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Mating system and floral scent in Arabis alpina

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Abstract

Flowering plants display an extraordinary floral- and reproductive diversity. Variation in the size, shape, colour and scent of flowers, and in systems and strategies of mating, is ubiquitous in comparisons of different species, but also exists among different conspecific populations. Diversity in these characters is central to the evolution of flowering plants and the formation of new species. In this thesis, I use the arctic-alpine plant *Arabis alpina* to explore various causes of intraspecific variation in floral scent, and consequences of evolutionary shifts in plant mating system. By combining experiments in the greenhouse, genomic data and studies in the field, I examine how mating system, natural selection, genetic differentiation and phenotypic plasticity shape intraspecific floral scent variation, and investigate the impact of mating system shifts for the build-up of reproductive isolation. Comparing the floral scent of different *A. alpina* populations distributed across Europe, I found that self-compatible populations had a lower floral scent emission rate and partly different scent composition compared to self-incompatible populations. For both self-compatible and self-incompatible populations, there was limited phenotypic plasticity in floral scent, with some effect of nutrient availability, but not of water availability, on scent emission rates. Comparing the genomic and phenotypic differentiation among self-incompatible populations, it was evident that closely related populations could differ considerably in floral scent. Estimating selection on floral scent, I found some evidence that patterns of selection differed between populations. By crossing plants from self-compatible populations with plants from self-incompatible populations, I found considerable reproductive isolation, consistent with parental conflict over seed provisioning being higher in self-incompatible than in self-compatible populations. Taken together, the results of my thesis reveal some of the complex patterns behind floral scent diversification, and demonstrate the importance of mating system shifts for the evolution of floral signalling and reproductive isolation among flowering plants.

Key words *Arabis alpina,* floral scent, intraspecific variation, mating system, parental conflict, phenotypic plasticity, phenotypic selection, reproductive isolation, speciation, volatile organic compound (VOC),

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Denna art är en bland våra vackrare fjellväxter då den blommar. Genom sin tufvighet kläder den också väl de ställen, der den bosatt sig, och der man stundom kan få se den hänga sina blommiga grenar från någon bergvägg eller brant klippa särdeles prydligt. Blommorna lära lukta väl.

Utkast till svenska växternas naturhistoria I – C. F. Nyman (1867)

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Author contributions

I.   M.F., P.T., J.Å. and **H.P.** planned and designed the research; **H.P.**, M.F. and P.T. collected data; **H.P.** performed statistical analyses; **H.P.** and M.F. wrote the manuscript with input from P.T. and J.Å.

II.   M.F., V.J.L. and **H.P.** planned and designed the research; V.J.L. collected data; **H.P.** and V.J.L. performed statistical analyses; V.J.L., **H.P.** and M.F. wrote the manuscript.

III.   **H.P.**, M.F., A.R. and J.Å. planned and designed the research; **H.P.** collected and analysed field data with contributions from M.F., J.M.H., L.P. and S.B.; K.S., A.R., and **H.P.** collected and analysed genomic data; **H.P.** wrote the manuscript with input from M.F. and J.Å., and contributions from all authors.

IV.   **H.P.**, M.F., P.T. and J.Å. planned and designed the research; **H.P.**, M.F. and H.T. collected data; **H.P.** performed statistical analyses with advice from J.Å and M.S.; **H.P.** wrote the manuscript with input from M.F., J.Å. and M.S., and contributions from all authors.

All authors in the list of papers have given their consent for the use of their work in the thesis.

Abstract

Flowering plants display an extraordinary floral- and reproductive diversity. Variation in the size, shape, colour and scent of flowers, and in systems and strategies of mating, is ubiquitous in comparisons of different species, but also exists among different conspecific populations. Diversity in these characters is central to the evolution of flowering plants and the formation of new species. In this thesis, I use the arctic-alpine plant *Arabis alpina* to explore various causes of intraspecific variation in floral scent, and consequences of evolutionary shifts in plant mating system. By combining experiments in the greenhouse, genomic data and studies in the field, I examine how mating system, natural selection, genetic differentiation and phenotypic plasticity shape intraspecific floral scent variation, and investigate the impact of mating system shifts for the build-up of reproductive isolation. Comparing the floral scent of different *A. alpina* populations distributed across Europe, I found that self-compatible populations had a lower floral scent emission rate and partly different scent composition compared to self-incompatible populations. For both self-compatible and self-incompatible populations, there was limited phenotypic plasticity in floral scent, with some effect of nutrient availability, but not of water availability, on scent emission rates. Comparing the genomic and phenotypic differentiation among self-incompatible populations, it was evident that closely related populations could differ considerably in floral scent. Estimating selection on floral scent, I found some evidence that patterns of selection differed between populations. By crossing plants from self-compatible populations with plants from self-incompatible populations, I found considerable reproductive isolation, consistent with parental conflict over seed provisioning being higher in self-incompatible than in self-compatible populations. Taken together, the results of my thesis reveal some of the complex patterns behind floral scent diversification, and demonstrate the importance of mating system shifts for the evolution of floral signalling and reproductive isolation among flowering plants.

Svensk sammanfattning

Blommor utgör ett av de mest tydliga exemplen på biologisk mångfald. Bland de cirka 300 000 kända arterna av blomväxter finns det en enorm diversitet i blommornas färg, form, storlek och doft. Hur och varför all denna mångfald har uppkommit är en fråga som fascinerat evolutionsbiologer, ekologer och botaniker under lång tid.

För att reproducera sig sexuellt behöver en växt föra över pollen från en ståndare till märket på en pistill, så att fröämnet befruktas och frön produceras. En stor del av alla blomväxter tar hjälp av olika pollinatörer, främst insekter men också andra djur, för att transportera pollen. Blommorna erbjuder föda i form av nektar och pollen, och när pollinatörerna tar sig mellan olika blommor i jakt på denna överförs pollen från ståndare till pistiller, vilket möjliggör reproduktionen. Genom sin storlek, färg och doft signalerar blommorna förekomsten av nektar och pollen, och lockar på så vis till sig pollinatörerna.

Vad och hur mycket en blomma doftar kan ha stor betydelse för en växts interaktioner med andra organismer, men evolutionen och den ekologiska betydelsen av blommors doft har inte studerats i samma utsträckning som andra blomkaraktärer. Blomdoft är blandningen av små organiska molekyler som släpps ut från en blomma. Doften kan variera både i mängd (hur mycket doftämnen som släpps ut) och sammansättning (vilka doftämnen som släpps ut och i vilka proportioner). Precis som för en blommas färg och form så fungerar doften främst som en signal för att locka till sig pollinatörer. Olika typer av pollinatörer kan föredra olika dofter. Om olika arter eller populationer inom samma art besöks av olika pollinatörer kan det genom naturlig selektion leda till att växterna utvecklar skillnader i hur mycket och vad de doftar. Förutom sådana evolutionära förändringar kan blommors doft också variera på grund av variation i den omgivande miljön. Till exempel kan mängden näring och vatten som en enskild planta har tillgång till påverka hur dess blommor doftar, vilket kan ha betydelse för attraherande av pollinatörer för enskilda individer. Generellt sett är mångfalden bland blomväxters blommor, inklusive deras doft, troligen till stor del ett resultat av naturlig selektion orsakad av pollinatörer, vilket kan leda till diversifiering och i slutänden bidra till bildandet av nya arter.

Förutom variation i färg, form och doft hos blommorna har blomväxterna också utvecklat en rad olika strategier för att optimera och säkerställa reproducering. Den viktigaste distinktionen är den mellan korspollinering (korsbefruktning) och självpollinering (självbefruktning). En stor del av alla blomväxter reproducerar sig främst genom korspollinering, vilket betyder att befruktning sker med pollen från andra individer. Bland dessa arter är självinkompatibilitet också vanligt, vilket innebär att olika genetiska och fysiologiska mekanismer hindrar pollen från samma individ att befrukta det egna fröämnet. Även om självinkompatibilitet är vanligt och

förekommer hos arter i många olika familjer av blomväxter, så har det försvunnit hos många andra arter. Förlusten av självinkompatibilitet är ofta irreversibel, och dessa arter blir istället självkompatibla. Självkompatibla arter kan pollineras av sitt eget pollen, och utvecklar ofta också autonom självpollinering, där blommorna pollinerar sig själva utan hjälp av pollinatörer. I många miljöer gynnas självinkompatibilitet och korspollinering eftersom det förhindrar eventuella problem med inavel. I andra fall, främst när det finns ont om pollinatörer i omgivningen, är istället självpollinering fördelaktigt, eftersom det säkerställer att frön kan bildas även utan pollinatörer. Ur ett evolutionärt perspektiv balanseras de potentiella fördelarna med självpollinering mot nackdelarna med inavel, vilket leder till variation mellan arter och populationer i förekomsten av de olika strategierna.

Att föröka sig genom korspollinering eller självpollinering kan ha stora konsekvenser för en blomväxts ekologi och evolution. Självpollinerande arter som inte längre behöver locka till sig pollinatörer utvecklar ofta mindre blommor, minskar det fysiska avståndet mellan ståndare och pistill, producerar mindre nektar och doftar mindre. På så vis blir självpollineringen mer effektiv, och mindre resurser spenderas på att tillverka blommor med mycket nektar och doft. Ett skifte från korspollinering till självpollinering kan också leda till att populationer eller närbesläktade arter blir reproduktivt isolerade, så att de inte kan producera avkomma tillsammans. En potentiellt viktig mekanism som kan orsaka sådan isolering är skillnader i föräldrakonflikt kring resursfördelning till frön mellan korspollinerande och självpollinerande växter. Under utvecklingen får varje frö resurser från moderplantan. Hos korspollinerande arter är fröets far en annan individ än dess mor, och andra frön som utvecklas samtidigt kan ha andra fäder. Detta göra att fadern gynnas av att fröet får så mycket resurser som möjligt, medan modern gynnas av att fördela resurserna mer lika. Resultatet blir en konflikt mellan föräldrarna, som utspelar sig via skilda genuttryck i generna från modern och fadern inuti fröet. Hos självbefruktande arter, där modern och fadern till ett frö är samma individ, blir konflikten betydligt mindre. Föräldrakonflikten är balanserad vid korsningar inom populationer, men vid korsningar mellan populationer eller arter med olika grader av korspollinering kan fröutvecklingen bli obalanserad och skilja sig åt beroende på om modern kommer från en mer eller mindre korspollinerande population än fadern. Detta, i sin tur, kan resultera i frön som inte gror, vilket bidrar till reproduktiv isolering och i slutänden artbildning.

