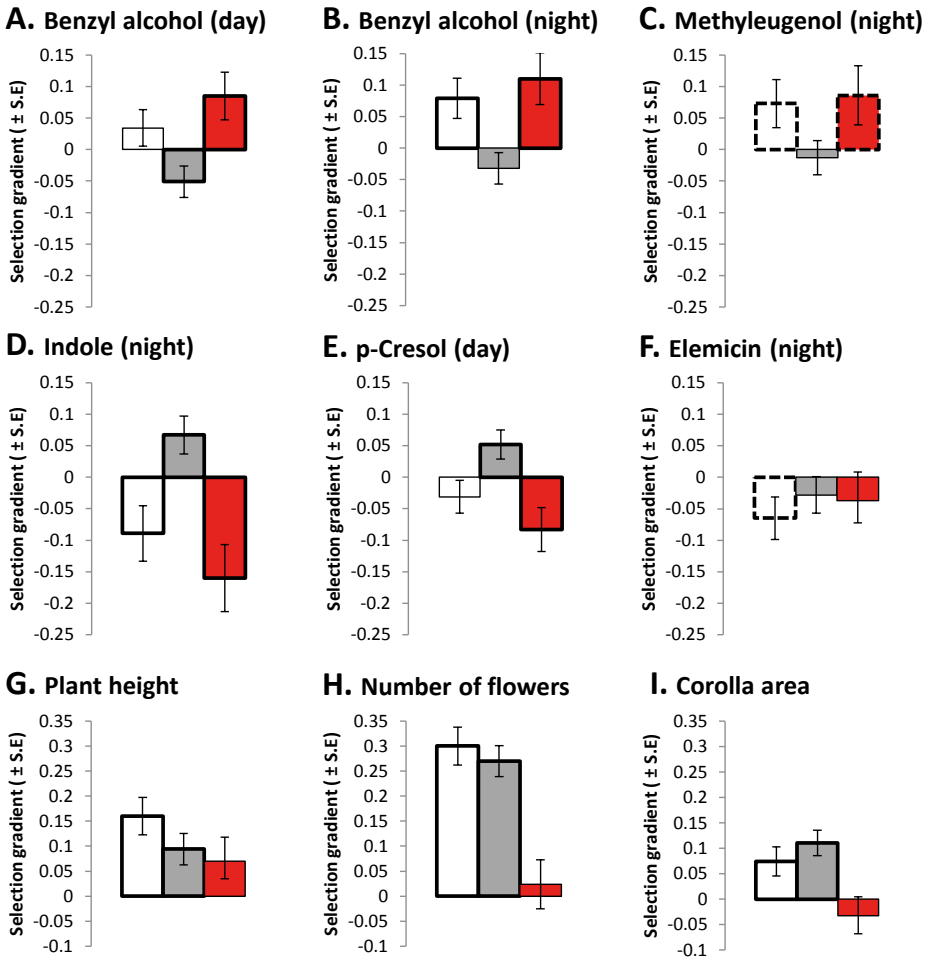


Figure 7. Selection gradients on six floral scent traits (A-F) and three visual display traits (G-I, note the different scale of the y-axes) among open-pollinated plants (net selection, β_C, white bars), hand-pollinated plants (non-pollinator-mediated selection, β_{HP}, grey bars), and attributed to interactions with pollinators (pollinator-mediated selection, $\Delta\beta_{poll}$ estimated as $\beta_C - \beta_{HP}$, red bars) in the *Gymnadenia conopsea* s.s. population at Folkeslunda in 2016 (**paper IV**). Significant ($P < 0.05$) and marginally significant ($P < 0.07$) gradients are indicated by thicker solid and dashed outlines, respectively. For the floral scent compounds, the period of emission is indicated between brackets. Only traits for which a significant or marginally significant gradient was detected in at least one of the pollination treatments are shown.

More surprisingly, I also detected significant non-pollinator mediated selection on several of the compounds, which in most of the cases opposed the pollinator-mediated selection (*Figure 7*). While selection by antagonists is likely to be important in many systems where scent has been shown to attract or repel antagonists (Theis 2006; Andrews et al. 2007; Kessler et al. 2008, 2013; Galen et al. 2011; Theis and Adler 2012), we do not have any

evidence of damage by florivores or other herbivores in our study population. The non-pollinator mediated selection is thus unlikely to be caused by antagonists, but could rather be mediated by abiotic factors or correlations with traits not included in the analysis. This **prevalence of conflicting selection on scent traits** suggests that compounds that influence plant-pollinator interactions often have multiple functions, or are correlated with traits influencing plant fitness via other mechanisms.

It is noteworthy that the **targets of selection were not the major compounds** of the floral bouquet of *G. conopsea* s.s.. This is consistent with two previous studies that quantified phenotypic selection on individual scent compounds (Ehrlén et al. 2012; Parachnowitsch et al. 2012). The fact that several studies have reported selection on relatively minor compounds and also that the direction of selection varies among compounds (this study; Ehrlén et al. 2012) suggest that **studying single compounds provides additional insights compared to approaches measuring selection on total scent or principal components,** since the latter are driven by the major constituents of the floral bouquet. However, it should be noted that I only quantified directional selection, and it is thus possible that major compounds are under stabilizing selection. More generally, the major compounds may always be present in quantities above the saturation point for pollinator attraction, but response curves of pollinator behavior to compound concentration remain unknown.

Interestingly, I showed in **paper II** that **pollinators can also mediate selection on combinations of traits**, and were in fact responsible for most of the detected correlational selection (some examples are given in *Figure 8*). At Melösa, the positive correlational selection on number of flowers and plant height suggests that **these two display traits act synergistically on pollinator attraction** (Campbell et al. 2014) while the positive gradient for number of flowers and spur length rather suggests a **multiplicative effect of pollinator attraction and pollination efficiency on fitness** (Sletvold and Ågren 2011b). Correlational selection on pairs of display and efficiency traits may be common as it has been reported in all previous studies documenting correlational selection on floral traits (O'Connell and Johnston 1998; Benitez-Vieyra et al. 2006; Cuartas-Domínguez and Medel 2010; Reynolds et al. 2010; Bartkowska and Johnston 2012). At Långlöt, no linear estimate of pollinator-mediated selection was significant, but pollinators mediated selection for earlier flowering in combination with longer spurs, and against later flowering combined with smaller corollas. In *Lobelia cardinalis*, pollinators also mediated selection on trait combinations in a population with no significant linear pollinator-mediated selection (Bartkowska and Johnston 2012), indicating that **it may be necessary to consider trait combinations to detect pollinator-mediated selection**. Due to the large number of variables included in the selection analysis in **paper IV**, I could not estimate correlational selection, but the results of **paper II** suggests that more

traits could be targets of selection. This is plausible given that scent compounds can act in synergy with each other (Kessler et al. 2008; Raguso 2008) or with visual signals (Raguso and Willis 2005; Burdon 2016).

