Evolutionary conflicts and ecological constraints at reproduction in the dioecious plant *Silene latifolia*

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Evolutionary conflicts and ecological constraints at reproduction in the dioecius plant *Silene latifolia*

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A mon frère

Dans le ciel j'ai vu la lumière
De ton étoile qui chaque jour éclaire
Et guide mes pas sur le chemin
Menant à de radieux lendemains

Ta petite soeur

La plus belle chose que nous puissions éprouver,
C'est le côté mystérieux de la vie.
C'est le sentiment profond qui se trouve
Au berceau de l'art et de la science véritable.

Albert Einstein

Contents

Key words

Plant reproduction ; dioecy ; sexual dimorphism ; flower number ; flower size ; nursery pollination ; mutualism ; seed predation ; phenotypic selection ; plant defences ; fruit abortion ; siring success ; microsatellite DNA ; paternity ; seed mass ; pollen tube growth ; stigma wilting ; stigma size ; *Silene latifolia ; Silene alba ;* white campion ; *Hadena bicruris.*

Mots-clés

Reproduction des plantes; dioécie ; dimorphisme sexuel ; nombre de fleurs ; taille des fleurs ; « nursery pollination » ; mutualisme ; prédation de graines ; sélection phénotypique; défenses des plantes; fruit abortion ; succès reproducteur; ADN microsatellite; paternité ; masse des graines ; croissance des tubes polliniques; fanaison du stigmate; taille du stigmate ; *Silene latifolia ; Silene alba ;* compagnon blanc ; silène des prés ; *Hadena bicruris.*

Thesis summary

Flowering plants show an astonishing diversity of flower shapes and colours. Among plant reproductive systems, dioecy is of particular interest because each sex can be subjected to independent selection pressures that may lead to the evolution of different floral traits in the two sexes (sexual dimorphism; **chapter 1**). On the one hand, many studies have confirmed the role of pollinators as selective agents on floral traits. Pollinators are attracted by plants with large flowers and with many open flowers (large floral display), and have been shown to contribute to selection on these traits. Plant fitness via pollination will not only depend on the probability of attracting pollinators but also on the actual dynamics of pollen deposition and reception, which are influenced by the floral phenology and characteristics of the receptive surfaces. On the other hand, natural enemies of flowers can directly affect plant fitness, and may also influence the evolution of floral traits and their dimorphism. Some plants are pollinated by insects that also act as natural enemies by consuming part of the seeds produced (nursery pollination). To reduce the cost of seed predation, female plants may use fruit abortion as a defence mechanism. However defences can be costly themselves, and defence costs may contribute to selection on floral traits. Thus pollinators and seed predators may select for opposite optima in floral traits in male and female plants. In this thesis I investigate the effect of the pollinating-seed predator *Hadena bicruris* on the fitness of the dioecious plant *Silene latifolia*, the contributions of pollinators and seed predators to selection on sexually dimorphic floral traits, the role of fruit abortion in mediating floral trait selection, the effect of flower size and fruit abortion on the insect performance, and at the flower level, the effect of floral phenology and characteristics of the receptive surfaces on the fitness of male and female plants.

In **chapter 2**, I investigated the effects of flower size, seed provisioning and fruit abortion on the fitness of larvae of the pollinating-seed predator. Using selection lines that varied in flower number and size (large- vs. small-flowered plants), I infested two flowers per plant with eggs of *H. bicruris*, measured the growth of the resulting larvae, fruit abortion, and seed provisioning of one non-attacked fruit. Fruits from large-flowered plants (LF) contained significantly more seeds and more nutrients, and gave rise to significantly larger larvae than small-flowered plants (SF). Fruit abortion had a dramatic effect on larval growth, reducing significantly the mass and time at emergence of larvae from the fruit. Also the success of larval establishment was higher on LF plants and these plants had a lower probability of fruit abortion (significant for the second fruit). Thus fruit abortion is detrimental for larval development- and likely reduces the amount of resources lost by the plant, and therefore may help to stabilize this plant-nursery pollinator mutualism.

In **chapter 3**, I investigated the effect of varying floral display and flower size on plant fitness, and pollinator and seed predator selection on floral traits. I conducted two common garden experiments. In, the first experiment, I exposed plants from SF and LF selection lines to naturally occurring pollinators and seed predators, and recorded fruit predation, abortion and seed production, and selection on floral traits. In the second experiment, I measured the risk of infestation by eggs of *H. bicruris* on plants that displayed only flowers. I found positive total selection but no seed predator selection on flower number. Total seed production, number of eggs received, and proportions of predated or aborted fruits did not differ significantly between lines. SF plants reared significantly more larvae, tended to have a larger parasite load, and lost more fruits due to predation and abortion than LF plants. Interestingly, fruit abortion was negatively selected, and seed predator selected against abortion in the SF but not in the LF plants. Fruit abortion was significantly more common in plants with high parasite load. While pollinators or non-ovipositing *H. bicruris* may select for

large floral display in males, the seed predator may contribute indirectly to selection for small floral display in females by imposing higher abortion costs to plants with a large floral display.