I min avhandling använder jag mig av den korsblommiga växten fjälltrav (*Arabis alpina*) för att genom experiment i växthus, genetiska analyser och mätningar i fält, studera inomartsvariation i blomdoft och andra blomkaraktärer, och undersöka effekterna av ett skifte från korspollinering till självpollinering.

I min första studie jämförde jag blomdoft och blomstorlek bland 17 olika europeiska populationer av fjälltrav. Vissa av populationerna är självinkompatibla och korspollinerade, andra är självkompatibla, men har svårt att bilda frön utan att en insekt flyttar pollen mellan ståndare och pistill inom den egna plantan, och

ytterligare andra är självkompatibla och kan bilda frön utan hjälp från pollinatörer. Frön från de olika populationerna odlades i växthus, och doft samlades in från många individer från varje population. Resultaten visade att individer från självinkompatibla populationer hade större blommor som doftade mer och till viss del annorlunda än individer från självkompatibla populationer. Däremot var det inga eller små skillnader i blomstorlek och doft mellan självkompatibla populationer med låg och hög grad av självpollinering. Resultatet är lite förvånande, eftersom självkompatibla populationer med hög autonom självpollinering är den enda gruppen som inte behöver attrahera pollinatörer, och kan möjligtvis förklaras av skillnader i ekologiska förhållanden mellan de båda kategorierna, eller begränsad genetisk diversitet hos populationerna med hög autonom självpollinering.

Den andra studien undersökte effekten av varierande miljöförhållanden på blomdoft. Frön från tio olika självkompatibla och självinkompatibla fjälltravspopulationer planterades i växthus, och enskilda individer fick mycket eller lite vatten, som innehöll mycket eller lite näring. Doft samlades in från de olika plantorna, och blomdoften jämfördes för individer i olika behandlingar. Behandlingarna hade en begränsad effekt för individer från både självkompatibla och självinkompatibla populationer. Individer som fick mycket näring doftade överlag lite mer, men det fanns ingen skillnad mellan de som fick mycket eller lite vatten. Ingen av behandlingarna påverkade heller sammansättningen av doften. Sammantaget verkar alltså variation i vatten- och näringstillgång ha en begränsad effekt på blomdoft hos fjälltrav, och hur betydelsefull sådan variation är beror sannolikt på vilket sätt doft attraherar pollinatörer.

I den tredje studien analyserades först det genetiska släktskapet hos några av fjälltravspopulationerna i den första studien, för att undersöka om populationer som hade en liknande doft också var genetiskt lika. DNA-sekvensering gjordes på individer från två grekiska och fyra italienska självinkompatibla populationer, för att undersöka hur de skiljde sig åt genetiskt. Jag undersökte också naturlig selektion på blomdoft och andra växtkaraktärer genom fältarbete i en grekisk och en italiensk population som doftar olika, för att se om specifika dofter eller karaktärer påverkade hur mycket frön individer producerade (deras reproduktiva fitness). Resultaten från den genetiska analysen visade att vissa populationer med olik doftsammansättning var genetiskt lika, vilket indikerar att även om olika populationer är genetiska nära besläktade så kan de ändå evolvera olika blomdoft. Fältundersökningarna visade dock på begränsad naturlig selektion på blomdoft, och många doftämnen och blomkaraktärer hade ingen effekt på reproduktiv fitness. Bland doftämnena var fenylacetaldehyd positivt förknippat med frösättning i den grekiska men inte i den italienska populationen, vilket betyder att grekiska men inte italienska individer som utsöndrade mer fenylacetaldehyd hade en högre reproduktiv fitness. Pollinatörerna som pollinerade fjälltrav skilde sig delvis åt mellan populationerna. Det skulle det kunna vara en anledning till skillnaderna i naturlig selektion, och kan ha bidragit till att blommorna i den grekiska och italienska populationen doftar olika.

I min fjärde studie undersökte jag om graden av föräldrakonflikt var högre hos självinkompatibla korspollinerande populationer, än hos självkompatibla mer självpollinerande populationer av fjälltrav. Individer från tre självinkompatibla och tre självkompatibla populationer odlades i växthus. När de blommade korsades individer inom och mellan olika populationer i alla möjliga kombinationer genom att pollinera individer för hand med hjälp av en pincett. Jag vägde och testade grobarheten hos de frön som bildades, och räknade ut graden av reproduktiv isolering mellan olika populationer. Resultaten indikerade att graden av föräldrakonflikt var högre hos de självinkompatibla populationerna, vilket överensstämmer med teorin. Frön från korsningar mellan två självinkompatibla eller två självkompatibla populationer hade en hög grobarhet. Däremot hade frön från korsningar mellan en självkompatibel och en självinkompatibel individ överlag mycket låg grobarhet, vilket innebär en hög grad av reproduktiv isolering mellan de grupperna på grund av skillnaden i föräldrakonflikt. Graden av reproduktiv isolering var faktiskt så hög att självkompatibla och självinkompatibla populationer av fjälltrav skulle kunna anses tillhöra olika arter.

Sammantaget visar min avhandling att blomdoft kan variera avsevärt mellan olika populationer av samma art, hur olika processer kan ge upphov till sån variation, och hur skillnader mellan korsbefruktande och självbefruktande populationer kan resultera i blomdoftsvariation och reproduktiv isolering. På en mer övergripande nivå illustrerar detta några av de evolutionära och ekologiska mekanismerna som genererar den stora mångfalden bland blomväxter.

Introduction

One of the most striking characteristics of biodiversity on Earth are the endless forms displayed by the flowers of angiosperms. Among the approximately 300 000 known species of flowering plants (Christenhusz and Byng 2016), there is great variety not only in the size, shape, colour and scent of flowers, but also in the mating strategies and systems that have evolved to achieve mating success. Studies of floral and reproductive diversity in angiosperms are central in investigations of natural selection and of how and why new species are formed. A natural question following the observation of this diversity is to ask why it exists. Why do flowers, reproductive structures with the main function of promoting mating by movement of pollen from anthers to stigmas, show this extraordinary diversity? One dominant hypothesis is that most floral evolution is the result of interactions with the organisms, mostly insects, which are pollinating a large majority of the flowering plants (Darwin 1877; Stebbins 1970; Harder and Johnson 2009). Differences in selection imposed by different groups of pollinators can generate variation in floral morphology important for pollination efficiency, and in the visual and chemical signals that flowers use to advertise the presence of rewards such as nectar and pollen to the pollinators (Fenster *et al.* 2004; Rosas-Guerrero *et al.* 2014). The reproductive diversity among angiosperms does not only include adaptations to pollinators, but also concerns various strategies affecting levels of self- and cross-fertilization. A large proportion of species across the angiosperm phylogeny have evolved different mechanisms to increase outcrossing and reduce or prevent self-fertilization (Barrett 2002; Igic and Kohn 2006). Under other ecological circumstances, selection can instead act against self-incompatibility, favouring autonomously selfing plants that no longer rely on pollinators for reproduction (Baker 1955; Wright *et al.* 2013). It is also becoming increasingly clear that pollinator and mating system shifts are often important in generating reproductive isolation and contributing to the formation of new plant species (Stebbins 1957; Johnson 2006; Kay and Sargent 2009; Wright *et al.* 2013; Van der Niet *et al.* 2014).

In this thesis, I study this floral and reproductive diversity. Specifically, I focus on the evolution of floral signalling, most importantly scent, and plant mating system separately and in combination, investigating some of the ecological and evolutionary drivers of floral diversification, and the evolutionary consequences of mating system shifts.

Plant mating and breeding systems

Flowering plants have evolved numerous adaptations affecting how they reproduce. The most central distinction regarding this reproductive diversity is that between self- and cross-fertilization (Lloyd and Schoen 1992; Barrett 2002), where plants are fertilized by pollen from themselves, or from other conspecific individuals, respectively. Traditionally, most species were considered to be either primarily outcrossing or primarily selfing (Schemske and Lande 1985). More recent research emphasizes that outcrossing rates, the proportion of seeds produced by cross-fertilization, is continuously distributed, varying from selfing, to mixed mating, to outcrossing both within and among species (Vogler and Kalisz 2001; Goodwillie *et al.* 2005; Whitehead *et al.* 2018). In line with Neal and Anderson (2005), I refer to this variation in outcrossing rate as *mating system* in my thesis.

Different mechanisms to ensure cross-fertilization and reduce or prevent self-fertilization are found in a large proportion of plant species across the angiosperm phylogeny (Igic and Kohn 2006). This includes, for example, the evolution of dichogamy, heterostyly and separate sexes (Barrett 2002). However, the most common of these mechanisms is self-incompatibility, whereby plants have the ability to recognize and reject their own pollen (Nasrallah 2002). Self-incompatibility is found in some 40% of angiosperm species, having evolved independently many times (Igic and Kohn 2006; Igic *et al.* 2008). I refer to the presence or absence of self-incompatibility as the *breeding system* of a plant. The presence of a self-incompatibility system ensures high levels of outcrossing (although some species are only partially self-incompatible; Raduski *et al.* 2012). This reduces the risk of inbreeding depression, where the fitness of selfed offspring is reduced relative to that of outcrossed offspring (Charlesworth 2006). The cost of inbreeding is regarded as the main selective pressure favouring self-incompatibility (Porcher and Lande 2005).

Although self-incompatibility is common, it is frequently lost, in what has repeatedly been called the most common evolutionary transition in flowering plants (Stebbins 1974; Barrett 2010; Wright *et al.* 2013). Such shifts from self-incompatibility to self-compatibility influence outcrossing rates, which can vary widely among self-compatible plants (Goodwillie *et al.* 2005; Whitehead *et al.* 2018), and have multiple genomic and phenotypic consequences (Igic *et al.* 2008; Wright *et al.* 2008). Often, a shift to self-compatibility is followed by evolution of autonomous selfing, where pollen is transferred from anther to stigma within the same flower without the need of pollen vectors, a strategy found in 10-20% of angiosperm species (Vogler and Kalisz 2001; Barrett 2002; Goodwillie *et al.* 2005). There are two main factors predicted to promote the evolution of selfing (Busch and Delph 2012). First, it provides a genetic transmission advantage over outcrossing (Busch and Delph 2012; Wright *et al.* 2013), because individuals can simultaneously self-pollinate and contribute with pollen for outcrossing. Second,

selfing provides reproductive assurance when the availability of pollinators or mates is rare or unpredictable (Darwin 1876; Baker 1955; Eckert *et al.* 2006). The advantages of selfing are balanced against the potential cost of inbreeding depression, pollen discounting (reduced outcross pollen success) and reduced genetic diversity (Barrett 2002). Collectively, these balancing factors influence under which circumstances mating system shifts occur.