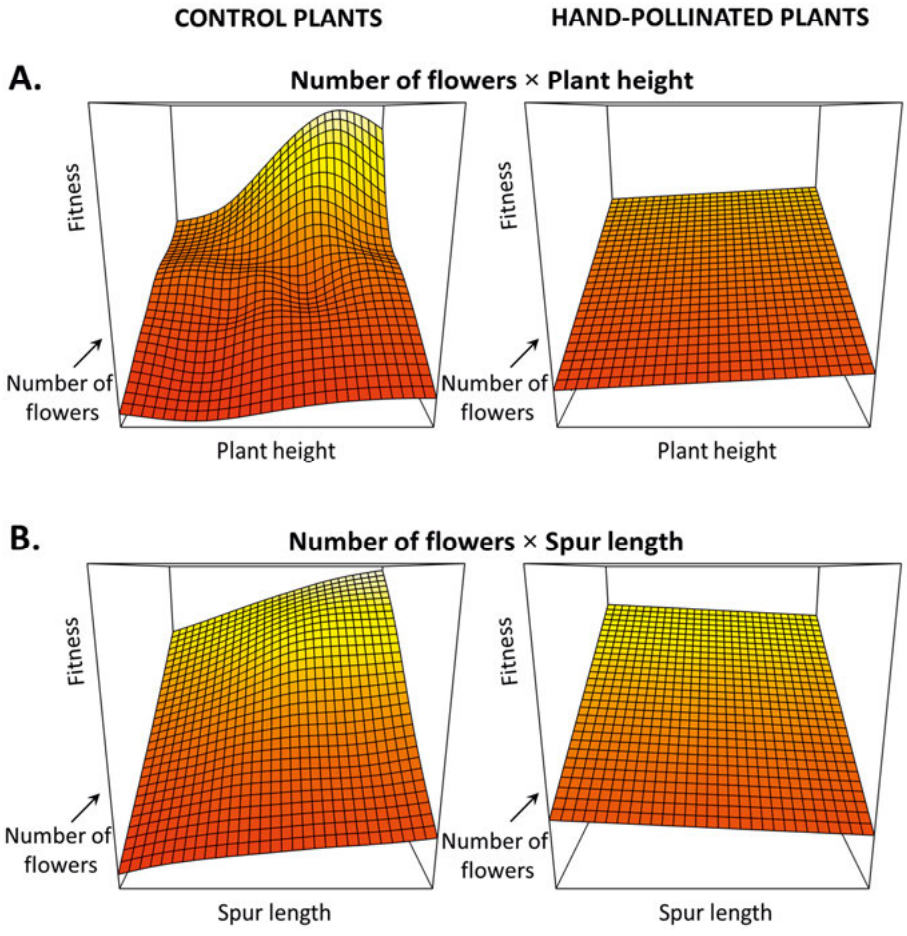


Figure 8. Fitness surfaces illustrating selection on combinations of traits (correlational selection) detected among open-pollinated control plants in the *Gymnadenia conopsea* s.s. population at Melösa in 2012 (**paper II**). There was no correlational selection among hand-pollinated plants (the fitness surface is a plan), indicating that correlational selection in control plants was pollinator-mediated.

Stronger pollinator-mediated selection is expected when the intensity of plant-pollinator interactions is lower, because less plants in the population are likely to be fully pollinated (Benkman 2013; Vanhoenacker et al. 2013; Trunschke et al. 2017). The intensity of plant-pollinator interactions can be measured by pollen limitation, which is quantified as 1 – (Fitness of control plants/Fitness of hand-pollinated plants): low pollen limitation indicates high intensity of pollination. **Contrary to expectation**, in **paper II**, pollinator-

mediated selection varied considerably despite similar pollen limitation in all four populations, while in **paper IV**, I detected relatively strong pollinator-mediated selection on several scent traits in spite of low pollen limitation. These results indicate the **importance of the functional relationship between pollinators and plant traits in driving strength and variation in pollinator-mediated selection** (Sletvold and Ågren 2014).

Studies that attempt to quantify selection on floral scent are still rare, and paper IV provides the first experimental quantification of pollinator-mediated selection on this complex trait. Taken together, the results of paper II and IV indicate that pollinators are the main selective agent on pollination efficiency traits, whereas their contribution to selection on phenology, visual and olfactory display traits is variable. More generally, I show that the targets, direction and strength of pollinator–mediated selection can be difficult to predict and that it can frequently oppose non-pollinator-mediated selection, strongly advocating for the experimental identification of agents of selection on floral traits.

Diurnal and nocturnal pollinators do not contribute equally to fitness, mediate different patterns of selection on floral traits, and are associated with differences in scent composition (II, III, IV)

In the three populations studied in **papers II** and **IV,** the selective exclusion experiment showed **that plants pollinated by nocturnal pollinators had higher fitness than those pollinated by diurnal pollinators in southern Sweden**, but this difference was significant only in the Folkeslunda population in 2016 (*Figure 9*). The difference in contribution of diurnal pollinators to fitness between **papers II** and **IV** could reflect **spatial or temporal variation in diurnal pollinators**, as the experiment was conducted in a different population and year in **paper IV**. It is worthy to note that in **paper II**, plants pollinated exclusively by diurnal or nocturnal pollinators had similar fitness as open-pollinated plants, showing that **each pollinator category in isolation can efficiently pollinate *G. conopsea* s.s.** Previous selective pollinator exclusion experiments demonstrated that nocturnal pollinators were more important than diurnal pollinators for seed set in two German populations at 51°N (Meyer et al. 2007), whereas the contrary was true in two populations in central Norway at 62-63°N (Sletvold et al. 2012b). Together with the results of **papers II** and **IV**, which were conducted at 56°N, this suggests a **latitudinal gradient in the relative importance of diurnal *vs.* nocturnal pollinators for *G. conopsea* s.s.**. With increasing latitude, day length in-

creases and night temperature decreases, which may be less favorable for nocturnal pollinators.

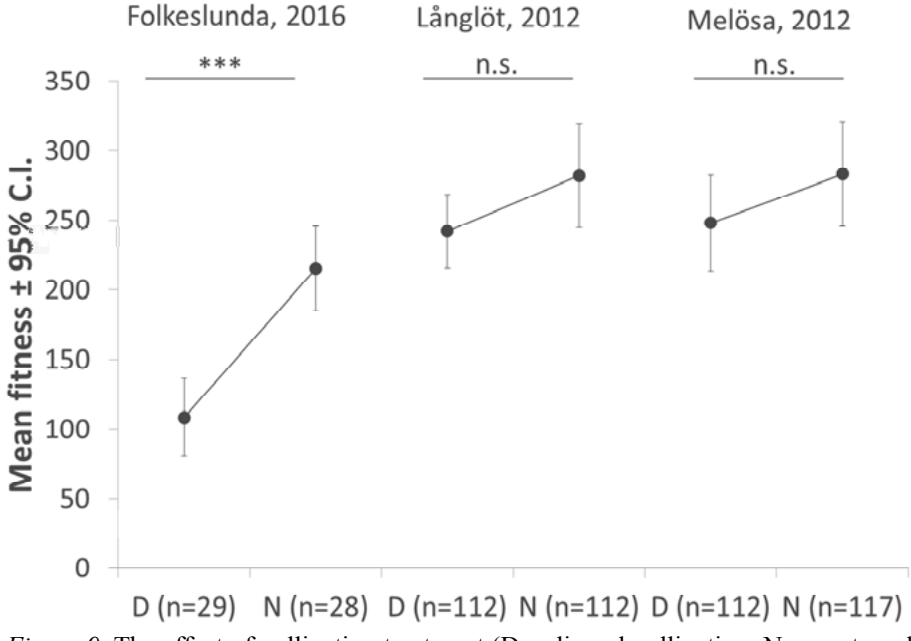


Figure 9. The effect of pollination treatment (D = diurnal pollination; N = nocturnal pollination) on female fitness in the *Gymnadenia conopsea* s.s. populations at Folkeslunda in 2016 (**paper IV**), and at Långlöt and Melösa in 2012 (**paper II**). C.I.: confidence interval estimated as ±1.96 S.E. The statistical significance of the effect of pollination treatment (D *vs.* N) is indicated at the top (n.s., not significant; ***, P<0.001).

In **paper II**, I **experimentally demonstrate** that **diurnal and nocturnal pollinators can mediate different patterns of both directional and correlational selection** by estimating selection in each exclusion treatment while **papers III** and **IV** provide **indirect evidence** that the two categories of pollinators mediate different patterns of selection on **floral scent**.

Plants pollinated both at day and at night encounter two guilds of pollinators belonging to different functional groups (sensu Fenster et al. 2004), and thus, if the evolution of floral scent has been shaped by plant-pollinator interactions, it can be expected that the composition of the floral scent bouquet should differ between day and night. Consistent with this hypothesis, in **paper III**, I show **a change in the composition of the floral scent bouquet of *G. conopsea* s.s. between day and night** (*Figure 10*). This was also the case in all studies that have characterized floral scent in plants pollinated both at day and night (Huber et al. 2004; Morinaga et al. 2008; Dötterl et al. 2012; Jürgens et al. 2014; van der Niet et al. 2015; Prieto-Benítez et al. 2016). However, **paper III** only provides correlative evidence and does not prove

that this change in floral scent throughout the day is adaptive, especially given that floral scent can change due to variation in temperature, light and humidity between day and night. This is why I tested for pollinator-mediated selection on floral scent in **paper IV**.