In **chapter 4**, I investigated how traits at the flower level affect the within fruit genetic diversity, a component of male and female fitness. As shown in a field survey fruits of *S. latifolia* are usually sired by multiple fathers but the mechanisms were not known. In greenhouse experiments I studied the effect of timing between hand-pollination with the pollen of two males on paternity and seed mass, the effect of pollen load on seed set, the time needed for pollen tubes to reach the ovary, and stigma wilting after pollen deposition. The first-arriving pollen sired significantly more seeds than later-arriving pollen, and this advantage was seen several hours before the pollen tubes could reach the ovary. The stigma papillae wilted simultaneously with pollen tube growth. A more even share of paternity between the two males resulted in a significantly lower individual seed mass. Thus multiply sired fruits of *S. latifolia* as usually found in the field are likely to result from simultaneous deposition of pollen from several male plants (pollen carry over) or from multiple pollinator visits within a short time interval. Male and female function may have conflicting interest over the duration of stigma receptivity, forward wilting would benefit the first-arriving pollen donor by increasing its paternity share compared to later-arriving donors, while wilting may be costly to the female function by reducing the genetic diversity of offspring and/or the number of pollen grains captured by the stigma.

In **chapter 5**, together with Sara Teixeira, I investigated whether the size of the receptive stigma surface or the size of the ovary affect the shares of paternity between two competing pollen donors. Sara hand-pollinated the flowers of two sisters and of one unrelated female with the pollen of two males, and determined paternity. I counted the number of stigma lobes, measured stigma surface and ovary size on unpollinated flowers of these females. Flowers had four to seven stigma lobes, but most flowers had five lobes. I found significant variation between the two populations in stigmatic surface and ovary size. Those two traits were significantly positively correlated with each other, but did not correlate with the relative siring success of the two males. Thus a larger stigmatic surface apparently does not increase competition between two pollen donors, and results in similar shares of paternity. However, it may influence pollen capture under natural pollination.

In conclusion (**chapter 6**) my results suggest that the evolution of sexually dimorphic traits in *S. latifolia* flowers may be due to opposing selection on male and female plants. While pollinators may select for larger display in both sexes, females would pay a larger cost to fruit abortion with increasing display size. Thus its natural enemy is likely to contribute to selection for small floral display in females. Fruit abortion seem to play an important role in this plant-insect interaction. This resistance trait is likely to be beneficial to the plant or to its offspring on the long term because it is expected to reduce the seed predator population. However on the short term, it is costly to the female plant. The nature of this cost remains to be elucidated. At the flower level, my results suggest that only a small window of time is available for a male’s pollen to obtain siring success. The role of stigma surface and wilting for pollen capture and paternity, and thus for the fitness of male and female plants under natural conditions need further investigation. Stigma surface may increase pollen capture, and wilting may be used by male or female to manipulate the outcome of pollen competition. Alternatively, wilting may serve as a defence mechanism against venereal diseases or may render the flower unattractive to ovipositing females of seed predators. Future studies on the evolution of floral traits in plants should not be limited to pollinators, but should simultaneously consider natural enemies as well as the more subtle changes at the flower level.

Résumé de la thèse

Les fleurs des angiospermes sont d'une étonnante diversité de formes et de couleurs. Parmi les systèmes de reproduction, la dioécie recèle un intérêt particulier étant donné que chaque sexe peut être soumis à des pressions de sélection différentes, pouvant amener à l'évolution de traits floraux différents chez les deux sexes (dimorphisme sexuel ; **chapitre 1**). D'un côté, de nombreuses études ont confirmé le rôle joué par les pollinisateurs en tant qu'agents de sélection sur les traits floraux. Les pollinisateurs sont attirés par des plantes qui possèdent de grandes fleurs ou de nombreuses fleurs ouvertes (floral display), et leur rôle dans la sélection de ces traits est connu. Le succès reproducteur d'une plante via la pollinisation ne dépend pas seulement de la probabilité d'attirer les pollinisateurs, mais aussi de la dynamique réelle du dépôt de pollen et de sa réception, lesquels sont influencés par la phénologie florale et par les caractéristiques des surfaces réceptives. D'un autre côté, les ennemis des fleurs peuvent directement affecter le succès reproducteur des plantes, et pourraient aussi influencer l'évolution des traits floraux et leur dimorphisme. Certaines plantes sont pollinisées par des insectes qui jouent aussi le rôle d'ennemi en consommant une partie des graines produites (nursery pollination). Pour réduire le coût de la prédation de graines, les plantes femelles pourraient se servir de l'avortement des fruits en tant que mécanisme de défense. Néanmoins les défenses peuvent elles-mêmes être coûteuses, et ces coûts pourraient contribuer à la sélection sur les traits floraux. Ainsi les pollinisateurs et les prédateurs de graines pourraient sélectionner des optimaux opposés pour les traits floraux des plantes mâles et femelles. Dans cette thèse j'examine l'effet du pollinisateur-prédateur de graines *Hadena bicruris* sur le succès reproducteur de la plante dioïque *Silene latifolia,* les contributions des pollinisateurs et des prédateurs de graines à la sélection des traits floraux sexuellement dimorphiques, le rôle

de l'avortement des fruits en tant qu'intermédiaire dans la sélection des traits floraux, l'effet de la taille des fleurs et de l'avortement des fruits sur la performance de l'insecte, et au niveau de la fleur, l'effet de la phénologie florale et des caractéristiques des surfaces réceptives sur la fitness des plantes mâles et femelles.