Most often, a shift to autonomous selfing is followed by a set of changes to the function and morphology of flowers, referred to as the selfing syndrome (Sicard and Lenhard 2011; Shimizu and Tsuchimatsu 2015; Cutter 2019). Compared to outcrossing relatives, selfing species have smaller flowers, reduced herkogamy (shorter distance between anthers and stigma), reduced pollen to ovule ratio, reduced nectar production and reduced scent emission. Collectively, these changes act to increase the efficiency of autonomous selfing, and reduce the metabolic and ecological costs of floral signalling when the need to attract pollinators is reduced (Goodwillie *et al.* 2010; Sicard and Lenhard 2011; Cutter 2019).

Mating system shifts are important in plant speciation for two main reasons. First, due to the underlying genetic architecture, shifts from self-incompatibility to self-compatibility are much more common than *vice versa* (Igic *et al.* 2008), which would seemingly make self-incompatibility a rare trait. However, it has been suggested that a selfing strategy may represent an "evolutionary dead-end", with higher rates of extinction among selfing than outcrossing lineages (Stebbins 1957). This idea is supported by empirical evidence suggesting that diversification rates differ between self-compatible and self-incompatible lineages (Goldberg *et al.* 2010). The second reason that mating system shifts are important in plant speciation is that the transitions themselves are associated with speciation. By the various changes associated with the shift to selfing, gene flow between different populations might be reduced, allowing for the accumulation of reproductive barriers and subsequently speciation (Foxe *et al.* 2009; Wright *et al.* 2013).

Plant mating system and reproductive isolation

Formation of new species requires the evolution of reproductive isolation between populations that allows the build-up and maintenance of genetic and phenotypic differences between these populations (Coyne and Orr 2004). Both prezygotic barriers, such as differences in pollinator preferences and flowering phenology, and postzygotic barriers, such as pollen infertility and hybrid unviability, may contribute to the evolution of reproductive isolation (Martin and Willis 2007; Widmer *et al.* 2009; Briscoe Runquist *et al.* 2014; Willis and Donohue 2017). In the case of intrinsic postzygotic reproductive barriers, these are often attributed to genetic incompatibilities between nuclear genes with different evolutionary histories, as described by the classic Bateson-Dobzhansky-Muller model (Bateson 1909; Dobzhansky 1936; Muller 1942; Rieseberg and Blackman 2010).

A curious pattern that is often observed in nature is the presence of asymmetric reproductive barriers, which occurs when the strength of reproductive isolation between two species or populations differs between reciprocal cross directions (Tiffin *et al.* 2001; Turelli and Moyle 2007). In other words, the result of a cross is dependent on which species was the pollen parent (father), and which was the ovule parent (mother). Already described in the 18th century by the German botanist J. G. Kölreuter (Mayr 1986), and later noted by Darwin (1859), the occurrence of such asymmetries is widespread in angiosperms (Tiffin *et al.* 2001). Asymmetries in reproductive isolation are especially common for postzygotic barriers when crossing species of different mating system (Lewis and Crowe 1958; Tiffin *et al.* 2001; Lowry *et al.* 2008; Pickup *et al.* 2019). The Bateson-Dobzhansky-Muller model cannot explain the asymmetries, as incompatibilities between nuclear genes from different species should occur equally regardless of cross direction. Therefore, other explanations are required to explain these patterns.

One potential explanation for the presence of asymmetric reproductive isolation between plants of different mating system is differences in the conflict between parents (and siblings) over allocation of resources to seeds (Brandvain and Haig 2005; Lafon-Placette and Köhler 2016). The developing embryo within the seed receives nutrients from the triploid endosperm, which is a seed tissue composed of one paternal and two maternal genome copies (Li and Berger 2012). In turn, the endosperm receives resources from the maternal plant that the seed is developing on, which should be under selection to distribute resources equally among developing seeds. In outcrossing species or populations, the father of a seed only supplies the pollen, and thus does not pay the cost of providing resources to its offspring. Selection would then be predicted to act for the pollen parent (father) to maximise resource allocation from the maternal plant to seeds sired by itself, at the cost of other developing seeds. Conversely, the ovule parent (i.e. the mother) would be favoured by allocating resources equally to all her seed offspring, which may have different fathers. This scenario generates an evolutionary conflict between the maternal and paternal genomes in each seed, resulting in adaptations for the paternal genome to exploit as much of the maternal resources as possible, and the maternal genome to counteract such adaptations (Queller 1983; Haig and Westoby 1989, 1991; Brandvain and Haig 2005; Burt and Trivers 2006; Raunsgard *et al.* 2018). In the literature, this situation is referred to as genomes having a certain "strength", depending on the local level of parental conflict. The conflict takes place via genomic imprinting in the endosperm, where the paternally inherited part of the genome expresses genes to increase resource allocation to the developing endosperm, while the maternally inherited part of the genome acts to counteract this (Lafon-Placette and Köhler 2016; Gehring and Satyaki 2017). In predominantly selfing species or populations, the maternal and paternal plant are more likely to be the same individual, which should lower the levels of conflict (Brandvain and Haig 2005; Lafon-Placette and Köhler 2016).

The parental conflict should be balanced for crosses within populations, where the strength of the maternal and paternal genomes should have coevolved to be similar. However, in crosses between populations or species of different mating system, differences in outcrossing rates, and hence the level of parental conflict, could contribute to reproductive isolation. This is explained by the weak inbreeder/strong outbreeder (WISO) hypothesis (Brandvain and Haig 2005). This hypothesis predicts that crosses between a maternal outcrossing (outbreeder) and paternal selfing (inbreeder) plant should result in reduced resource allocation to the endosperm, resulting in smaller seeds. In the reciprocal cross, with a selfing maternal and outcrossing paternal genome, the pattern should be opposite, resulting in comparatively larger seeds. In this case, however, development of the endosperm may fail, producing malformed seeds that do not germinate (Brandvain and Haig 2005; Lafon-Placette and Köhler 2016; Gehring and Satyaki 2017). Combined with the fact that seed size itself is important for germination also under normal conditions (Westoby *et al.* 1996), this suggests that parental conflict differences can be an important factor generating reproductive isolation in flowering plants. I examine the importance of plant mating system and parental conflict for reproductive isolation in Chapter IV.

Floral scent

Floral traits such as the size, colour and scent of flowers act as signals that advertise the presence of a reward (nectar and/or pollen) in the flower, and therefore attract pollinators with an innate or learned preference for the signal (Chittka *et al.* 1999; Raguso 2008; Schiestl and Johnson 2013). The evolution of these traits is often the result of interactions with the pollinators, or in the case of mating system shifts from outcrossing to selfing, the result of a reduced need for pollinators (Harder and Johnson 2009; Sicard and Lenhard 2011). While many studies have investigated the evolution of visual floral signalling (size, colour) in this context, chemical floral signalling (scent) has received less attention, despite constituting a vital part of the floral phenotype, often important for pollinator attraction (Raguso 2008; Wright and Schiestl 2009).

Floral scent is the mix, fittingly called the bouquet, of volatile organic compounds emitted from a flower. This mix normally consists of a few to over a hundred different small molecules of different classes of compounds, which are produced by several biosynthetic pathways within the plant and emitted from different parts of the flower (Muhlemann *et al.* 2014; Junker and Parachnowitsch 2015). There are multiple functions of floral scent. Most importantly, it is a signal to pollinators. There are numerous examples from both generalized and specialized pollination systems of how scent attracts pollinators over both short and long distances, and acts as a landing and feeding cue (e.g. Dobson *et al.* 1999; Wright *et al.* 2005; Riffell

et al. 2014; Byers *et al.* 2014; Friberg *et al.* 2014). Importantly, in these examples, both the emission rate, the composition of the bouquet, and the presence or absence of certain compounds can affect the pollinator response, demonstrating the complexity of scent as a floral signalling trait. While pollinator attraction is most often the main function of floral volatiles, the chemicals released can serve also other functions. These include deterrence of herbivores (Junker and Blüthgen 2010), mediation of plant-microbe (Burdon *et al.* 2018) and plant-plant (Caruso and Parachnowitsch 2016) interactions, and protection against environmental stresses (Farré-Armengol *et al.* 2020). Further, floral volatiles may also attract antagonistic insects such as herbivores and florivores (Theis 2006), indicating that scent emission may invoke ecological costs to the plant and therefore be under selection from both mutualists and antagonists (Schiestl 2015).

There is a remarkable diversity in floral scent among angiosperms. In their review, Knudsen *et al.* (2006) identified more than 1700 different chemical compounds from almost 1000 species of flowering plants. Just as for visual floral signals (Fenster *et al.* 2004), this scent variation is often attributed to selection imposed by different pollinators, with preferences for different compounds (Dobson *et al.* 1999; Raguso 2008; Friberg *et al.* 2013; Farré-Armengol *et al.* 2020), although as mentioned above, other factors are likely also important in shaping floral scent evolution. A prerequisite for the evolution of such interspecific (between species) variation is the presence and heritability of intraspecific (within species) variation (Raguso 2008). Variation in floral scent within species is common, varying between individuals within and between populations at small and large geographical scales (Dötterl *et al.* 2005; Delle-Vedove *et al.* 2017; Chapurlat *et al.* 2018; Friberg *et al.* 2019). Just as variation between species, many cases of intraspecific floral scent differences have been attributed to differences in pollinator communities and the divergent selection these might impose (Gross *et al.* 2016; Delle-Vedove *et al.* 2017; Chapurlat *et al.* 2019). However, floral scent and pollinator variation sometimes do not correspond, with floral scent variation occurring between populations despite no notable differences in pollinator communities (Delle-Vedove *et al.* 2017). Other factors than pollinator-mediated selection, such as selection by herbivores and florivores (Galen *et al.* 2011), genetic drift (Dötterl *et al.* 2005), biochemical constraints (Majetic and Sinka 2013), phylogenetic constraints (Steiner *et al.* 2011), sensory drive (Koski 2020) and phenotypic plasticity (Majetic *et al.* 2009a) could contribute to generating intraspecific variation. Below, I go through a subset of these factors that are most relevant to this thesis.

Selection on floral scent

Phenotypic selection occurs when individuals with different phenotypes, for example floral scent, differ in fitness (Kingsolver and Pfennig 2007). If individuals within a population vary in scent, and the local pollinators prefer to visit and

pollinate flowers that smell a certain way, this could increase the number of offspring produced by the preferred individuals, thereby increasing their fitness. If the scent variation has a heritable genetic basis (Zu *et al.* 2016), this selection might lead to evolutionary change, shifting the average scent composition and/or emission rates in the population. Differences between populations in which species of insects are pollinating the plants could generate divergent selection between populations, resulting in intraspecific variation in floral scent.