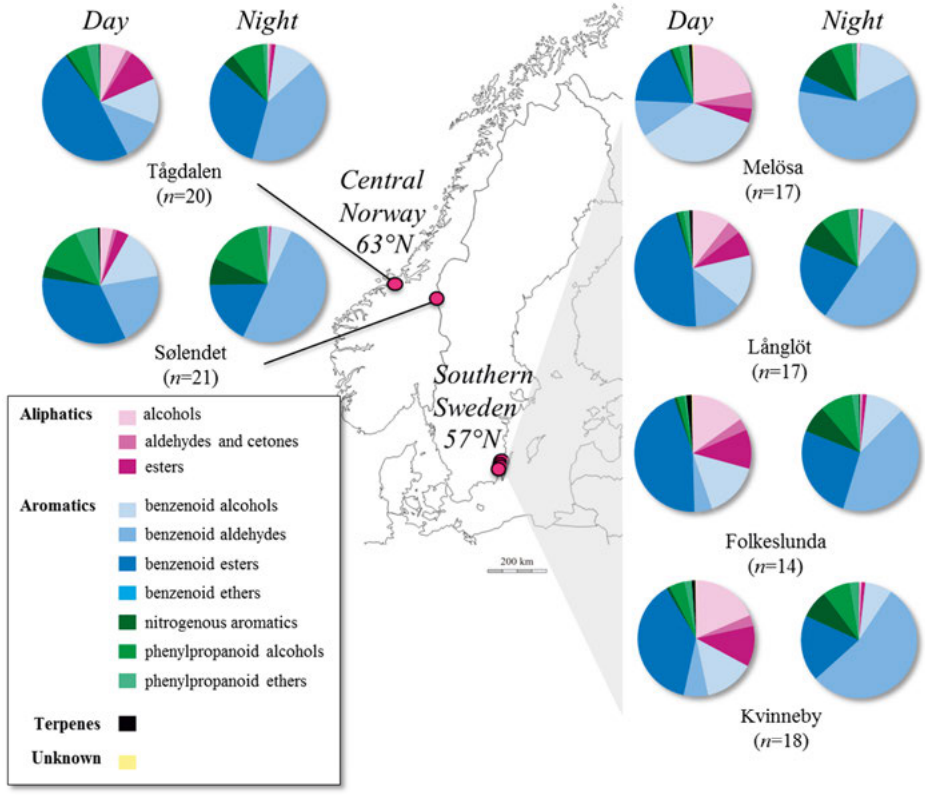


Figure 10. Floral scent variation between the diurnal (Day) and nocturnal samples (Night) in the six study *Gymnadenia conopsea* s.s. populations sampled in the field in 2013 in **paper III**.

In **paper IV**, I showed that **pollinators mediated selection on nocturnal and diurnal scent emission rates** of different scent compounds (*Figure 7*). Contrary to **paper II**, I did not estimate selection in pollinator exclusion treatments, and thus I cannot conclusively link selection on scent during day and night to each respective pollinator category. Still, it seems likely that pollinator-mediated selection on nocturnal or diurnal rates is mediated by the species that are active during the respective period of the day and thus, to the exception of benzyl alcohol, **each pollinator category appears to target different compounds** (*Figure 7*). However, patterns of selection between day and night did not fully correspond to what could be expected based on observed differences in absolute emission rates between day and night in *Gymnadenia conopsea* s.s. described in **paper III**. Selection for higher noc-

turnal emission rates of methyl eugenol and benzyl alcohol is in line with an increase of emission of these compounds at night, but selection for higher diurnal emission of benzyl alcohol suggests that this compound is also important for attracting diurnal pollinators. More surprisingly, I observed selection for reduced nocturnal emission rates of indole and elemicin, two compounds that increase at night. The **lack of congruence between diel patterns of scent emission and current net selection** suggests that the observed difference in emission rate between day and night does not necessarily represent an equilibrium or that there is temporal variation in selection on scent, as observed in a closely related species (Gross et al. 2016). Furthermore, the prevalence of conflicting selection by pollinators and other agents of selection on floral scent demonstrated in **paper IV** suggests that this floral trait is shaped by multiple factors and the results of **paper III** should not be interpreted solely in the light of pollinator-mediated selection.

Taken together, the results of papers II, III and IV suggest that even pollinators that are less important for reproductive success can exert selection on floral traits, including floral scent. Additionally, selection mediated by diurnal and nocturnal pollinators differed, indicating that variation in the relative importance of the two pollinator categories may contribute to spatial variation in selection.

Spatial variation in pollinator communities causes spatial variation in selection and is associated with genetic variation in floral scent rhythms (II, III)

In **paper II**, I was able to directly link spatial variation in selection to interactions with pollinators by experimentally quantifying current pollinator-mediated selection in multiple populations. In **paper III**, I took a longer-term perspective and indirectly tested for the hypothesis that the latitudinal gradient in relative importance of nocturnal pollinators has driven evolution of different scent emission rhythms between southern Sweden and Norway.

Pollinator communities often vary across the distributional range of plant species with generalized pollination systems, potentially causing variation in selection on floral traits associated with attraction or mechanical fit (Gómez et al. 2008, 2009; Sletvold and Ågren 2010, 2014). In *Erysimum mediohispanicum*, variation in the direction of selection on corolla shape and tube width has been associated with geographical variation in the composition of the pollinator community (Gómez et al. 2009), and the strength of selection on floral traits has been shown to vary spatially in several species with semi-generalized pollination systems (Caruso et al. 2003; Weber and Kolb 2011).

In **paper II**, I experimentally showed that **among-population variation in strength of directional selection** and in **the targets of correlational selection** in *G. conopsea* s.l. could largely be **attributed to variation in pollinator-mediated selection** (*Figure 11*). Based on these results, it seems likely that pollinators are at least partly causing the selection patterns observed in **paper I** and **pollinators may thus have contributed to the floral divergence between *G. conopsea* s.s. and *G. densiflora***.

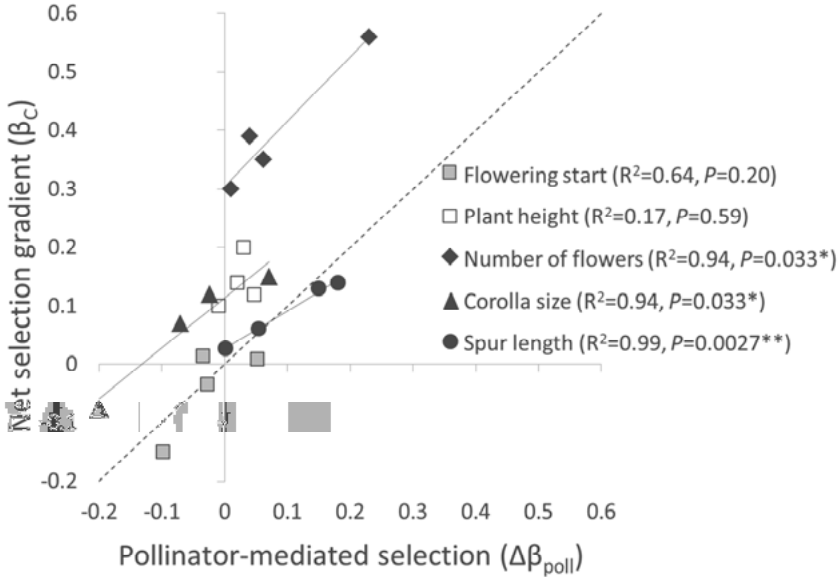


Figure 11. Correspondence between pollinator-mediated selection and net selection for each floral trait in the four study populations of **paper II**. Each symbol is the estimate of selection in one of the populations. Solid lines represent significant linear regressions (R^2 and P of each regression given within brackets). The dashed line represents a 1:1 relationship between β_C and $\Delta\beta_{poll}$.

In **paper III**, I found that scent emissions were greater at night than at day in the four Swedish populations but the opposite was not true in the two Norwegian populations (*Figure 12*). The rate, rhythm and composition of floral scent emissions all are known to show plasticity in response to environmental factors (Hansted et al. 1994; Jakobsen and Olsen 1994; Gouinguene 2002; Friberg et al. 2014; Farré-Armengol et al. 2014; Majetic et al. 2016), which makes it important to complement field observations of scent variation with data from a controlled environment. The difference in emission rate between day and night was larger in the Swedish populations compared to the Norwegian populations also after transfer to the growth chamber (*Figure 12*). My results thus suggest a **genetic component to variation in diel scent rhythms among *G. conopsea* s.s. populations**.