Au **chapitre 2,** j'examinai les effets de la taille des fleurs, de l'approvisionnement des graines, et de l'avortement des fruits sur le succès reproducteur du pollinisateur-prédateur de graines. En utilisant des lignées de sélection qui variaient dans la taille et le nombre de fleurs (plantes à larges- ou petites fleurs), j'infestai deux fleurs par plante avec des oeufs de *H. bicruris,* mesurai la croissance des larves en résultant, l'avortement des fruits, et l'approvisionnement des graines d'un fruit non attaqué. Les fruits des plantes à larges fleurs (LF) contenaient significativement plus de graines et plus de nutriments, et les larves qui émergèrent de ces fruits étaient plus grandes comparativement aux plantes à petites fleurs (SF). L'avortement des fruits eut un effet considérable sur la croissance des larves, réduisant significativement la taille des larves et le temps nécessaire à leur émergence du fruit. Aussi la probabilité qu'avaient les larves de s'établir était plus élevée chez les plantes LF, et ces plantes avaient aussi une plus faible propension à l'avortement (significatif pour le deuxième fruit) que les plantes SF. Ainsi l'avortement des fruits est préjudiciable au développement de la larve et vraisemblablement diminue la perte de ressources pour la plante, et par conséquent pourrait aider à stabiliser le mutualisme entre cette plante et ce pollinisateur-prédateur de graines.

Au **chapitre 3**, j'étudiai l'effet de la variation du nombre de fleurs ouvertes et de la taille des fleurs sur le succès reproducteur de la plante, et la sélection sur les traits floraux par les pollinisateurs et les prédateurs de graines. Je conduisis deux études en jardin expérimental. Dans la première étude, j'exposai des plantes des lignées de sélection SF et LF aux pollinisateurs et prédateurs de graines naturellement présents, et mesurai la prédation et l'avortement des fruits, la production de graines, et la sélection sur les traits floraux. Dans la seconde expérience, je mesurai le risque d'infestation des fleurs avec des oeufs de *H. bicruris* sur des plantes ne déployant que des fleurs. Je trouvai de la sélection totale positive mais pas de sélection de la part du prédateur de graines sur le nombre de fleurs. La production totale de graines, le nombre d'oeufs reçus, et les proportions de fruits attaqués ou avortés ne différèrent pas de manière significative entre les lignées. Les plantes SF élevèrent significativement plus de larves, eurent tendance à avoir une charge parasitaire plus élevée, et perdirent plus de fruits dus à la prédation et à l'avortement que les plantes LF. Curieusement, l'avortement des fruits était négativement sélectionné, et le prédateur de graines sélectionna contre l'avortement chez les plantes SF mais pas les LF. L'avortement des fruits était significativement plus courante chez les plantes avec une charge parasitaire élevée. Alors que les pollinisateurs et les *H. bicruris* ne pondant pas d'oeufs pourraient sélectionner un grand nombre de fleurs ouvertes chez les mâles, le prédateur de graines pourrait contribuer indirectement à la sélection d'un petit nombre de fleurs ouvertes chez les femelles en imposant des coûts plus élevés pour l'avortement chez les plantes avec un grand nombre de fleurs.

Au **chapitre 4** j'examinai comment les traits au niveau d'une fleur influencent la diversité génétique à l'intérieur d'un fruit, une composante du succès reproducteur mâle et femelle. Une étude de terrain a démontré que les graines d'un fruit de *S. latifolia* sont généralement de plusieurs pères différents, mais les mécanismes n'en étaient pas connus. J'étudiai lors d'expériences en serre l'effet du temps écoulé entre les pollinisations manuelles avec le pollen de deux mâles sur la paternité et la masse des graines, l'effet de la charge pollinique sur la production de graines, le temps nécessaire aux tubes polliniques pour atteindre l'ovaire, et la fanaison du stigmate après le dépôt du pollen. Le pollen arrivé en premier contribua à la paternité des graines de façon significativement plus importante que le pollen arrivé plus tard, et cet avantage était visible plusieurs heures avant l'arrivée des tubes polliniques à l'ovaire. Les papilles des stigmates se fanèrent simultanément avec la croissance des tubes polliniques. Un partage de paternité plus équilibré entre les deux mâles impliqua une réduction de la masse individuelle des graines. Ainsi les fruits résultant de plusieurs pères de *S. latifolia* connus sur le terrain sont vraisemblablement le résultat du dépôt simultané de pollen de plusieurs plantes mâles (pollen carry over) ou de la visite de plusieurs pollinisateurs durant un court intervalle de temps. La fonction mâle et femelle pourraient avoir un conflit d'intérêt quant à la durée de la réceptivité du stigmate, une fanaison précoce pourrait bénéficier au premier donneur de pollen arrivé sur le stigmate en augmentant sa contribution à la paternité comparativement à des donneurs de pollen arrivés plus tard, alors que la fanaison pourrait être coûteuse pour la femelle en réduisant la diversité génétique de ses descendants et/ou le nombre de grains de pollen capturés par le stigmate.