A limited number of studies have investigated selection on floral scent (Majetic *et al.* 2009b; Schiestl *et al.* 2011; Parachnowitsch *et al.* 2012; Ehrlén *et al.* 2012; Gross *et al.* 2016; Chapurlat *et al.* 2019; Joffard, *et al.* 2020b). All of these have found positive selection on at least some (combination of) scent compounds, suggesting these might be important for pollinator attraction, or in other ways increase plant fitness. Still, many compounds were under no detectable or negative selection, indicating that these are not important or even repellent for pollinators, or attractants of local herbivores or florivores. Additionally, scent evolution could be constrained by what variation already exists within a lineage, which could be important in limiting effects of phenotypic selection (Whitehead and Peakall 2009). In this case, differentiation in floral scent could reflect genetic differentiation among populations, and phylogenetic relationships may explain variation between different species instead of, or in addition to, pollinators (Steiner *et al.* 2011; Joffard *et al.* 2020a). However, we still know relatively little about the importance of these factors in shaping floral scent variation, especially between different populations of the same species. I address some of these questions in Chapter III.

Scent and plant mating system

One special case of selection on floral scent regards the effect of mating system shifts. A shift from outcrossing to autonomous selfing does not only affect selection on floral morphology, but can also affect selection on floral scent signals. As the need to attract pollinators is reduced following a shift to selfing, selection might act to decrease floral scent emission to reduce the ecological and metabolic costs of its production. Likewise, genetic drift could contribute to reducing floral scent if it is no longer important for plant fitness. Only a handful of studies have investigated the effect of mating system shifts by comparing the scent of autonomously selfing or self-compatible populations, subspecies or species to that of outcrossing or self-incompatible relatives (Raguso *et al.* 2007; Doubleday *et al.* 2013; Sas *et al.* 2016; Majetic *et al.* 2019). In general, these studies found that scent emission rates were to some extent decreased in selfing plants, although the strength of the effect varied considerably. None of these studies compared plants encompassing the full variation in mating system (outcrossing and selfing) and breeding system (self-compatible and self-incompatible), and a more complete understanding on the matter is still lacking. I perform a comprehensive investigation of this subject in Chapter I.

Phenotypic plasticity in floral scent

Phenotypic plasticity is the change in an individual's phenotype due to different environmental conditions (Price *et al.* 2003). Such plasticity could generate intraspecific floral scent variation in addition to, or in the absence of, any genetically based differentiation, although comparatively few studies have examined this (Majetic *et al.* 2009a). Previous research that has tested the effect of environmental variation such as temperature, drought, nutrients, carbon dioxide, irradiance and humidity on floral scent emission rate and composition has often, but not always, found an effect (Jakobsen and Olsen 1994; Farré-Armengol *et al.* 2014; Friberg *et al.* 2017; Glenny *et al.* 2018). Focusing on nutrient and water availability, two central environmental factors that can vary substantially across time and space, effects vary. Previous studies suggest that nutrient availability has no or only a small effect (Majetic *et al.* 2016; Friberg *et al.* 2017). Water availability on the other hand, seems to have an effect on floral scent in some plant species, often with increasing scent emission rates in plants experiencing drought, and sometimes with effects on the composition of the scent bouquet (Burkle and Runyon 2016; Glenny *et al.* 2018; Campbell *et al.* 2018; Rering *et al.* 2020). As of yet, no study has investigated the simultaneous effect of both nutrient and water availability on floral scent. This is examined in Chapter II.

Aims of the thesis

In this thesis, I use the perennial herb *Arabis alpina* to explore causes of intraspecific variation in floral scent, and consequences of evolutionary shifts in plant mating system. I combine greenhouse, genomic and field approaches to investigate the effects of mating system shifts, environmental factors, genetic differentiation and phenotypic selection on floral scent, and the effect of mating system shifts on parental conflict and reproductive isolation. Specifically, I address the following questions:

1. Are visual and chemical floral signals reduced in self-compatible compared to self-incompatible populations? Alternatively, is signalling only reduced in self-compatible populations with a high capacity for autonomous selfing? (Chapter I)

2. Do populations with different mating system vary in the composition of floral scent? (Chapter I)

3. Does floral scent show any phenotypic plasticity with regard to emission rate or composition under varying water and nutrient conditions? Does the level of plasticity differ between self-compatible and self-incompatible populations? (Chapter II)

4. Is differentiation in floral scent reflected in genetic differentiation among populations, so that genetically similar populations also have similar scent? (Chapter III)

5. Is there phenotypic selection on floral scent and other traits, and do patterns of selection differ among populations? (Chapter III)

6. Do populations with different mating systems differ in levels of parental conflict over seed provisioning? (Chapter IV)

7. What is the effect of parental conflict differences for reproductive isolation in crosses between populations of similar or different mating systems? (Chapter IV)

Methodology

The study species – *Arabis alpina*

Arabis alpina (L.), alpine rock-cress, is a short-lived perennial herb in the Brassicaceae family. It is widely distributed in the northern hemisphere, occurring in arctic, alpine and subalpine regions across parts of Europe, North America, North and East Africa and parts of the Middle East (Koch *et al.* 2006). Often occurring in mountain areas with calcareous soil, individuals are typically found growing in rocky, disturbed and moist habitats, such as along scree slopes, on rock ledges and next to small streams. It reproduces sexually with seeds, although stoloniferous growth also occurs (Toräng *et al.* 2015). Seeds germinate throughout the growing season, forming a leaf rosette. Soon after snowmelt in the spring or summer, plants grow one or several flowering stems. Individuals typically flower for a few weeks, producing varying numbers of white, scented flowers that each remain open for pollination by insects for a few days (Figure 1). Fruits mature one to two months after flowering, producing numerous seeds that disperse as the ripe fruit opens up.

Across its distribution in Europe, *A. alpina* differs in both mating and breeding system. Populations in Greece and central Italy are self-incompatible outcrossers (Ansell *et al.* 2008; Tedder *et al.* 2011; Laenen *et al.* 2018). In contrast, populations from Scandinavia, Spain and the French, Italian and Swiss Alps are self-compatible (Ansell *et al.* 2008; Tedder *et al.* 2011; Buehler *et al.* 2012; Toräng *et al.* 2017). These populations show evidence of the selfing syndrome and have lower outcrossing rates, especially in Scandinavia where plants show high degrees of autonomous self-pollination (Tedder *et al.* 2015; Toräng *et al.* 2017). For Chapters I, II and IV, I studied both self-compatible and self-incompatible populations from these areas, while in Chapter III, I examined only self-incompatible populations.

Figure 1. Photo of an *Arabis alpina* plant growing on a moss-covered rock face. This individual is from a Greek population, and has recently started flowering.

Plant cultivation (I, II, III, IV)

All chapters included cultivation of *A. alpina* plants from different populations across Europe. Seeds were collected from populations in France, Greece, Italy, Scandinavia and Spain. In brief, seeds were sown either on agar plates or directly on soil in pots, and stratified for one week in cold and dark conditions to increase germination. They were then moved into a greenhouse, and the emerging seedlings were grown in separate pots. Thereafter, either leaf material was collected for DNA extraction (Chapter III), or plants were moved to cold conditions for overwintering to induce flowering (Chapter I, II and IV). For Chapter I, III and IV, plants were

watered regularly with water containing a moderate amount of nutrients, allowing for normal plant growth. For Chapter II, plants were treated with either high or low amounts of water, and high or low amounts of nutrients in a full factorial design. After approximately three months of overwintering, plants were put back into the greenhouse, which induced flowering. Thereafter, floral scent was collected from the flowers (I, II) or plants were cross-pollinated by hand within and between different populations (IV).

Floral scent sampling (I, II, III)

Floral scent was collected from plants using dynamic headspace sampling (Raguso and Pellmyr 1998). This method allows for the identification of the different chemical compounds present in the scent bouquet, while simultaneously estimating their emission rate. For Chapter I and II this was done in a greenhouse, while in Chapter III this was done in the field. In all cases, scent was collected during daytime. For each plant, the inflorescence with a known number of open flowers was enclosed in an oven bag with a large and a small opening. A Teflon tube scent trap was then attached to the large opening in the oven bag. The air surrounding the inflorescence was pulled through the scent trap with an air pump at a flow rate of 200 ml/min, monitored by a flow meter, for three hours. At each sampling occasion, a control sample was also collected. After sampling, scent traps were eluted directly into 300 µl hexane (I, II), or sealed with PTFE tape, stored in a freezer and later eluted into hexane (III). In the lab, the 300 µl hexane samples containing the floral volatiles were concentrated to 50 µl, and an internal standard of 5 µl 0.03% toluene was added to each sample.

To identify the floral scent compounds and estimate their emission rates, I used gas chromatography coupled with mass spectrometry (GC-MS). The gas chromatograph separates the volatiles in the samples, while the mass spectrometer fragments the scent molecules into ions, whose pattern and quantity are used to identify the compound and together with the internal standard quantify their emission rate. Compounds were identified with library suggestions, confirmed with Kovats retention indices from the literature, and in some cases through comparisons with synthetic standards. The area under each floral scent peak in the chromatograms were manually integrated using the MS-manufacturer's software. Finally, the emission rate of the different compounds was quantified in units of nanograms per hour per flower, using the internal standard (*cf.* Friberg *et al.* 2013). Total floral scent emission rate per hour per flower for individual plants was calculated by summing the emission rate of the individual compounds.

Floral scent and mating system (I)

In Chapter I, I examined the effect of mating system and breeding system variation on floral scent. I collected floral scent and measured the flower size of 575 individual plants from 17 different *A. alpina* populations. This encompassed nine self-incompatible populations from Greece and Italy, four self-compatible populations with a low capacity for autonomous selfing from France and Spain, and four self-compatible populations with a high capacity for autonomous selfing from Scandinavia. I compared flower size, floral scent emission rate and composition between populations within and between these three mating system categories. Differences in flower size and emission rate were tested with linear mixed models. To investigate variation in scent composition, I calculated Bray-Curtis dissimilarities and visualised patterns with non-metric multidimensional scaling (NMDS) and hierarchical cluster analysis. Permutational multivariate analysis of variance (PERMANOVA) was used to statistically test for differences in the composition of floral scent between mating system categories and populations. Statistical analyses in all chapters were conducted in R 3.5.2 (R Core Team 2018).