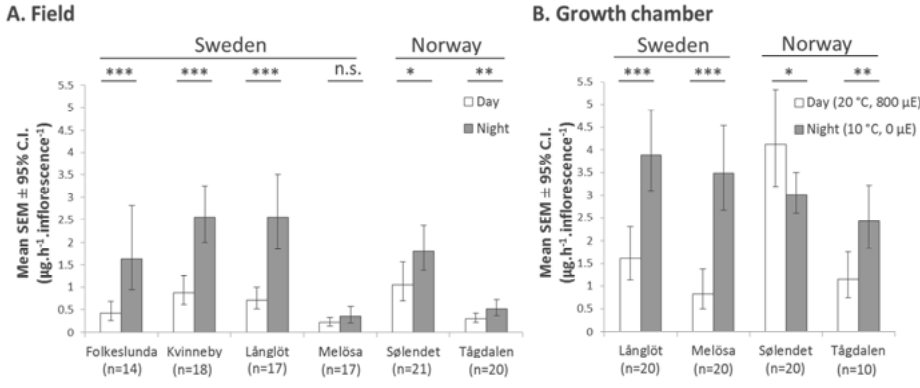


Figure 12. Mean floral volatile standardized emission rate (SEM) per inflorescence ± 95% confidence interval (back-transformed from the log-transformed data) in the field (A) or in the growth chamber (B) and in diurnal (open bars) and in nocturnal (gray bars) conditions for the two sets of *Gymnadenia conopsea* s.s. study populations located in Sweden and Norway. The statistical significance of the difference between diurnal and nocturnal SEM within each population (tested with a paired t-test on log-transformed data) is indicated. n.s. $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

However, the observed among-population differences in diel rhythms were only **partly consistent with the prediction that the timing of floral scent emissions should match the peak period of pollinator activity**. Floral scent **may be a more important signal at night than at day**, while visual display may be more important for attracting day-active pollinators. Studies on several lepidopteran species indicate that the relative importance of visual and olfactory signals at night may be interaction-specific (Raguso and Willis 2002; Balkenius et al. 2005; Hirota et al. 2012). Furthermore, I showed in **paper IV** that pollinators do not necessarily select for increased scent signalling. Still, the observation that plants from the Sølendet population emit significantly more diurnal scent compared to the other populations suggests that floral scent could be important for attracting the diurnal *Empis* flies that visit this population.

It is important to note that **neutral processes could also have contributed to divergence in floral scent rhythms** between the study populations included in **paper III**. The four Swedish populations with similar diel scent rhythms are closely located and likely to be well connected by gene flow, while the two Norwegian populations, which differ from each other and from the southern ones in their scent emission rhythms, may be more isolated, less connected by gene flow and thus potentially more influenced by genetic drift. It would be important to **clarify the population genetics** of *G. conopsea* s.s. in Scandinavia to conclude about the relative importance of adaptive *vs.* non-adaptive processes in the evolution of floral scent in this system.

The results of **paper III** are thus consistent with a scenario where **spatial variation in relative importance of nocturnal and diurnal pollinators has resulted in selection for different floral scent rhythms** within *G. conopsea* s.s but further investigations are needed to directly test this hypothesis.

Taken together, the results of paper II and III indicate that even subtle variation in pollinator communities may result in differences in selection on floral traits in a plant with a semi-generalized pollination system. By manipulating the pollination environment in multiple populations in paper II, I show that it is possible to test rigorously for the presence of a geographic mosaic of pollinator-mediated selection.

Conclusion

In this thesis, I explore natural selection processes within and between species that can explain evolution and maintenance of differentiation in floral traits. I have used the fragrant orchids *Gymnadenia conopsea* s.s. and *Gymnadenia densiflora* to investigate the role of pollinators as agents of selection on floral traits and to test whether they cause spatial variation in selection among populations.

My first study is one of few to test whether variation in selection on floral traits can explain the maintenance of floral trait divergence between closely related species (but see Johnston 1991; Campbell 2003; Joffard 2017). My results suggest that the difference in flowering time between these two species is adaptive while non-adaptive processes may play a role in the differentiation of the other floral traits. Alternatively, selection has driven this differentiation historically but is not strong any longer (Harder and Johnson 2009). Experimental manipulations of floral traits are needed to fully understand whether floral differentiation between *G. conopsea* s.s. and *G. densiflora* is adaptive (Meléndez-Ackerman and Campbell 1998; Harder and Johnson 2009).

In my second and fourth study, I tested whether pollinators were agents of selection on various floral traits by manipulating the pollination environment. Studies that attempt to quantify selection on floral scent are still rare (Delle-Vedove et al. 2017), and my last study provides the first experimental quantification of pollinator-mediated selection on this complex trait. Taken together, my results indicate that pollinators were the main selective agent on pollination efficiency traits, whereas their contribution to selection on phenology, visual display and floral scent was variable and could be conflicting with non-pollinator mediated selection. I have also shown that pollinator-mediated selection could target not only single traits but also combinations of traits. More generally, my results suggest that the targets, direction and strength of pollinator-mediated selection and its contribution relative to that of other selective agents can be difficult to predict, strongly advocating for the experimental identification of agents of selection on floral traits. However, to complete this approach and reveal the underlying mechanisms generating directional and correlational pollinator-mediated selection, studies of trait effects on pollinator visitation and pollination efficiency are needed (e.g. Campbell 2009; Trunschke 2018).

I also examined whether diurnal and nocturnal pollinators contributed equally to reproductive success of *G. conopsea* s.s in southern Sweden and whether they exerted different selection patterns on floral traits using exclusion experiments. Taken together, my results suggest that even pollinators that are less important for reproductive success can exert selection on floral traits, including floral scent, contrary to the "most effective pollinator principle" (Stebbins 1970). Additionally, selection mediated by diurnal and nocturnal pollinators differed, indicating that variation in the relative importance of the two pollinator categories may contribute to spatial variation in selection, and thus potentially to intraspecific differentiation in floral traits.

Finally, I also investigated evidence of a geographic mosaic of pollinator-mediated selection. In my second study, I directly linked spatial variation in selection to interactions with pollinators by experimentally quantifying pollinator-mediated selection in multiple populations, while in my third study, I indirectly tested for the hypothesis that the latitudinal gradient in relative importance of nocturnal pollinators has driven evolution of different scent emission rhythms between southern Sweden and Norway. Taken together, the results of these studies indicate that even subtle variation in pollinator communities may result in differences in selection on floral traits in a plant with a semi-generalized pollination system. By manipulating the pollination environment in multiple populations in paper II, I show that it is possible to test rigorously for the presence of a geographic mosaic of pollinator-mediated selection. However, this should be repeated at wider geographic scales and in various systems, in particular where spatial variation in pollinators is likely to cause divergent selection, to understand whether such a selective mosaic is common and likely to drive intraspecific floral differentiation.

It should be noted that in my thesis I used one-season reproductive output as a proxy for female fitness, while *Gymnadenia* orchids are perennials. A full understanding of the consequences of plant-pollinator interactions in this system will require the use of demographic data, to integrate costs of reproduction, which have been demonstrated in *G. conopsea* s.s. (Sletvold and Ågren 2011a), and to estimate lifetime fitness.

Taken together, the results support the hypothesis that pollinators cause spatial variation in selection on floral traits, including floral scent, and suggest that pollinators may have contributed to the floral divergence between *G. conopsea* s.s. and *G. densiflora*, and more generally supports the idea that pollinators can drive floral evolution. They also highlight the importance of experimentally identifying sources of selection to reveal conflicting and reinforcing selection by multiple agents and thus advance our understanding of the evolutionary ecology of floral traits.

Svensk sammanfattning

Har du någonsin undrat varför det finns en sådan mångfald i storlek, antal, form, färg eller doft av blommor? Den frågan har fascinerat forskare länge och är fortfarande en central fråga i växternas evolutionära biologi.