Au **chapitre 5**, avec la collaboration de Sara Teixeira, j'étudiai si la taille de la surface réceptive du stigmate ou la taille de l'ovaire influence le partage de paternité entre deux donneurs de pollen en compétition. Sara fit les pollinisations manuelles des fleurs de deux soeurs et d'une femelle non apparentée avec le pollen de deux mâles, et détermina la paternité. De mon côté, je comptai les lobes du stigmate, mesurai la surface du stigmate et la taille de l'ovaire sur des fleurs non pollinisées de ces mêmes plantes femelles. Les stigmate des fleurs avaient de quatre à sept lobes, mais la plupart en avaient cinq. Je trouvai de la variation significative entre les deux populations dans la surface du stigmate et la taille de l'ovaire. Ces traits étaient significativement corrélés l'un avec l'autre, mais aucun n'était corrélé avec le succès de paternité relatif des deux mâles. Ainsi, une surface de stigmate plus grande semble ne pas augmenter la compétition entre deux donneurs de pollen, et se traduit par un partage de paternité similaire. Néanmoins, cela pourrait augmenter la capture de pollen dans des conditions naturelles de pollinisation.

En conclusion (**chapitre 6**), mes résultats suggèrent que l'évolution des traits sexuellement dimorphiques chez les fleurs de *S. latifolia* pourraient être due à la sélection opposée chez les plantes mâles et femelles. Alors que les pollinisateurs pourraient sélectionner pour un plus grand nombre de fleurs ouvertes chez les deux sexes, les femelles paieraient des coûts croissants pour l'avortement des fruits à mesure qu'elles augmentent leur nombre de fleurs. Son ennemi naturel contribue ainsi vraisemblablement à la sélection des petits nombres de fleurs ouvertes chez les plantes femelles. L'avortement des fruits semble jouer un rôle important dans l' interaction entre cette plante et cet insecte. Ce trait de résistance devrait être bénéfique pour la plante et ses descendants sur le long terme car il devrait réduire la population du prédateur de graines. Néanmoins à court terme ce trait est coûteux pour la plante femelle. La nature de ce coût demeure mystérieuse à ce jour. Au niveau de la fleur, mes résultats suggèrent que le pollen d'un mâle ne dispose que d'une petite fenêtre temporelle pour obtenir la paternité. Le rôle de la surface et de la fanaison du stigmate pour la capture du pollen et pour la paternité, et donc pour le succès reproducteur des plantes mâles et femelles en conditions naturelles reste à explorer. La surface du stigmate pourrait augmenter la capture du pollen, et la fanaison pourrait être utilisée par le mâle ou la femelle pour manipuler le résultat de la compétition pollinique. D'un autre côté, la fanaison pourrait servir de mécanisme de défense contre les maladies vénériennes ou pourrait rendre la fleur inattractive pour des femelles pondeuses de prédateurs de graines. Les prochaines études sur l'évolution des traits floraux des plantes ne devraient pas se limiter aux pollinisateurs mais devraient simultanément considérer les ennemis naturels ainsi que les changements plus subtiles qui ont lieu au niveau des fleurs.

CHAPTER 1: General introduction

Silene latifolia

♂

and

♀

with an egg of *Hadena bicruris*

Dioecy and sexual dimorphism

Flowering plants exhibit a variety of reproductive systems (Barrett, 2002; Charlesworth, 2006). The vast majority of plants are hermaphroditic with perfect flowers (Barrett, 2002), but about 7.6 % of genera and 6 % of species are dioecious (Renner & Ricklefs, 1995), with male and female flowers on different plants (Sakai & Weller, 1999). The general explanations for the evolution of dioecy include the avoidance of inbreeding depression (Lloyd, 1975; Lloyd, 1976; Charlesworth & Charlesworth, 1978; Charlesworth, 1999) and changes in the resource allocation that allow females to produce over twice as many seeds than hermaphrodites (Charlesworth, 1999; Sakai & Weller, 1999).