Phenotypic plasticity in floral scent (II)

Chapter II aimed to investigate phenotypic plasticity in floral scent. Here, scent was collected from 391 *A. alpina* individuals from ten populations, eight of which were the same as in Chapter I, including four self-compatible populations and six self-incompatible populations. This time, after germination and an initial period of growth, plants from the same seed family were randomly assigned to different water and nutrient treatments in four combinations: high water × high nutrients, high water × low nutrients, low water × high nutrients and low water × low nutrients. Treatments of high water and nutrients were largely similar to those in Chapter I, III and IV, allowing for normal plant growth. The low water treatment received half the amount of water as the high water treatment, while the low nutrient treatment received only 10% as much NPK fertilizer as the high nutrient treatment. These treatments continued through the whole experiment. Floral scent was collected two to three days after a plant's first flower opened. Following scent collection, all current and subsequent open flowers for plants in the two low nutrient treatments were hand-pollinated. To investigate if there was phenotypic plasticity in floral scent in regards to variation in water and nutrient conditions, linear mixed models were used to test for differences in scent emission rate between treatments. PERMANOVAs were used to test for effects on scent composition, which was also visualised with NMDS. Additionally, linear mixed models were constructed to examine if there was a trade-off between scent and seed production in the low nutrient treatments.

Genetic differentiation, phenotypic selection and floral scent (III)

Chapter III considered the effects of genetic differentiation and phenotypic selection on floral scent variation among self-incompatible populations, and was divided into two parts.

In the first part, nine to ten plants from each of two Greek and four Italian populations had their genome resequenced. These populations were the same or closely located to the populations examined in Chapter I, enabling a comparison of genetic differentiation and floral scent differentiation. Among these, the two Greek populations were located close to each other (6 km apart), as were three populations in central Italy (35-80 km apart), while the last Italian population was located in northern Italy (325 km from the closest other population analysed). Individual plants were grown from seeds collected in the field, and DNA was extracted from leaf material. Whole genome resequencing was done by SciLifeLab (Solna, Sweden). Sequence data was mapped to the *A. alpina* reference genome (Willing *et al.* 2015). Variant calling and genotyping was done with GATK (McKenna *et al.* 2010), and indels were filtered out so that only bi-allelic single nucleotide polymorphisms (SNPs) were used in subsequent steps. The SNPs were then filtered in order to remove low quality sites and produce a final data set for further analyses.

Several population genetic analyses were performed. Principal Component Analysis (PCA) was used to examine the genetic structure of individuals from the different populations. Admixture analysis was used to more closely examine the number of potential clusters in the data set. Finally, genetic differentiation was estimated by calculating pairwise F_{ST} values between all populations. The overarching goal of these analyses was to investigate whether the extensive floral scent differentiation among populations discovered in Chapter I reflected the genetic differentiation.

In the second part of the chapter, I quantified linear phenotypic selection on floral scent and other traits in one Italian and one Greek population. This was done during two field seasons, in 2018 and 2019. In each population, up to around 200 plants were marked at the beginning of the flowering season, which began in early April in the Greek population and early May in the Italian population. For each marked plant, flower number, flower size, flowering start and plant height were measured. Floral scent was collected from a subset of the marked individuals. A subset of individuals was also hand-pollinated, in order to estimate pollen limitation. Additionally, pollinators visiting the *A. alpina* plants were observed, and visitation rates of different groups of pollinators were calculated. Approximately two months after the flowering period, populations were visited again and the number of fruits the marked plants had produced were counted. A subset of fruits was collected to examine seed production, so that the total number of seeds produced by individual

plants could be estimated and used as measures of female fitness in subsequent analyses.

I estimated phenotypic selection on floral traits, i.e. the relationship between relative fitness and trait expression, using multiple regression models following Lande and Arnold (1983). Analyses were done using relative fitness as the response variable and variance-standardized traits as the predictor variables. Linear phenotypic selection (β) was estimated separately for each population and year. Selection was estimated for the measured plant traits and on individual floral scent compounds. The two populations emitted partly different scent compounds, and selection was only estimated for the most commonly occurring compounds in each population. As a result, 4-oxoisophorone, benzaldehyde, phenylacetaldehyde and phenylethyl alcohol were included in the analyses of the Greek population. Benzaldehyde, benzyl alcohol, phenylacetaldehyde, phenylethyl acetate and phenylethyl alcohol were included in the analysis of the Italian population. For the Italian population, I could only estimate selection on floral scent in 2018, since in 2019 bank voles (*Myodes glareolus*) consumed a large proportion of the marked plants. Finally, I compared pollinator visitation rates and community composition between the populations, and quantified pollen limitation.

Mating system, parental conflict and reproductive isolation (IV)

In Chapter IV, I investigated the effect of plant mating system on parental conflict and reproductive isolation. A total of 228 individual *A. alpina* plants from three self-compatible populations with low outcrossing rates (one French, one Spanish, one Swedish), and three self-incompatible populations (one Greek, two Italian), presumably with high outcrossing rates, were crossed in all possible combinations within and among populations. For each cross-combination, three to six pairs of plants were crossed with each other in both directions by hand-pollination of three flowers per plant with pollen from the other plant in the pair. After fruit maturation, I counted the number of seeds per fruit produced from the hand-pollinated flowers. All seeds were weighed, and a subset was sown in pots, where the proportion of seeds that germinated was scored during two weeks after sowing.

I analysed the number of seeds per fruit, seed mass and proportion of seeds germinating to investigate the effect of the type of cross and the cross direction on these traits. The aim was to test if seed size differed between crosses in a way that would suggest that parental conflict was higher in the more outcrossing populations, and to quantify the level of postzygotic reproductive isolation for crosses between populations of similar and different mating system. First, using (generalized) linear mixed models, seed number, seed mass and the proportion of seeds that germinated

were compared for crosses between self-compatible populations (SC×SC), crosses between self-incompatible populations (SI×SI), crosses between a self-compatible maternal and a self-incompatible paternal population (SC×SI; crosses are noted as maternal × paternal), and crosses between a self-incompatible maternal and a self-compatible paternal population (SI×SC). Second, I examined the combined effect of cross type and seed mass on germination for the four types of crosses above using generalized linear mixed models. Finally, I calculated reproductive isolation (RI) for each type and direction of cross (Lowry *et al.* 2008), to quantify the strength of the reproductive barriers for different traits and crosses.

Results and Discussion

Floral scent and mating system (I)

Examining the effect of mating system on floral scent in *A. alpina*, I found that, in general, plants from the self-incompatible populations produced larger flowers with a higher emission rate of floral scent than plants from the self-compatible populations (Figure 2). In contrast, there were no or only minor differences between the self-compatible populations with a high and a low capacity for autonomous selfing. This finding is somewhat surprising, as one could expect that only plants with high autonomous selfing would be under selection to decrease floral signalling. Instead, also the self-compatible populations with low autonomous selfing showed a significantly decreased flower size and scent emission compared to the self-incompatible populations. Possible explanations could include low levels of pollen limitation in the self-compatible populations with low autonomous selfing, which could relax selection on floral signalling. Additionally, the Scandinavian populations with high autonomous selfing have a recent origin and very limited genetic variation (Laenen *et al.* 2018), which could have prevented further reductions in floral signalling.
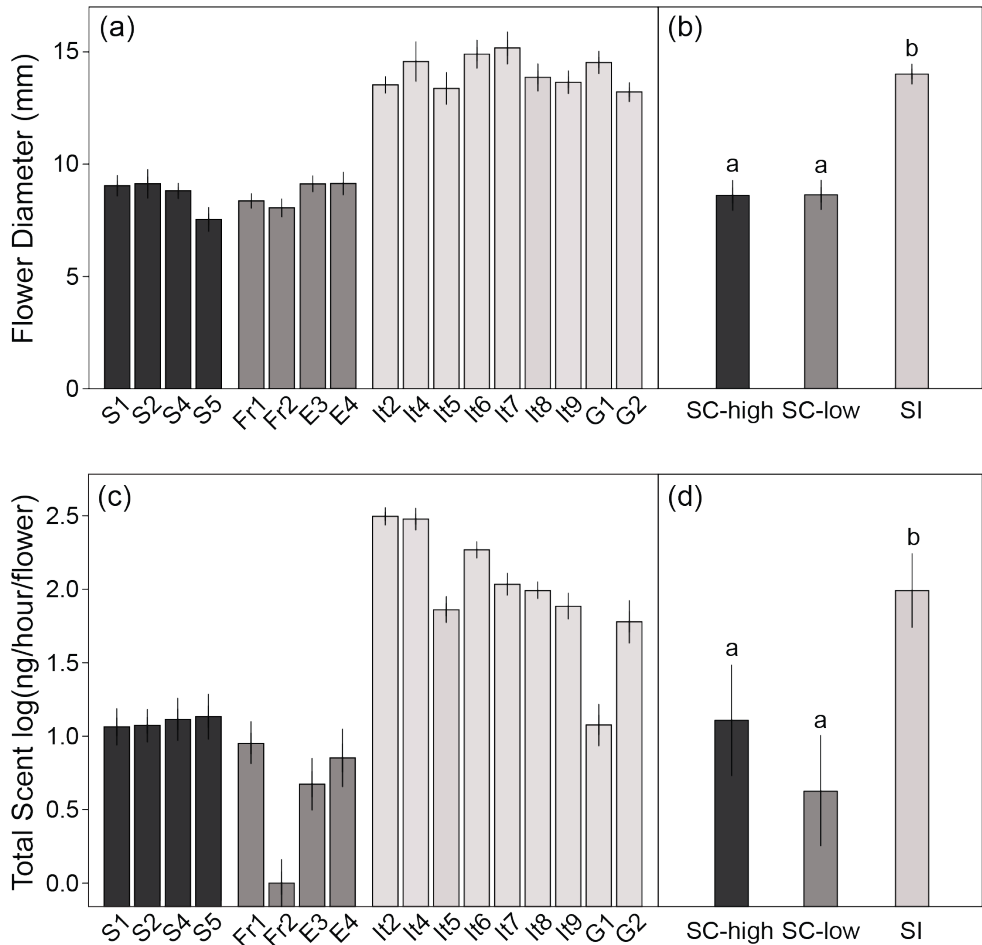
Figure 2. Flower diameter (a-b) and total scent emission rate per flower (c-d) of plants from 17 different *Arabis alpina* populations. Shown are means and 95% CI (confidence intervals) for individual populations (a, c) and for the three categories of populations (b, d). SC-high: Scandinavian (S) self-compatible populations with high capacity for autonomous selfing; SC-low: French (Fr) and Spanish (E) self-compatible populations with low capacity for autonomous selfing; SI: Italian (It) and Greek (G) self-incompatible populations. Colours indicate to which category different populations belong. Letters above bars in (b) and (d) indicate significant differences between mating system categories.