Dynamiska interaktioner mellan växter och pollinatörer

Den stora majoriteten av växter i världen är blomväxter. Detta tyder på att blommor, de reproduktiva strukturerna hos dessa växter, har varit en nyckelfaktor i växternas evolution. Eftersom växter är stationära organismer, kräver deras reproduktion att pollen från en blomma transporteras till en annan där fröämnet befruktas och frön produceras. För 88% av de blommande växterna sker denna transport med hjälp av ett djur, kallat en pollinatör. Forskare har observerat denna mekanism, vilket har lett dem till att testa och visa att blommans storlek, färg eller doft är viktiga för att locka pollinatörer, och att blommans form påverkar hur effektiv pollinationen är.

Interaktioner mellan växter och pollinatörer är inte statiska i tid och rum. För det första, liksom människor varierar i storlek, kroppsform eller hårfärg, varierar växter av samma art i blomstorlek, form eller färg. Pollinatörer kan till exempel vara mer attraherade av de största växterna i en population och pollinera dem oftare än andra individer. Större individer kommer därför att producera fler frön och kommer följaktligen att ha större chanser att överföra sina gener till nästa generation, vilken kommer att tendera att bestå av större växter, vilka återigen kommer att gynnas av pollinatörer, o.s.v... Det här kallas naturligt urval, enligt Darwins definition. Växterna och deras pollinatörer evolverar ständigt som svar på detta urval.

För det andra, kan enskilda individer inom samma växtart pollineras av olika arter beroende på om de växer i söder eller i norr, på låg eller hög höjd, i skog eller i gräsmark. Olika pollinatörer, till exempel en kolibri och ett bi, kan skilja sig åt i sina preferenser eller i kroppsformer som kan påverka pollinationens effektivitet. Alltså, om kolibrier utför det naturliga urvalet av blommor på en plats kan större och rödare blommor gynnas där, medan om bin pollinerar samma växt på en annan plats kanske mindre och gulare blommor gynnas. I detta scenario gynnar den naturliga selektionen olika typer av blommor på olika platser, vilket är det som kallas divergerande

urval. Det divergerande urval leder till evolution av olika blommor som är anpassade till sina lokala pollinatörer, och leder därmed till mer mångfald.

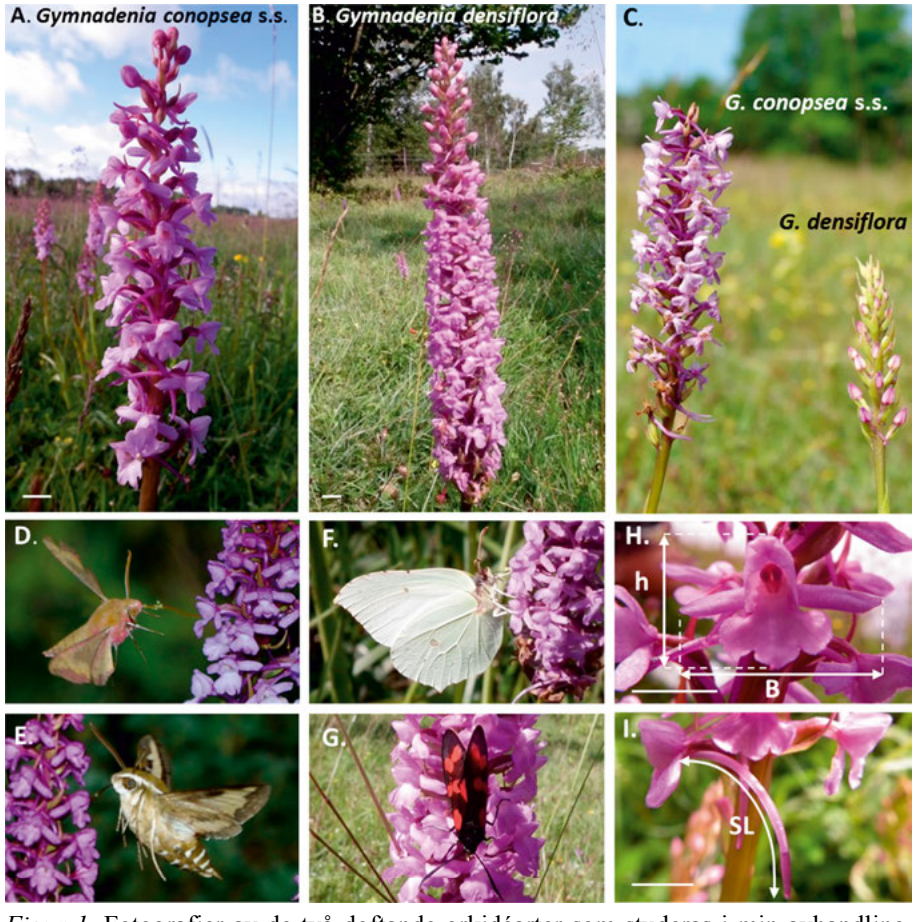
Av alla dessa skäl är en nyckelhypotes att interaktioner mellan växter och pollinatörer driver blommors evolution. Kan vi se när naturligt urval av blommor sker i naturen? Orsakar pollinatörer variation i den naturliga selektionen?

Studiesystemet

I min avhandling tar jag mig an dessa frågor med hjälp av två arter av doftande orkidéer, brudsporre (*Gymnadenia conopsea*, *Figur 1A*) och praktsporre (*Gymnadenia densiflora*, *Figur 1B*). Dessa två arter har relativt lika rosa blommor med smala sporrar (*Figur 1H-I*), alltså rör som innehåller nektar (en söt vätska som pollinatören äter). Sporren är oerhört viktig för pollineringseffektiviteten i dessa orkidéer eftersom pollinatören tvingas att sträcka in tungan i sporren för att komma åt nektar, och därmed samla det pollen som ligger vid sporrens öppning. Ju längre sporren är desto effektivare är pollineringen. De två *Gymnadenia*-arterna skiljer sig i höjd, blomstorlek och sporrens längd, men främst i blommans doft och blomningstid. Praktsporre *G. densiflora* är den som blommar senare (*Figur 1C*). Det finns mycket variation i alla dessa egenskaper även inom arterna, vilket betyder att det finns råmaterial för den naturliga selektionen. Dessutom pollineras dessa två orkidéer av många fjärilsarter, både dag- och nattaktiva (*Figur 1D-G*). Pollinatörerna varierar mellan olika populationer inom och mellan de två orkidéarterna, vilket, som jag förklarat tidigare, troligen medför variation i selektion mellan platser och mellan arter.

Finns det divergerande urval mellan de två arterna?

I min första studie mättes det naturliga urvalet av blomningstid, växthöjd, antal blommor, blomstorlek och sporrens längd i flera populationer av varje art. Jag hittade selektion för tidigare blomning i två populationer av den tidigblommande arten (brudsporre, *G. conopsea*), och selektion för senare blomning i två av populationerna av den senblommande arten (praktsporre, *G. densiflora*). Alltså, det finns divergerande urval i blomningstid mellan dessa två arter, men inte för de andra blomningsegenskaperna. Sammanfattningsvis verkar det naturliga urvalet bidra till skillnaden i blomningstid mellan de två arterna, vilket tyder på att denna skillnad är adaptiv. Men är det här en anpassning till pollinatörer eller till något annat?



Figur 1. Fotografier av de två doftande orkidéarter som studeras i min avhandling, **A.** brudsporre *Gymnadenia conopsea* och **B.** praktsporre *Gymnadenia densiflora* som **C.** skiljer sig i blomningstid och av **D. - G.** de vanligaste pollinatörerna på Öland, där jag gjorde de flesta av mina studier. Det som visas är två av brudsporrens nattliga pollinatörer: mindre snabelsvärmare (*Deiliphila porcellus*, **D.**) och brunsprötad skymningssväarmare (*Hyles gallii*, **E.**) och två av praktsporrens dagsaktiva pollinatörer : citronfjäril (*Gonepteryx rhamni*, **F.**) och sexfläckig bastardsvärmare (*Zygaena filipendula*, **G.**). Man kan se pollinaria, de strukturer som innehåller orkidéernas pollen, kopplade till pollinatörernas tungor, särskilt i bild **D**. De två arternas blommor har liknande form, och jag mätte **H.** deras storlek som produkten av deras höjd (h), bredd (B) och **I.** sporrens längd (SL). De vita skalstrecken indikerar 0,5 cm.