Dioecy allows sexes to respond differently to selection on traits other than gender itself, and this can lead to different phenotypic optima in homologous characters in the two sexes (Meagher, 1992; Geber, 1999). Sexual dimorphism can be found in secondary sex characters (Geber, 1999) such as flower and inflorescence traits (Delph *et al.*, 1996; Eckhart, 1999), life history traits (Delph, 1999), vegetative morphology and physiology (Dawson & Geber, 1999), and ecological traits such as competitive ability and susceptibility to herbivores and pathogens (Ågren *et al.*, 1999). Also, greater costs of reproduction paid by females compared to males (Bell, 1980; Delph, 1999; Obeso, 2002) can lead to sexual dimorphism in attributes related to resource acquisition. Sexual dimorphism evolves through natural– or sexual selection (Geber, 1999). However, response to selection on a trait will be determined by the level of additive genetic variation for this trait, by the degree of within-sex genetic correlations between that trait and other traits subject to other selection pressures, and also by the degree of between-sex correlations of homologous traits expressed in both sexes

(Meagher, 1992; Geber, 1999). Finally, in animal pollinated plants, pollinators will select for similarities rather than differences between individuals in secondary sex characters (Geber, 1999), thus limiting dimorphism to some degree.

Flowers attract pollinators

The diversity in angiosperm flowers is thought to have evolved to attract certain animals to disperse pollen (Fenster *et al.*, 2004; Bronstein *et al.*, 2006). There are many floral traits that play a role in attracting pollinators, such as flower size, shape, colour, texture, scent, and rewarding nectar (Whitney & Glover, 2007). Pollinators are more attracted by plants with large flowers (Galen, 1996; Vaughton & Ramsey, 1998; Thompson, 2001) or by plants with a large floral display (number of open flowers; Davis, 1981; Eckhart, 1991; Ohara & Higashi, 1994; Conner & Rush, 1996; Grindeland *et al.*, 2005; Sandring & Ågren, 2009).

Moreover, many attractants to pollinators are sexually dimorphic. Males usually have larger flowers in temperate regions, more numerous flowers per inflorescence, and their flowers emit a stronger scent than female plants (Delph *et al.*, 1996). Nectar is also a dimorphic trait, but there are as many cases with one sex producing more nectar than the other (Eckhart, 1999). Attractive parts of the perianth and nectar are likely to have evolved by sexual selection (Stephenson & Bertin, 1983; Stanton & Young, 1994; Grant, 1995), and may explain the evolution of large flowers in males which fitness depends more strongly on mating success than that of females (Bell, 1985), in accordance with Bateman's principle (Bateman, 1948). An increase in flower number in a given sex may result from fecundity selection if flower size and number trade-off, and if that sex is constrained in the number of pollen grains/ ovules it can pack inside a single flower (Frey *et al.*, 2007).

There is ample evidence that different pollinating groups exert different selection pressures on floral traits (Fenster *et al.*, 2004). Furthermore, several studies have shown pollinator selection on floral traits (e.g. Galen, 1996; Irwin & Strauss, 2005; Arista & Ortiz, 2007; Gomez *et al.*, 2008). Nevertheless the vast majority of flowering plants are visited by several pollinators, and each of the flower visitor may contribute differently to selection on floral traits, so that floral traits that seem to be an evolutionary response to one pollinator species may in fact reflect a more diverse pollination history (Waser *et al.*, 1996).

Flower and pollinator traits that affect the quality of pollination

Potential selection on floral traits may additionally depend on the pollinator behaviour. Pollinators may affect the timing of pollen deposition on the stigma (Skogsmyr & Lankinen, 1999), its quantity (Spira *et al.*, 1992) and genetic diversity (Mitchell *et al.*, 2005; Karron *et al.*, 2006). Sequential visits by several pollinators or pollen carry-over on several flowers by a single pollinator may lead to multiply sired fruits (Delph & Havens, 1998; Bernasconi, 2003). However successful pollination will depend on the viability of pollen grains and on their interaction with the female structures. In many plant species the stigma surface is not always receptive to pollen germination (Edlund *et al.*, 2004), and there is natural variation in style length (Barrett *et al.*, 2000; Nishihiro *et al.*, 2000), thus stigma surface may affect the intensity of pollen competition (Delph & Havens, 1998) or the likelihood of pollen capture (Cruden & Millerward, 1981). Chances of ovule fertilization will depend on the speed of pollen tube growth and on interactions with the female sporophytic tissues (Walsh & Charlesworth, 1992; Cheung, 1996; Heslop-Harrison, 2000; Bernasconi *et al.*, 2004).

Upon fertilization, flowers wilt (senesce) in many plant species (e.g. Lankinen *et al.*, 2006; Abdala-Roberts *et al.*, 2007; Castro *et al.*, 2008), and exhibit traits that render them less attractive to pollinators, such as cessation of scent production, colour change, flower closure, and petal abscission (van Doorn, 1997). Flower senescence affects floral display, plant attractiveness to pollinators, and pollen export in hermaphroditic plants (Stanton, 1994; Harder & Johnson, 2005). In dioecious plants, female flowers usually are longer lived than male flowers, and senescence may reduce the metabolic cost of flower maintenance and nectar production (Primack, 1985). In addition, senescence is likely to prevent pollen tube growth, and early senescence may be advantageous to the first-arriving pollen while decreasing the chance of collecting more diverse pollen for the female, thus leading to a sexual conflict (Bernasconi *et al.*, 2004; Lankinen *et al.*, 2006). In other words, the timing of pollen arrival, ovule fertilization, and flower senescence, as well as stigma surface may affect paternity success and the fitness of the resulting offspring. These factors may limit the opportunity of pollinator-mediated selection on floral traits to certain conditions, that need to be investigated.