In total, I detected 32 different compounds in the floral scent of *A. alpina*. As indicated by the pie charts (Figure 3a), NMDS plot (Figure 3b) and clustering analysis (Figure 3c), there was substantial variation in the composition of floral scent between plants of different populations, both between and within the three mating system categories. In most cases, samples from the same populations clustered closely together (Figure 3b), indicating that floral scent varied comparatively less within populations than between populations. There were larger differences in scent composition between self-compatible and self-incompatible populations, compared to that between self-compatible populations with high and

32

low autonomous selfing. This indicates that the transition from self-incompatibility to self-compatibility did not simply reduce the overall scent emission rate, but instead shows that the emission rate of some compounds has been reduced more than others.
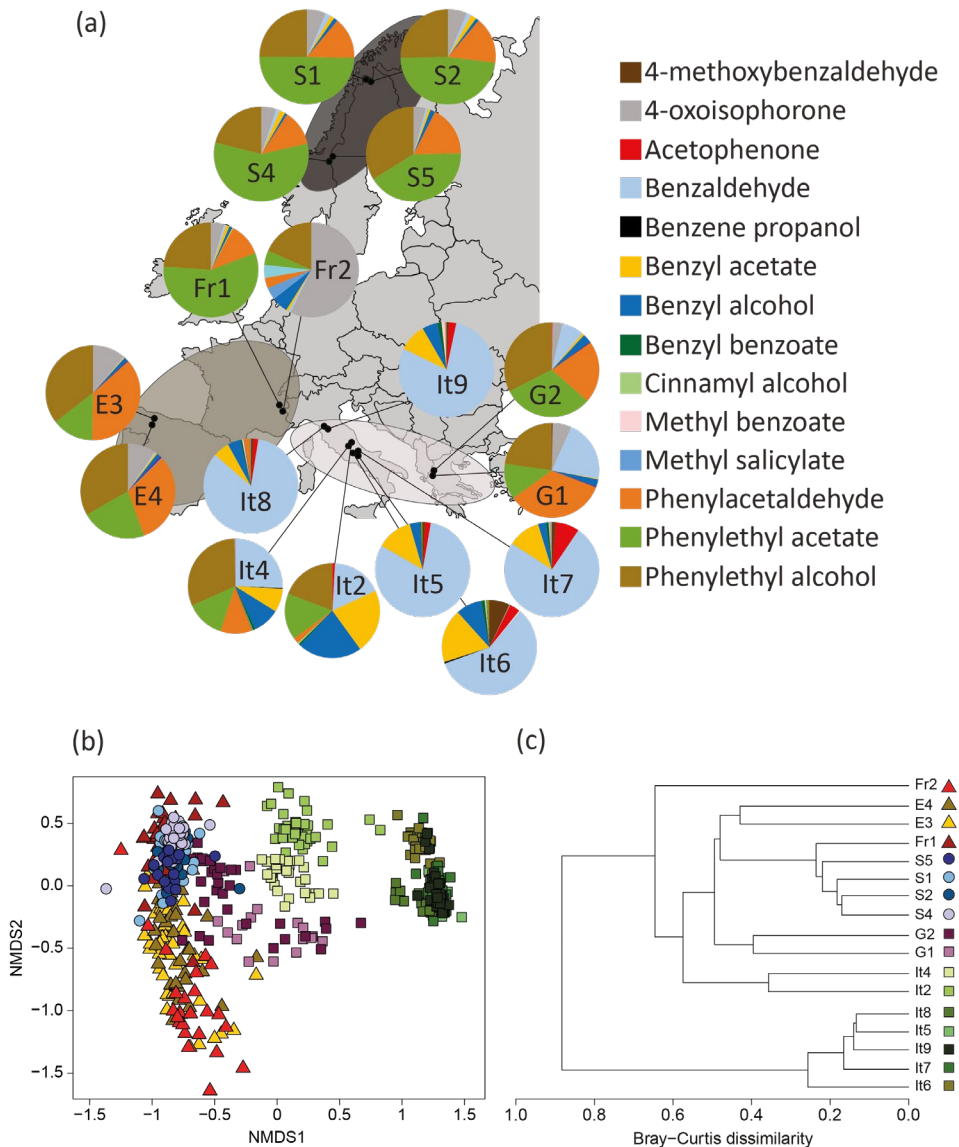

Figure 3. Visualisation of variation in the composition of the floral scent bouquet among 17 *Arabis alpina* populations. (a) Map with the location of each population, with ellipses indicating the mating system categories (same as in Figure 2). Pie charts show the average scent composition for each population. (b) Non-metric multidimensional scaling plot indicating the distribution of all floral scent samples from the 17 populations. Symbols are indicated in (c), with data points located close to each other having a similar scent composition. (c) Clustering cladogram showing population level variation in floral scent composition.

On a population level, the four Scandinavian populations, with a high capacity for autonomous selfing, all had a very similar scent composition dominated by phenylethyl acetate and phenylethyl alcohol. Among the French and Spanish populations, with a low capacity for autonomous selfing, there were larger differences between and within populations. The self-incompatible Greek and Italian populations also largely differed between populations, although some populations had an overall similar composition (Figure 3). Interestingly, the scent composition of some but not all Italian populations was completely dominated by benzaldehyde. These differences in scent composition between populations of the same mating system suggest that also other factors such as genetic drift and selection by pollinators and herbivores (Delle-Vedove *et al.* 2017) are important in shaping scent variation in *A. alpina*. In conclusion, this chapter provides an example of extensive intraspecific floral scent variation at varying geographical scales, and demonstrates the potential importance of both mating system shifts and other factors as causes of such diversification.

Phenotypic plasticity of floral scent (II)

Chapter II investigated the effects of environmental conditions on floral scent. For both self-compatible and self-incompatible populations, plants in the high nutrient treatment emitted somewhat more scent per flower than plants in the low nutrient treatment (Figure 4a-b). In contrast, the water treatment had no effect on the scent emission rate. Scent composition, on the other hand, was unaffected by both treatments. Instead, samples clustered largely by population, which as a factor explained much more of the variation than the treatments did (Figure 4c-d). Additionally, there was no trade-off between scent production and seed production in the low nutrient treatments.
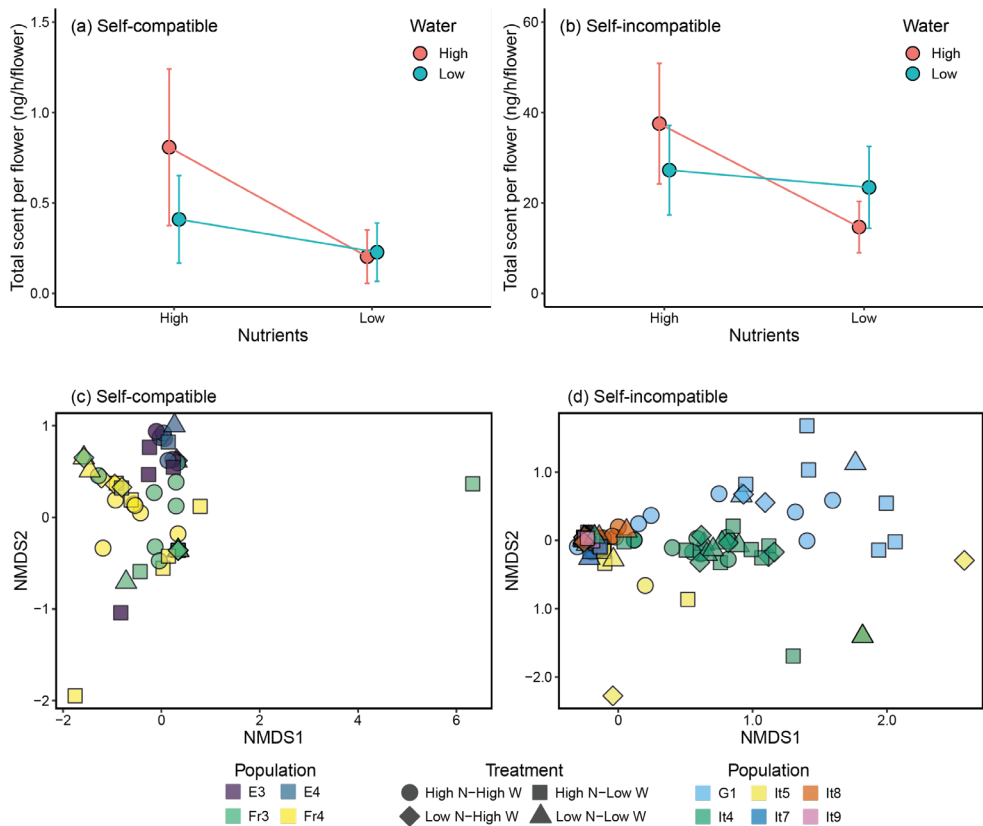
Figure 4. Effects of nutrient and water treatments on the emission rate per flower (a-b) and composition (c-d) of floral scent among self-compatible (a, c) and self-incompatible (b, d) *Arabis alpina* plants. Scent emission rate (a-b) represent back-transformed mean ± SE (standard error) estimates from linear mixed models. Composition (c-d) is indicated by NMDS plots, with data points representing individual plants from different populations and treatments as indicated by the shapes and colours in the legend (N – nutrients, W – water).

The effect of nutrient but not water treatment on emission rate partly contrasts with previous studies. These have found no or very limited effects of nutrients (Majetic *et al.* 2016; Friberg *et al.* 2017), but often an effect of drought (Burkle and Runyon 2016; Glenny *et al.* 2018; Campbell *et al.* 2018; Rering *et al.* 2020). This variability emphasizes the difficulty of making any general conclusions about the effects of environmental conditions on floral scent, and suggests that results are often species-specific.

While plants in the high nutrient treatment emitted more scent per flower, which in theory could increase pollinator visitation rates and subsequent seed set, the importance of this plasticity in nature is less certain. Plants in the high nutrient treatment produced approximately ten times more flowers in total than plants in the low nutrient treatment. Consequently, nutrient availability could have a larger effect on floral scent emission at the level of the whole inflorescence than at the level of

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the individual flower. Furthermore, floral scent could be important in both attracting pollinators to the plant from a distance, and as a short-distance cue making them land on individual flowers. This means that the significance of plasticity in floral scent could depend on what type of pollinators are the most important visitors. This chapter also points to the importance of increasing our understanding of environmental effects on floral signalling, especially as human-induced global change is increasingly influencing the biotic and abiotic environment experienced by flowering plants.