Orsakar pollinatörer variation i selektion av blommor?

Blommor interagerar inte bara med pollinatörer, utan också med andra djur eller mikroorganismer som kan skada blommorna, vilket påverkar fröproduktionen och därigenom orsakar naturligt urval i blommorna. Vissa växter kan också vara bättre än andra på att t.ex. samla resurser i torra jordar, vilket

gör det möjligt för dem att producera större blommor och därmed locka fler pollinatörer, men mer resurser gör det också möjligt att producera fler frön. I det här fallet blir det svårt att avgöra om urvalet orsakas av pollinatörer eller genom resursförvärv.

I min avhandling har jag använt ett handpollineringsexperiment för att ta reda på i vilken utsträckning pollinatörer orsakar naturligt urval av blommor. Genom att pollinera alla växter lika, avlägsnade jag effekten av pollinatörer på fröproduktion. Urvalet som orsakas av pollinatörer är därmed skillnaden mellan urvalet hos växter som pollineras naturligt och urvalet hos handpollinerade växter.

I min andra studie använde jag detta handpollinationsexperiment i fyra av populationerna, bland vilka selektionen på blommor varierade. Mitt experiment visade att denna variation till stor del berodde på pollinatörerna. Jag demonstrerade alltså experimentellt en av de viktigaste hypoteserna i teorin som förklarar hur pollinatörer kan driva blommors diversifiering. Dessa resultat tyder på att det divergerande urvalet som observerats i blomningstid mellan mina två arter (första studien) åtminstone delvis orsakas av pollinatörer.

Hur var det med blommornas dofter?

Efter dessa första resultat bestämde jag mig för att studera blommornas doft. Det här är en aspekt av blommor som är komplex. Inte bara för att vissa blommor kan avge mer än hundra olika kemiska föreningar som utgör doften, utan också för att dofterna kan variera under tid (t.ex. doftar vissa blommor bara på natten) och påverkas av miljöförhållanden som torka eller temperatur. Av dessa skäl har blomdofter inte studerats mycket tidigare. I många fall har vi precis börjat upptäcka och beskriva variation i blomdofter inom och mellan naturliga växtpopulationer.

I min tredje studie fokuserade jag på en art, brudsporre *Gymnadenia conopsea*, och började med provtagning av blomdofter i flera populationer i fält. Tack vare tidigare experiment visste jag att dagaktiva pollinatörer är viktigare än nattaktiva pollinatörer i mellersta Norge, medan motsatsen gäller i södra Sverige. Därför bestämde jag mig för att provtaga blomdofter dag och natt i flera populationer i varje region för att testa hypotesen att växter i Norge avger mer blomdofter under dag än natt, och motsatsen i södra Sverige. Eftersom miljöförhållandena skiljde sig mellan populationer när jag utförde fältexperimentet bestämde jag mig för att ta med några växter från varje population till laboratoriet för att exponera alla för samma förhållanden och därmed kontrollera om skillnaderna jag såg i naturen var kvar. Även om det är mycket viktigt, görs detta mycket sällan i studier av blomdoft.

Som förväntat fann jag att växter i södra Sverige avgav mer doft på natten än på dagen, och i Norge doftade de mindre på natten än svenska växter.

Detta gällde även i de kontrollerade förhållandena på laboratoriet, vilket visar att dessa skillnader troligen är genetiska och därmed kanske anpassningar. I motsats till min hypotes såg jag en ökning av doftutsläpp dagtid i bara en av de norska populationerna. Mina resultat är därmed delvis förenliga med hypotesen att interaktioner mellan växter och pollinatörer har drivit utvecklingen av blommornas doftrytm i brudsporre. Denna studie är emellertid inte ett direkt test och beskriver endast doftvariationen hos arten.

Orsakar pollinatörer selektion av blomdofter?

I min sista studie följde jag upp den föregående studien och undersökte naturlig selektion av blomdofter med samma metod som tidigare (handpollination). För att veta vilka föreningar att mäta selektion på, identifierade jag först vilka doftföreningar som uppfattas av pollinatören med hjälp av en metod som analyserar antennresponser. Jag upptäckte att pollinatörer selekterar för starkare signaler av vissa föreningar, som förväntat om deras roll är att locka pollinatörer. Förvånansvärt, fann jag också att pollinatörerna selekterade för reducerade utsläpp av andra föreningar, vilket tyder på att dessa föreningar verkar frånstötande på fjärilarna och alltså har någon annan funktion än att attrahera pollinatörer. Dessutom visade mitt experiment att andra faktorer orsakade selektion av blomdofter i motsats till selektionen som orsakades av pollinatörer, vilket var ganska oväntat. Sammanfattningsvis visar denna studie att det är svårt att förutse och tolka det naturliga urvalet utan ett experimentellt tillvägagångssätt.

Slutsats

Sammanfattningsvis stödjer mina resultat hypotesen att pollinatörer orsakar variation i det naturliga urvalet hos blommor och därmed har de potential att öka blommornas mångfald. Resultaten framhäver också vikten av experimentellt identifierande urvalskällor för att öka vår förståelse av blommors evolution.

(Översättare: Fia Bengtsson)

Résumé en français

Ne vous êtes-vous jamais demandé pourquoi il y a une telle diversité de tailles, formes, couleurs ou parfums de fleurs? Cette question fascine les chercheurs depuis longtemps et reste une question centrale en évolution des plantes.

Des interactions dynamiques entre plantes et pollinisateurs

La grande majorité des espèces végétales sur Terre sont des plantes à fleurs. Cette observation suggère que la fleur, qui est la structure reproductrice de la plante, a été une innovation clé dans l'évolution végétale. Comme les plantes sont des êtres vivants immobiles, leur reproduction nécessite le transfert de pollen d'une fleur vers une autre où le pollen peut féconder les ovules pour produire des graines. Pour 88 % des plantes à fleurs, ce transfert a lieu grâce à un animal, qu'on appelle un pollinisateur. En observant ce mécanisme de pollinisation, les scientifiques ont fait l'hypothèse et démontré que la taille, la couleur ou le parfum des fleurs sont importants pour attirer les pollinisateurs, et que la forme des fleurs influence l'efficacité de la pollinisation.

Les interactions entre plantes et pollinisateurs ne sont pas immuables. Tout d'abord, tout comme les humains n'ont pas tous la même taille, corpulence ou couleur de cheveux, les fleurs d'une même espèce de plantes peuvent varier en taille, forme ou couleur. Les pollinisateurs peuvent, par exemple, être plus attirés par les plantes qui ont les plus grosses fleurs et les polliniser plus souvent que les autres. Les plantes avec les plus grosses fleurs produiront plus de graines que les autres et auront donc plus de chance de transmettre leurs gènes à la génération suivante, qui aura donc tendance à produire de plus grosses fleurs, qui seront encore favorisées par les pollinisateurs et ainsi de suite. C'est ce qu'on appelle la sélection naturelle, définie par Darwin. Les plantes et leurs pollinisateurs évoluent en permanence en réponse à cette sélection.

Ensuite, au sein d'une même espèce de plante, chaque plante peut rencontrer différentes espèces de pollinisateurs, selon si elle pousse au nord ou au sud, à basse ou haute altitude, dans une forêt ou dans une prairie, par exemple. Ces pollinisateurs, par exemple un colibri et une abeille, peuvent

différer dans leurs préférences ou leur morphologie, influençant l'efficacité avec laquelle ils vont polliniser les fleurs. Ainsi, dans une première localité, la sélection naturelle par les colibris peut favoriser des fleurs plus grosses et plus rouges alors que dans une deuxième localité, les abeilles sélectionnent des fleurs plus petites et plus jaunes. Dans ce cas, la sélection favorise différents types de fleurs dans différentes localités, ce qu'on appelle de la sélection divergente. La sélection divergente peut donc conduire à l'évolution de différentes fleurs adaptées chacune à leurs pollinisateurs, et donc aboutir à plus de diversité florale.

Pour toutes ces raisons, une hypothèse clé est que les interactions entre plantes et pollinisateurs sont au cœur de l'évolution des plantes à fleurs. Peut-on observer la sélection naturelle sur les fleurs en action dans la nature? Les pollinisateurs causent-ils de la variation dans cette selection?