Flowers attract enemies

Floral traits attractive to pollinators may also serve as cues to attract natural enemies. The consumption of flower structures, or florivory, is widespread and can be substantial (McCall & Irwin, 2006). Florivory can affect floral traits, including flower size (Mothershead & Marquis, 2000) and floral display (Karban & Strauss, 1993), and can reduce plant attractiveness to pollinators (Karban & Strauss, 1993; Cunningham, 1995; Mothershead & Marquis, 2000). In dioecious plants, florivory or herbivory on vegetative tissue is larger for males than females (Ågren *et al.*, 1999). Also, antagonists such as nectar thieves (Galen, 1999; Irwin, 2009), castrating fungi (Shykoff *et al.*, 1997) and seed predators (Brody & Mitchell, 1997) affect differentially plants with varying floral display or flower size. Most studies have measured how natural enemies affect plant fitness, but few have investigated their role as selective agent on floral traits (Giles *et al.*, 2006; Nuismer & Ridenhour, 2008). Sex-specific damage inflicted by natural enemies could play a role in the transition from hermaphroditism to dioecy (Ashman, 2002), and might also contribute to selection on sexually dimorphic traits in plants.

Nursery pollination : combining pollination with seed predation

Plants may depend for pollination service on insects that also act as natural enemies. This is the case in at least 13 nursery pollination interactions (Dufaÿ & Anstett, 2003), where female insects deposit pollen and one or several eggs in the flowers, and then larvae consume the developing seeds. The mutualistic relationship (Bronstein, 1994) between yucca/yucca moth (Pellmyr & Huth, 1994) and fig/fig wasp (Bronstein, 1992) are well-known examples of obligate interactions, but non-obligate interactions where plants use different pollinators, and seed predators different hosts are also known, as for example in the Caryophyllaceae (Kephart *et al.*, 2006). Not all of these interactions are mutualistic (Dufaÿ & Anstett, 2003), and the outcome can vary from mutualistic to antagonistic depending on plant populations (Thompson & Cunningham, 2002), insect densities (Holland & DeAngelis, 2001) and presence of co-pollinators (Thompson & Pellmyr, 1992). To avoid overexploitation by their partner, plants have evolved counter-adaptations such as flower or fruit abortion (Addicott, 1986; Pellmyr & Huth, 1994; Richter & Weis, 1995) that benefit the plant by limiting the insect abundance (Holland & DeAngelis, 2002).

In dioecious plants, floral traits that increase pollen export may be selected in males, while in females those traits may be under opposing selection to attract pollinators and to minimize the costs of the interaction with the pollinating seed-predator. Several studies have suggested that florivores and pollinators impose opposing selection on floral traits (Strauss *et al.*, 1999; Mothershead & Marquis, 2000; Adler *et al.*, 2001; Irwin *et al.*, 2003; Ashman *et al.*, 2004), but the contribution of pollinating seed-predator to selection on sexually dimorphic traits remains unexplored. Furthermore the insect may benefit from laying its eggs in flowers that provide the best resources for its offspring or that are the least likely to be aborted. Thus insect fitness may also depend on floral traits, which opens the perspective of coevolution between the two partners of the interaction (Thompson, 2005).

Study system

The plant : Silene latifolia

The white campion, *Silene latifolia* Poiret 1789 (= *S. alba* (Miller) Krause = *S. pratensis* (Rafn.) Godr. & Gren. = *Lychnis alba* (Miller) = *L. vespertina* Sibthorp = *Melandrium album* (Miller) Garcke) is a weedy short lived perennial occurring in open disturbed habitats (Goulson & Jerrim, 1997), native from Eurasia (Prentice, 1979), and introduced to America where it is considered a pest species (Baker, 1948; Wolfe, 2002). It is a diploid (2n = 24) dioecious species with chromosomal sex determination system (Westergaard, 1958). Populations are female-biased (Carroll & Mulcahy, 1993), and this sex-ratio is apparently due to the fact that females live longer than males (Lovettdoust *et al.*, 1987; Carroll & Mulcahy, 1993; Gehring & Linhart, 1993). In Europe, *S. latifolia* flowers from end of April to the end of October with two distinct flowering peaks in May and August (Jurgens *et al.*, 1996). Its white flowers open at dusk and emit scent (Jurgens *et al.*, 1996), composed mainly of lilac aldehyde (Dotterl *et al.*, 2005), that is very attractive to *Hadena bicruris* (Dotterl *et al.*, 2006), the main visitor and seed predator of *S. latifolia* (Jurgens *et al.*, 1996). Its flowers with deep corolla tubes and exerting stigma and anthers are adapted to long-tongued insects (Jurgens, 2006) such as moths (Noctuidae) and hawkmoths (Sphingidae) that presumably pollinate the plant at night, but hoverflies and bumblebees also visit the plant during the day (Shykoff & Bucheli, 1995; Jurgens *et al.*, 1996; Jurgens *et al.*, 2002; Young, 2002; Van Putten *et al.*, 2003).