Genomic differentiation, phenotypic selection and floral scent (III)

The population genetic analyses for the six Italian and Greek *A. alpina* populations indicated that the level of genetic differentiation between populations could be largely explained by the geographical distance between them. Individual plants clustered within populations in the PCA plot (Figure 5b). For both the PCA and F_{ST} analyses, populations grouped by geography, with the two Greek populations, and the three central Italian populations clustering together with each other (Figure 5b, Table 1). The Admixture analysis showed a slightly different pattern in regards to the Italian populations (Figure 5c). Combined with the floral scent data of some of these populations in Chapter I (Figure 3), these results indicate that genetically similar populations can differ considerably in floral scent. Most clearly, populations It2, It4 and It6 in central Italy belonged to the same genetic cluster, but still showed large differences in scent composition. Potentially, such differentiation between closely related populations could comparatively easily evolve in a species such as *A. alpina* where scent composition differences mostly regard relative proportions of different compounds produced by similar biosynthetic pathways.
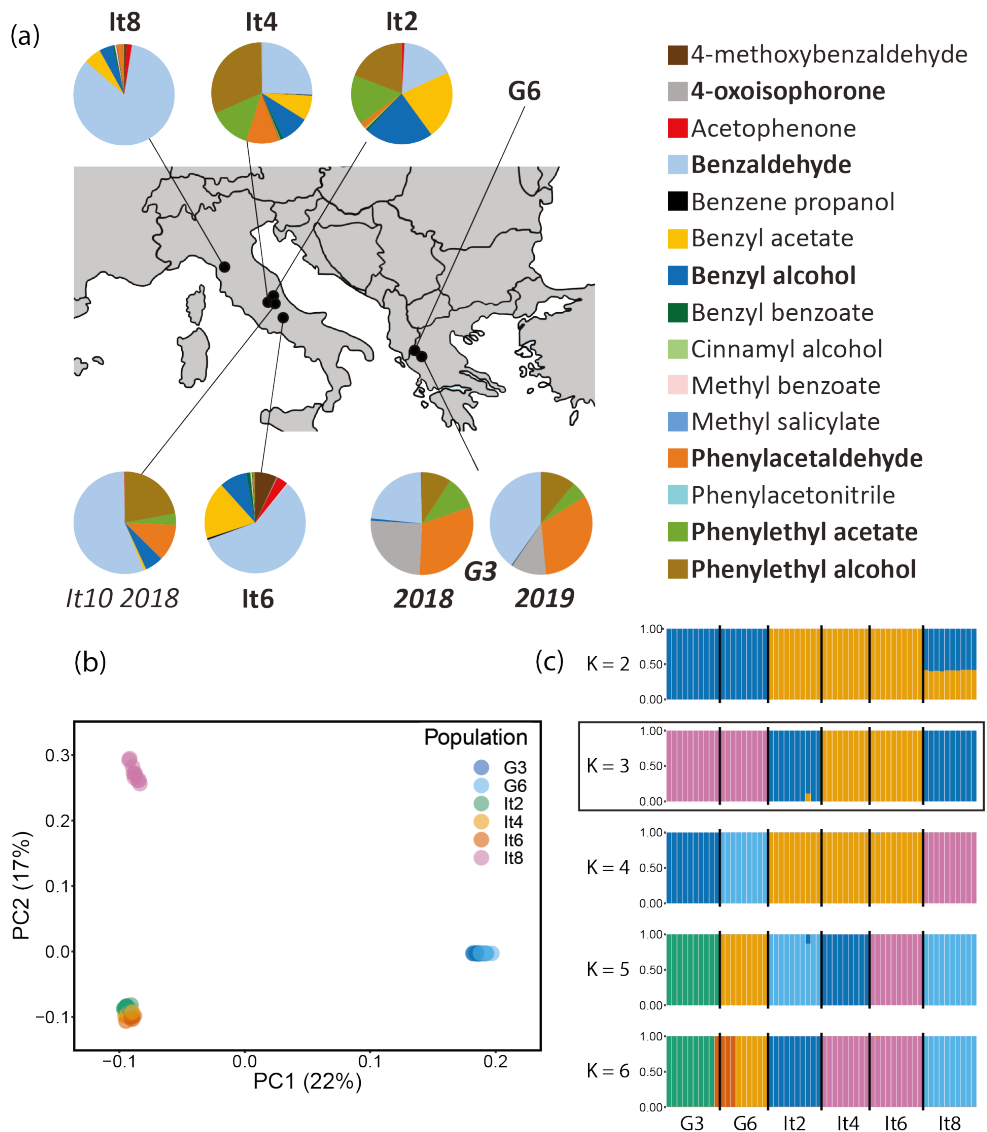
Figure 5. Floral scent and genetic differentiation among *Arabis alpina* populations included in genomic and selection experiments. (a) Map showing the location of the populations, and their average scent composition (with pie charts from It2, It4, It6 and It8 from Chapter I). Populations in bold were sequenced, populations in italic were investigated for phenotypic selection. The six compounds indicated in bold were included in estimates of selection. (b) Principal Component Analysis (PCA) showing the genetic clustering among individuals from the sequenced populations G3, G6, It2, It4, It6 and It8. (c) Admixture analysis indicating population structure based on K = 2-6 clusters. The black border around the analyses for K = 3 indicates that this was the most likely number of genetic clusters.

Table 1. Pairwise F_{ST} values between the Greek (G3, G6) and Italian (It2, It4, It6, It8) *Arabis alpina* populations that were sequenced. Low values indicate little genetic differentiation between populations, high values indicate strong genetic differentiation.

	G3	G6	It2	It4	It6
G6	0.060				
It2	0.452	0.436			
It4	0.421	0.403	0.241		
It6	0.413	0.395	0.257	0.182	
It8	0.451	0.434	0.351	0.322	0.317

Investigating selection on floral scent and other traits, I found some differences in selection patterns for the Greek and the Italian population, although most traits did not show evidence of experiencing phenotypic selection. In both populations, there was strong, positive selection on flower number during both years, indicating that plants producing more flowers had higher fitness (as estimated by total seed production). Additionally, there was selection for increased flower size in one year in the Greek population (Figure 6). For floral scent traits, selection favoured increased emission of benzaldehyde in the Italian population in 2018, and increased emission of phenylacetaldehyde and decreased emission of phenylethyl alcohol in the Greek population in 2018. However, selection was not consistent for phenylethyl alcohol across years, and only measured for one year in the Italian population, so only phenylacetaldehyde was shown to be under somewhat consistent selection both years. A number of different pollinators, of which *Bombylius* spp. (bee flies) were the most common in both populations, visited the *A. alpina* plants. Pollinator visitation rates were lower in the Italian than the Greek population, and the pollinator community differed between populations. The Italian population experienced higher levels of pollen limitation.
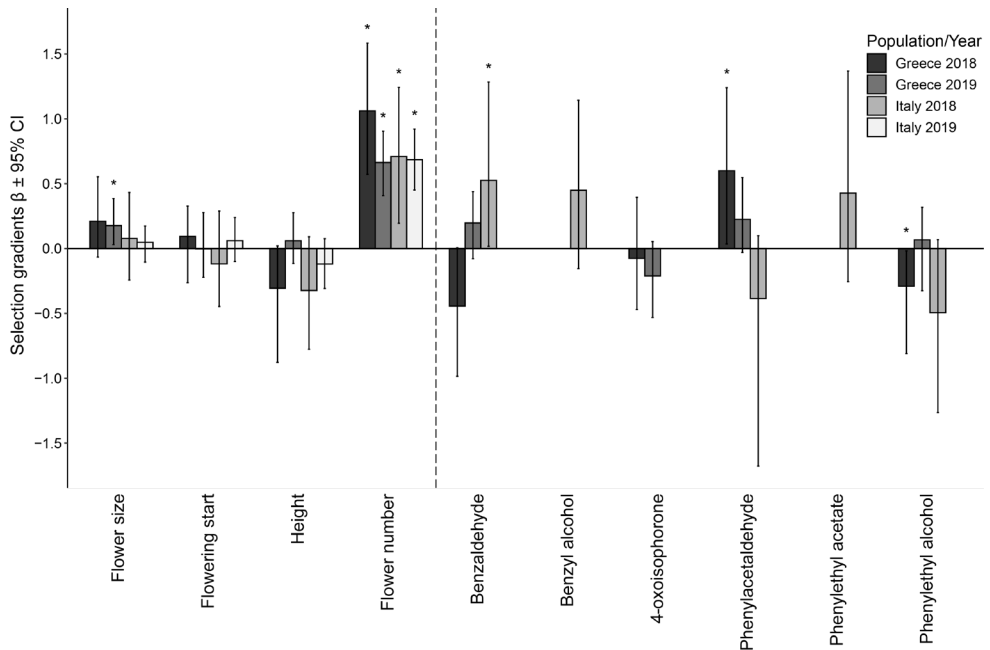
Figure 6. Linear phenotypic selection (β ± 95% CI) for flower size, flowering start, plant height, flower number and six floral scent compounds in the Greek and Italian *Arabis alpina* populations where selection was estimated in 2018 and 2019. Selection was estimated on partly different floral scent compounds in the two populations, and not in the Italian population in 2019. Asterisks above individual bars indicate selection gradients with 95% CI not overlapping zero.

The limited evidence of linear phenotypic selection on floral scent and other traits is perhaps not surprising, since this was also the case for many scent compounds in previous studies (Schiestl *et al.* 2011; Parachnowitsch *et al.* 2012; Gross *et al.* 2016; Chapurlat *et al.* 2019; Joffard *et al.* 2020b). In fact, estimates of directional selection in nature are often non-significant (Kingsolver *et al.* 2001). There could be several explanations for these patterns, including compounds not being important for pollinator attraction, conflicting selection by pollinators and herbivores, and presence of stabilizing rather than directional selection (Harder and Johnson 2009; Schiestl *et al.* 2011; Joffard *et al.* 2020b). Still, some traits and scent compounds were under statistically significant selection. As the pollinator communities were partly different in the two populations, this could contribute to shaping the patterns of selection, although other factors could also be important (Delle-Vedove *et al.* 2017; Farré-Armengol *et al.* 2020). In summary, this chapter demonstrates that close genetic relatedness between populations does not necessarily limit floral scent differentiation, and provides an example of partial differences in selection patterns on floral scent and other traits for different populations of the same species.