Système d'étude

Dans ma thèse, je répond à ces questions en utilisant deux espèces d'orchidées parfumées, *Gymnadenia conopsea* et *Gymnadenia densiflora* (*Figure 1*). Ces deux espèces ont des fleurs roses relativement similaires qui produisent un éperon (*Figure 1H-I*), c'est-à-dire un tube contenant du nectar (liquide sucré qui nourrit les pollinisateurs). Cet éperon est très important pour l'efficacité de la pollinisation de ces orchidées car il oblige les pollinisateurs à insérer leur langue jusqu'au fond du tube pour accéder au nectar, et ainsi augmente les chances qu'ils collectent le pollen situé à l'entrée de l'éperon. Plus l'éperon est long, plus l'efficacité de la pollinisation augmente. Les deux espèces de *Gymnadenia* diffèrent en hauteur, taille de fleur, et longueur d'éperon mais surtout dans les parfums qu'elles émettent et dans leur période de floraison, *G. densiflora* fleurissant plus tardivement (*Figure 1C*). Il y a aussi beaucoup de variation dans toutes ces caractéristiques au sein de chaque espèce, sur laquelle la sélection naturelle peut agir. De plus, ces deux orchidées sont pollinisées par de nombreuses espèces de papillons de jour et de nuit (*Figure 1D-G*). Les espèces de pollinisateurs varient entre les populations et entre les deux espèces, ce qui, comme expliqué plus haut, peut causer de la variation dans la sélection dans l'espace et entre les deux espèces.

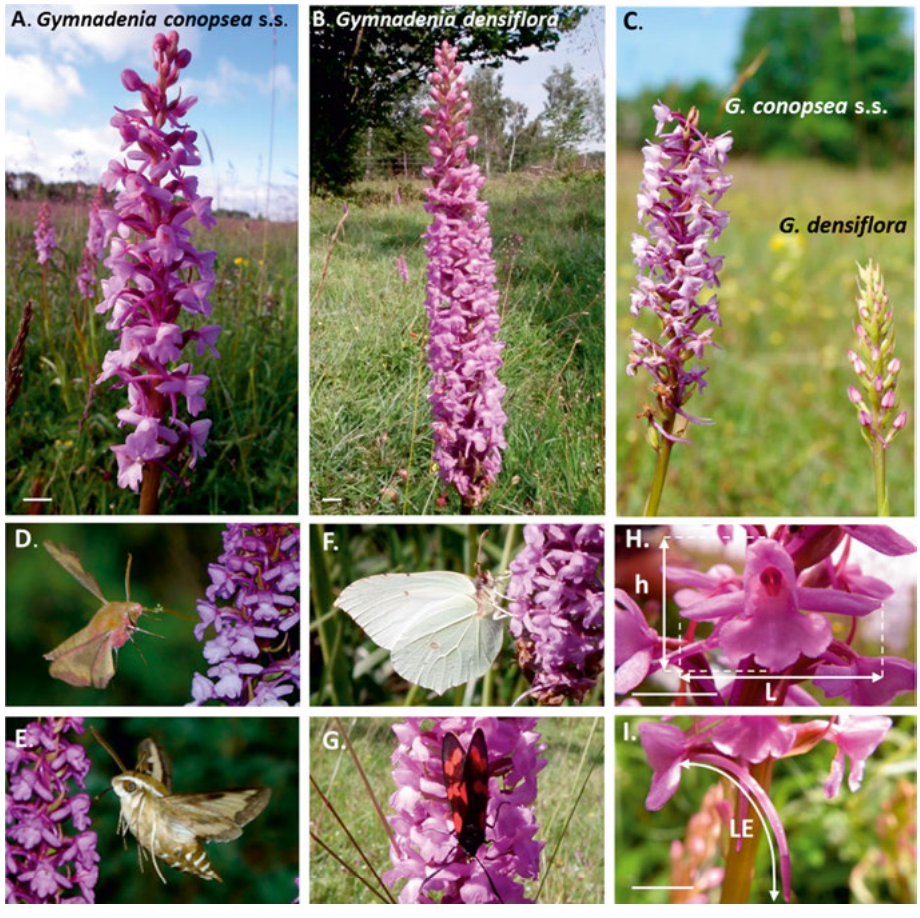


Figure 1. Illustration des deux espèces d'orchidées étudiées dans ma thèse: **A.** *Gymandenia conopsea* et **B.** *Gymnadenia densiflora* qui **C.** diffèrent dans leur période de floraison, et **D. – G.** des pollinisateurs les plus fréquents sur l'île d'Öland où j'ai effectué la plupart de mes études. Sont illustrés deux pollinisateurs nocturnes de *G. conopsea*: le petit sphinx de la vigne (*Deiliphila porcellus*, **D**.) et le sphinx de la garance (*Hyles gallii*, **E.**) ainsi que deux pollinisateurs diurnes de *G. densiflora*: le papillon citron (*Gonepteryx rhamni*, **F.**) et le zygène de la spirée (*Zygaena filipendula*, **G.**). On peut voir les pollinia, les structures qui contiennent le pollen des orchidées, attachées sur la trompe de chaque pollinisateur, en particullier sur la photo **D**. Les fleurs des deux espèces ont une forme similaire, et j'ai mesuré **H.** leur taille en multipliant leur hauteur (h) par leur largeur (L) ainsi que **I.** la longueur de leur éperon (LE). Les lignes blanches horizontales représentent une échelle de 0.5 cm.

Ces deux espèces sont-elles soumises à de la sélection divergente ?

Dans ma première étude, j'ai mesuré la sélection naturelle sur la date de floraison, la hauteur des plantes, le nombre de fleurs, la taille des fleurs et la longueur de l'éperon dans plusieurs populations de chaque espèce. J'ai observé qu'il y avait de la sélection pour une floraison plus précoce dans deux des populations de l'espèce qui fleurit plus tôt (*G. conopsea*), et à l'inverse de la sélection pour une floraison plus tardive dans deux des populations de l'autre espèce (*G. densiflora*). Il y a donc de la sélection divergente sur la date de floraison entre ces deux espèces, mais ce n'est pas le cas pour les autres caractéristiques mesurées. Ainsi, la sélection naturelle semble contribuer à la différence de période de floraison entre ces deux espèces, ce qui suggère que cette différence est une adaptation. Mais est-ce une adaptation aux pollinisateurs ou à autre chose ?

Les pollinisateurs causent-ils la variation dans la sélection sur les fleurs?

Les fleurs n'interagissent pas seulement avec les pollinisateurs, mais avec d'autres animaux ou micro-organismes qui peuvent les abimer, affectant leur production de graines et ainsi provoquer aussi de la sélection sur les fleurs. Certaines plantes peuvent aussi être plus performantes que d'autres pour acquérir des ressources dans des sols secs, par exemple, leur permettant de produire de plus grosses fleurs, et ainsi attirer plus de pollinisateurs mais aussi produire plus de graines. Dans ce cas, il sera difficile de dire si la sélection est causée par les pollinisateurs ou par la capacité à acquérir des ressources.

Dans ma thèse, pour déterminer dans quelle mesure les pollinisateurs causent la sélection naturelle sur les fleurs, j'ai utilisé une expérience de pollinisation dite « à la main ». En effet, en pollinisant à la main de la même manière toutes les plantes, j'ai enlevé l'effet des pollinisateurs sur la production de graines. La sélection due aux pollinisateurs est donc la soustraction entre la sélection parmi les plantes pollinisées naturellement, et la sélection parmi les plantes pollinisées à la main.

Dans ma deuxième étude, j'ai utilisé cette expérience de pollinisation à la main dans quatre populations entre lesquelles la sélection sur les fleurs variait. Mon expérience a montré qu'en effet cette variation de sélection était largement due aux pollinisateurs. J'ai ainsi démontré expérimentalement une des hypothèses clé dans la théorie expliquant comment les pollinisateurs peuvent induire la diversification des fleurs. Ces résultats suggèrent que la

sélection divergente observée sur la date de floraison entre mes deux espèces (première étude) est causée au moins en partie par les pollinisateurs.

Qu'en est-il du parfum des fleurs ?