Male and female plants are dimorphic for several traits, including plant size, flower number and size, and cost of reproduction. Males are smaller and produce fewer vegetative biomass (Lovettdoust *et al.*, 1987; Gehring & Linhart, 1993; Delph & Meagher, 1995; Delph *et al.*, 2005), up to 14 times more flowers (Carroll & Delph, 1996; Laporte & Delph, 1996; Delph *et al.*, 2002), and larger floral display than females (Meagher, 1992; Meagher & Delph, 2001). The cost of reproduction is larger for males due to increased CO_2 loss in flowering stems (Delph *et al.*, 2005), but females invest more biomass in reproduction than males because they have larger flowers and fruits with many seeds (Gross & Soule, 1981; Delph & Meagher, 1995; Laporte & Delph, 1996). Flower production increases with plant age, but decreases in females with pollination and initiation of fruit development (Meagher & Delph, 2001). Male plants with a larger floral display are more likely to receive visits by insects (Shykoff & Bucheli, 1995), and to become infested by the anther smut fungus *Microbotryum violaceum* as compared to females (Thrall & Jarosz, 1994). The fungus induces male and female flowers to produce spore-bearing anthers and thus sterilizes its host (Ruddat *et al.*, 1991).

Many floral traits are sexually dimorphic. Male flowers have smaller quantity of more concentrated nectar (Carroll & Delph, 1996; Shykoff, 1997; Gehring *et al.*, 2004), less dry biomass, narrower calyx, smaller petal diameter and limb length, and shorter corolla tubes (Meagher, 1992; Carroll & Delph, 1996) than female flowers. Flower size and number are negatively genetically correlated (Delph *et al.*, 2004b), and populations with the greatest number of flowers per plant have flowers with the narrowest calyxes (Delph *et al.*, 2002). Flower number, flower mass, calyx width and petal limb length are genetically correlated with each other, within and/or between sexes (Delph *et al.*, 2004a; Delph *et al.*, 2004b). Most homologous characters in the two sexes are positively genetically correlated (Meagher, 1992). Larger flowers contain more ovules, but not more pollen grains than smaller flowers (Delph *et al.*, 2004b).

Female flowers usually have five stigmatic lobes (Teixeira *et al.*, 2008), covered with papillae receptive to pollen germination (Lassere *et al.*, 1996) and are receptive to pollen for about five days (max= 7 days) if unpollinated, and wilt within 12 hours after pollination (Primack, 1985; Young & Gravitz, 2002). Each female produces several fruits with 480-670 ovules (Jurgens *et al.*, 1996; Jurgens *et al.*, 2002) and 48-408 seeds per fruit (Baker, 1947; Jurgens *et al.*, 1996; Young, 2002). Male flowers remain open for one day and then wilt (Primack, 1985), have 10 anthers (Delph *et al.*, 2004b) and 21’000-37’500 pollen grains per flower (Jurgens *et al.*, 2002). In the field, individual fruits are often multiply sired, with an average of about four sires per fruit (Teixeira & Bernasconi, 2007). There is heritable genetic variation among populations in pollen germination rate in vitro (Jolivet & Bernasconi, 2007a).

The pollinating and pre-dispersal seed predator : Hadena bicruris

Hadena (Schrank, 1802) species are Lepidoptera (Noctuidae) specialized on the Caryophyllaceae (Elzinga, 2005). The lychnis, *Hadena bicruris* Hufnagel 1766 (*Phalaena* Hufn.) (*Dianthoecia* B.) (*Capsincola* Schiff.) (*Capsincoloides* Standfuss) is strongly attracted by the smell of *S. latifolia* (Dotterl *et al.*, 2006). Other host plants include *Silene vulgaris*, *Silene dioica* and rarely *Saponaria officinalis* (Jurgens *et al.*, 1996; Bopp & Gottsberger, 2004). Larvae reared on *S. latifolia* attain a larger pupal weight than on *S. dioica* (Bopp & Gottsberger, 2004). In Europe, *H. bicruris* is present in 90 % of the *S. latifolia* populations, and destroys 25-50 % of all fruits (Biere & Honders, 1996; Wolfe, 2002; Elzinga *et al.*, 2005). Adults fly from mid-May until the beginning of September, in probably two overlapping generations (Elzinga *et al.*, 2005), and there are two peaks in fruit predation in early July and August (Biere & Honders, 1996). Late flowering *S. latifolia* plants suffer less from seed predation than early flowering plants (Biere & Honders, 1996).