Mating system, parental conflict and reproductive isolation (IV)

Comparing crosses between breeding systems (SC×SI, SI×SC), to crosses within each breeding system (SC×SC, SI×SI), I found that seed mass and germination (Figure 7b-c), but not seed number (Figure 7a) were decreased in crosses between self-compatible and self-incompatible populations. Importantly, there were also differences between the reciprocal cross directions for the crosses between breeding systems, with more and heavier seeds being produced by SC×SI crosses than by SI×SC crosses, but there was no difference in germination when comparing the reciprocal crosses. However, when examining the combined effect of type of cross and seed mass on germination, it became clear that although seeds from SI×SC crosses were on average smaller than the reciprocal cross (Figure 7b) they had higher germination at any given seed size (Figure 7d). The decreased seed size in crosses between self-compatible and self-incompatible plants, along with differences in seed size and germination between reciprocal crosses between breeding systems, suggest that the level of parental conflict over seed provisioning is higher in the self-incompatible populations (Brandvain and Haig 2005; Lafon-Placette and Köhler 2016). In such crosses, the endosperm may receive little resources (SI×SC crosses) resulting in very small seeds, or fail to develop correctly (SC×SI crosses), resulting in slightly larger but deformed seeds with lower germination.
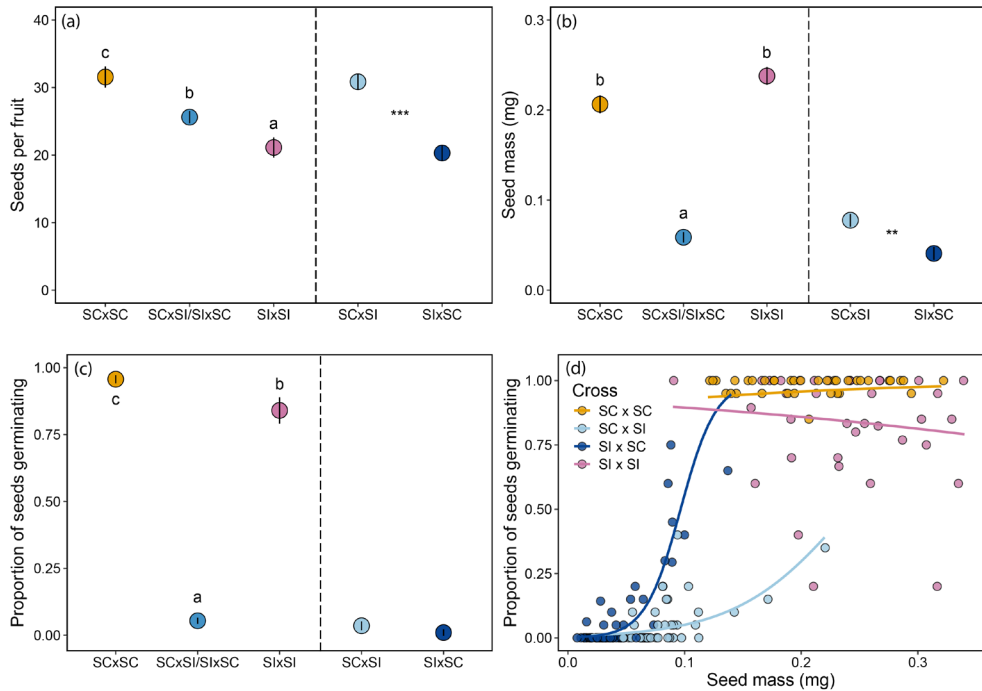
Figure 7. Effect of type of cross on the number of seeds per fruit (a), seed mass (b) and proportion of seeds germinating (c), for crosses between three self-compatible and three self-incompatible populations of *Arabis alpina*. Points indicate mean ± SE. The left parts of plots in (a-c) show outcomes of crosses between self-compatible (SC×SC) populations, between self-incompatible (SI×SI) populations and between populations of different breeding systems (SC×SI/SI×SC combined). Different letters above boxes indicate significant differences between groups. The right parts of the plots indicate the effect of cross direction in reciprocal crosses between breeding systems (SC×SI and SI×SC), with asterisks indicating a significant difference between cross directions (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (d) The proportion of seeds germinating as a function of type of cross and seed mass. Data points represent seed mass and germination of seeds from individual maternal plants, with lines indicating model predictions for each type of cross. Importantly, germination for SI×SC crosses (dark blue) is higher at any given seed mass than germination for SC×SI crosses (light blue).

As a result of the compromised seed development for crosses between self-compatible and self-incompatible plants, there was strong reproductive isolation between breeding systems (Figure 8). Total reproductive isolation (Figure 8c), which takes into account differences in both seed number and germination, was very strong for SC×SI and SI×SC crosses, while SC×SC and SI×SI crosses showed essentially no reproductive isolation at all. In fact, self-compatible and self-incompatible *A. alpina* populations were so strongly reproductively isolated that they could be considered different species. Overall, this chapter demonstrates the potential importance of mating- and breeding system shifts in generating reproductive isolation, with potential consequences for gene flow, hybridization and ultimately speciation.
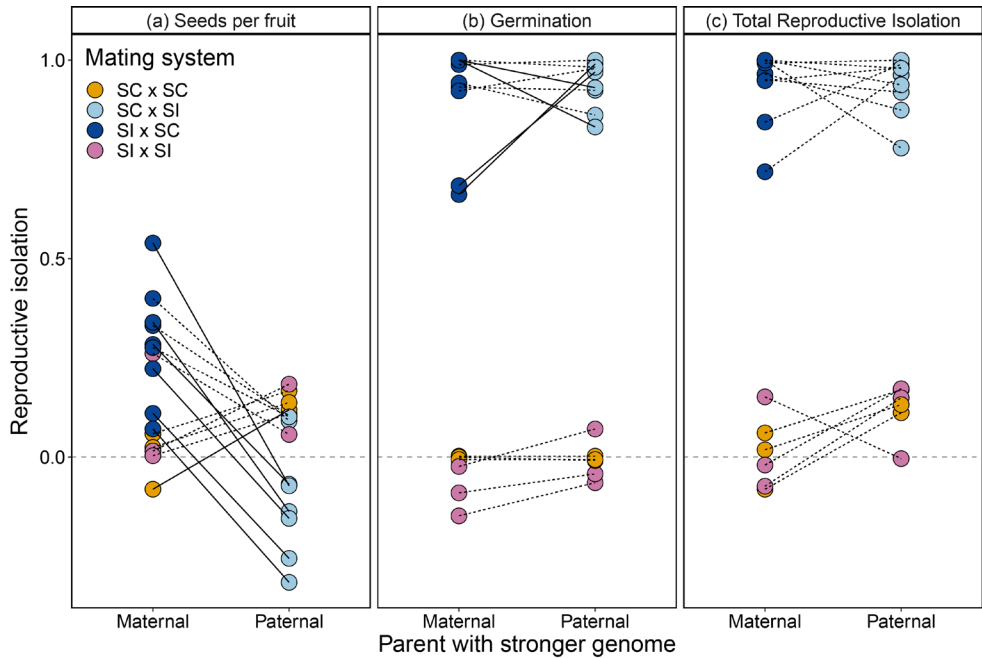
Figure 8. Estimates of reproductive isolation for (a) number of seeds per fruit, (b) proportion of seeds germinating and (c) total reproductive isolation in crosses between self-compatible (SC×SC), between self-incompatible (SI×SI) and between self-compatible and self-incompatible (SC×SI, SI×SC) *Arabis alpina* populations. A value of 0 indicates no reproductive isolation, with between population crosses producing similar numbers of seeds (a), or seeds with similar germination (b) as within population crosses. A value of 1 represents complete reproductive isolation, with between populations producing no seeds (a), or seeds that do not germinate (b). Total reproductive isolation (c) takes into account effects of both seed number and germination. Lines connect crosses between the same two populations in different directions, where either the maternal (left) or paternal (right) parent is predicted to have the stronger genome (based on comparisons of outcrossing rates and strength of self-incompatibility). Solid lines indicate statistically significant effects of cross direction.

Conclusions

Floral and reproductive diversity among angiosperms is ubiquitous. Understanding the origin of this diversity requires studies of the potential mechanisms generating this variation. In this thesis, using *A. alpina* as a study species, I have investigated causes of intraspecific variation in floral scent, and consequences of evolutionary shifts in plant mating system. I found evidence of some phenotypic plasticity in floral scent in response to varying environmental conditions, although the importance of such plasticity in natural populations remains to be investigated (Chapter II). Investigations of genomic patterns indicate that even genetically similar populations can differ substantially in floral scent. Further, phenotypic selection may be important in shaping differences in floral scent between conspecific populations (Chapter III). Comparing populations of different breeding- and mating system, I found differences in scent emission rate and composition mainly between self-compatible and self-incompatible populations, but also between populations of the same mating system. However, floral signalling was not further reduced in the self-compatible populations with high levels of autonomous selfing, a pattern that could have several explanations and should be further investigated in future studies (Chapter I). Moreover, mating systems shifts are important in generating reproductive isolation, with potential consequences for speciation. For *A. alpina* specifically, reproductive isolation between self-compatible and self-incompatible populations is so strong that they may even be considered different species (Chapter IV). Collectively, these studies on *A. alpina* provide an example of considerable intraspecific floral signalling variation, demonstrate the potential role of local ecological conditions in generating this diversity, and elucidate how mating system shifts are important in shaping the evolution of both floral scent and reproductive isolation. On a broader level, the results of my thesis illustrate some of the ecological and evolutionary mechanisms generating the extraordinary floral and reproductive diversity among flowering plants.

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List of papers

I. Petrén H., Toräng P., Ågren J., Friberg M. (2020). Evolution of floral scent in relation to self-incompatibility and capacity for autonomous self-pollination in the perennial herb *Arabis alpina.* Submitted manuscript.

II. Luizzi V.J., Friberg M., Petrén H. (2020). Phenotypic plasticity in floral scent in response to nutrient, but not water, availability in the perennial plant *Arabis alpina* (Brassicaceae). Manuscript.

III. Petrén H., Svensson K., Runemark A., Pace L., Halley J.M., Boutsi S., Ågren J., Friberg M. (2020). Phenotypic selection, genetic differentiation and floral scent variation in a widespread flowering plant. Manuscript.

IV. Petrén H., Thosteman H., Stift M., Toräng P., Ågren J., Friberg M. (2020). Differences in breeding system and predicted parental conflict affect postzygotic reproductive isolation in a perennial herb. Manuscript.