Après ces premiers résultats, j'ai décidé de me focaliser sur l'étude du parfum des fleurs. C'est un aspect des fleurs qui est très complexe : non seulement certaines fleurs peuvent émettre plus de cent composés, mais en plus les parfums floraux peuvent varier dans le temps (par exemple, certaines fleurs ne sentent que la nuit) ou être influencés par les conditions environnementales comme la sécheresse ou la température de l'air. C'est pourquoi, jusqu'à récemment, le parfum des fleurs a été très peu étudié. Dans bien des cas, on commence juste à découvrir et décrire la variation en parfum au sein et entre les populations naturelles de plantes.

Pour ma troisième étude, je me suis focalisée sur une seule espèce, *Gymnadenia conopsea*, et j'ai donc commencé par échantillonner les parfums floraux dans plusieurs populations sur le terrain. Grâce à de précédentes expériences, je savais qu'au centre de la Norvège, les pollinisateurs diurnes sont plus importants que les pollinisateurs nocturnes, alors que c'est l'opposé dans le sud de la Suède. J'ai ainsi décidé d'échantillonner les odeurs florales de *G. conopsea* le jour et la nuit dans plusieurs populations dans chacune de ces régions pour tester l'hypothèse qu'en Norvège, les plantes émettent un parfum plus important le jour que la nuit, et l'opposé au sud de la Suède. Comme les conditions environnementales variaient entre les populations lors de l'échantillonnage sur le terrain, j'ai décidé de prélever quelques plantes de chaque population pour les exposer en laboratoire aux mêmes conditions, et ainsi vérifier si les différences observées sur le terrain étaient toujours présentes. C'est un contrôle important à faire, rarement fait dans les études des odeurs florales.

Comme je m'y attendais, les plantes du sud de la Suède émettaient un parfum plus fort la nuit que le jour, et en Norvège, les plantes émettaient un parfum nocturne plus faible que les plantes suédoises. C'était aussi le cas en conditions de laboratoire, ce qui montre que ces différences sont probablement génétiques et donc peut-être des adaptations. Cependant, contrairement à mon hypothèse, il n'y avait d'augmentation claire des émissions d'odeurs le jour que dans une des populations de Norvège. Ainsi, mes résultats sont en partie en accord avec l'hypothèse que les interactions entre plantes et pollinisateurs ont joué un rôle dans l'évolution du rythme d'émissions des parfums floraux de *Gymnadenia conopsea*. Cependant, cette étude n'est pas un test direct et se limite à une description de la variation en odeurs florales dans cette espèce d'orchidée.

Est-ce que les pollinisateurs causent de la sélection sur les parfums floraux ?

Pour aller plus loin, dans ma dernière étude, j'ai étudié la sélection sur les parfums floraux, en utilisant l'expérience de pollinisation à la main décrite plus haut. Pour choisir les composés sur lesquels mesurer la sélection, j'ai tout d'abord identifié quels composés sont détectés par les pollinisateurs en utilisant une méthode qui analysent les réponses antennaires des insectes. J'ai trouvé que les pollinisateurs sélectionnaient pour une émission plus importante de certains composés, comme attendu si leur rôle est d'attirer les pollinisateurs. De façon plus surprenante, j'ai aussi trouvé que les pollinisateurs sélectionnaient pour une réduction des émissions d'autres composés, ce qui suggère que ces composés sont répulsifs et qu'ils remplissent une autre fonction que l'attraction des pollinisateurs. De plus, cette expérience a aussi montré que d'autres facteurs causaient la sélection sur les odeurs florales et pouvaient s'opposer à la sélection des pollinisateurs, ce qui était assez inattendu. Globalement, cette étude montre qu'il est difficile de prédire et d'interpréter la sélection sur les odeurs sans une approche expérimentale.

Conclusion

Pris dans leur ensemble, mes résultats soutiennent l'hypothèse que les pollinisateurs causent de la variation dans la sélection naturelle sur les fleurs et ainsi ont le potentiel d'augmenter la diversité florale. Ils soulignent aussi l'importance d'utiliser une approche expérimentale pour identifier les sources de selection naturelle et ainsi faire progresser notre compréhension de l'évolution des fleurs.

Acknowledgments

First, I would like to thank my supervisors Nina Sletvold and Jon Ågren for granting me the opportunity to do this PhD project. Nina, you have been an excellent mentor for me, I can now measure the progress I made in critical thinking, scientific writing and many other skills thanks to you. I highly appreciated your positive thinking, your flexibility and your availability for all the steps of the different projects, for discussing science but also, importantly for me, life in general. Jon, it was always great to have your challenging, critical and therefore valuable questions or comments that made the projects and manuscripts move forward in quality. I am also deeply grateful to Magne Friberg, who joined as co-supervisor along the way when I needed a new mentor, and who guided me through the traps and challenges of studying floral scent. The field sampling and labwork would not have been possible without you and far less fun.

I also want to thank all the assistants who helped me preciously throughout the years: Joseph A., Karl F., Anna H., Andreas J., Maria U.-P., Mattias V., Linus V. Special thanks to Joseph who has been an amazing MacGyver when it came to solving problems and even improving scent sampling or analyses. Always bringing fun, culture, optimism and hard work that sometimes saved the day, you were also a great master student and have become a real friend. Maria, you were a friend from the start and it was great to have the opportunity to work with you too. I am very grateful for your commitment and in particular for all the hard work you put into collecting pollinator data. I also appreciate the contributions of a French friend and very skilled master student, Iris L. R., to analyses and writing of my first manuscript.

I would like to thank the entire Department of Plant ecology and Evolution for the many years working there. In particular, I want to thank all former and current PhD colleagues, especially Andres C., Camille M., Fia B., Froukje P., Judith T., Lina L. and Maria U-P for sharing difficulties but mostly fun at and outside of work. Special thanks to the members of the writing club which helped me in the final phase of writing: Fia B., Charlie C., Anna-Malin L. and Matt, T. It was great to share the best and cosiest office of the department with Fia B. and Håkan H. all these years (maybe the secret is to have always one or two people on leave at a time!). Fia, I also want to thank you for the translation of the swedish summary, and just for being such good friends with Aaron. Special thanks also go to Amy P., Karin

G., Sophie K., Marion O. and Pascal M., Tom E. and Barbie E. for valuable discussion of science and other important life matters.

I am grateful to Dave K. from station Linné for his help with pollinator identification and to all the farmers who allowed me to work on their land. I am also greatly in debt with Glenn Svensson from Lund University for his precious help with the GC-EAD analysis. I want to thank the Pheromone Group at the Department of Biology, Lund University for welcoming me and allowing me to conduct these analyses in their premises.

My fieldwork, conference and educational travels were financially supported thanks to the amazing scholarships opportunities available to us and granted to me by the Swedish Phytogeographical Society, the Extensus, Lars Hiertas Minne and Linnéan scholarship foundations, the Liljewalch and Regnell's botanical travel scholarships and the Gradschool for Genomes and Phenotypes.

Tack så mycket Sverige och Uppsala Universitet för att välkomna alla och ge chansen att kombinera forskning och privatliv under bästa möjliga förhållanden.

Un grand merci à Camille M. et Alice M. pour avoir partagé les joies et les peines de la vie d'expatriés: par moment ça fait du bien de pouvoir se plaindre en français!

Merci à tous mes amis et ma famille en France, pour être venus nous rendre visite ou avoir gardé le contact pendant toutes ces années malgré la distance. Je voudrais remercier en particulier mes parents pour avoir toujours cru en moi, sans jamais me mettre la pression, au contraire, pour m'avoir fait relativiser quand il y en avait besoin tout en me laissant toute liberté dans mes choix de vie. Merci à ma petite soeur Camille pour son précieux soutien dans certains moments difficiles.

Moana et Poema, vous m'avez apporté beaucoup ~~de fatigue,~~ de joie et de force pour finir cette thèse, en me rappelant ce qui est vraiment important dans la vie et en me donnant un équilibre entre travail et vie privée. J'espère que vous serez fières de moi et que je vous transmettrai l'amour de la nature qui m'anime.

Amélie, il y a tellement de raisons de te remercier, de ton sacrifice aux moustiques pour m'aider sur le terrain à la superbe couverture de cette thèse, en passant par les heures à discuter de science et surtout de projets qui font rêver... Merci surtout pour ton soutien infaillible, je t'aime.

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