After drinking nectar from a *S. latifolia* flower, *H. bicruris* females may oviposit usually a single egg (Brantjes, 1976a) only in female flowers (Brantjes, 1976b). After hatching, the first instar larva enters the fruit (hereafter primarily infested or attacked fruit), consumes all the developing seeds, and excretes red frass out of the entrance hole. After consuming the seeds, the larva (usually fourth or fifth instar) leaves this primarily infested fruit through the enlarged entrance hole and starts feeding on other fruits on the same plant (hereafter secondarily infested or attacked fruits). On secondarily infested fruits larvae are often larger than the fruit, and parts of their body are thus exposed to parasitoids (Biere *et al.*, 2002). Larval development from eclosion to pupation lasts around four weeks (Elzinga *et al.*, 2002) and larvae destroy 5-7 fruits to complete development (Brantjes, 1976b; Bopp, 2003). Pupation takes place in the soil and the adult emerges after approximately three weeks or the pupa enters diapause and emerges after the winter (Elzinga *et al.*, 2002). Adult moths live about 10 days, and females lay 100-400 eggs during its lifespan (Brantjes, 1976b; Elzinga *et al.*, 2002). *S. latifolia* plants respond to experimental egg infestation by increased flower or fruit abortion, which suggests that abortion may be a plant response to reduce damage by *H. bicruris* larvae (Jolivet & Bernasconi, 2006). Also parasitoids may cause up to 50% mortality in *H. bicruris* larvae, but most of these parasitoids kill their host larvae when they are in the fourth or fifth instar (Elzinga *et al.*, 2005; Elzinga *et al.*, 2007c), thus reducing the seed predator population but not substantially the damage suffered by the plant.

Scope and outline of the thesis

The general aim of this thesis is to investigate the role of a pollinating-seed predator on the evolution of sexually dimorphic floral traits in a dioecious plant. More precisely, I study the effect of floral display and flower size on the fitness of *Silene latifolia* and *Hadena bicruris*, the costs and benefits of fruit abortion for both partners, the contribution of the insect to selection on floral traits, and several temporal or physical factors during pollination that may influence the genetic diversity of plant offspring.

In my thesis I combined greenhouse and common garden experiments, and used different methodologies such as measuring natural levels of fruit predation, experimentally infesting flowers with eggs, performing hand-pollinations, using microscopy and analyzing images, or using microsatellite genetic markers. In chapters 2 and 3, I used artificially selected plants which provide extreme phenotypes in flower size and number, and in chapter 4 and 5 greenhouse grown plants stemming from natural populations. Both plants and insects are common in Switzerland, and insects were naturally present in the common garden, where I collected eggs for the experiment described in chapter 2.

In **chapter 2** I investigate the effects of flower size, seed provisioning and fruit abortion on the fitness of larvae of the pollinating-seed predator. To answer this question I conducted a greenhouse experiment where I infested two flowers from plants that vary in flower size with eggs of *H. bicruris*, measured fruit provisioning of one non-attacked fruit, the growth of two larvae per plant and fruit abortion. The measurement of two larvae per plant allowed to investigate potential induced plant responses to seed predation.

Plant fitness was investigated in **chapter 3**, where I report the results of two garden experiments where female plants that vary in floral display and flower size were exposed to pollinating-seed predators and co-pollinators. On a set of plants I measured the risk of infestation by eggs of *H. bicruris*, and on a different set of plants I recorded which fruits were predated, aborted, or healthy, and how many seeds were produced. In addition, I investigate the contribution of the pollinating seed predator to selection on floral traits. To do this, I combine flower measurements from greenhouse-grown plants with measurements of fruit damage and abortion in the garden experiment described above.

Floral traits likely to affect the genetic diversity among plant offspring are studied in two greenhouse experiments. In the first experiment (**chapter 4**) I varied the timing of hand-pollination with the pollen of two males as a surrogate of different timing of pollen deposition by pollinators, and measured the proportion of offspring sired by each of the two males, and offspring performance. These results are compared with estimates of saturating pollen load, fertilization timing and flower senescence to explore some mechanisms that could explain paternity.

In the second experiment (**chapter 5**), together with Sara Teixeira, I investigate whether stigma surface or ovary size affects the shares of paternity between two competing pollen donors. More specifically I measured ovary size, the number of stigma lobes and stigma surface and correlated these traits with paternity and seed-related traits measured by Sara Teixeira.

Finally in **chapter 6**, I discuss the patterns that are found and how this pollinating seed-predator may contribute to the evolution of sexually dimorphic floral traits. Also I explain the limitations of my experiments and I make suggestions for future experiments.

CHAPTER 2 : Benefits and costs to pollinating, seed-eating insects : the effect of flower size and fruit abortion on larval performance

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Plants in the greenhouse

Larva after emergence from the fruit

Abstract

Plant-pollinator interactions are well-known examples of mutualism, but are not free of antagonism. Antagonistic interactions and defenses or counter-defenses are expected particularly in nursery pollination. In these systems, adult insects, while pollinating, lay their eggs in flowers, and juveniles consume the seeds from one or several fruits, thereby substantially reducing plant fitness. The outcome of such interactions will depend, for the plant, on the balance between pollination vs. seed predation and for the larvae on the balance between the food and shelter provided vs. the costs imposed by plant defenses, e.g., through abortion of infested fruits. Here, we examine the costs and benefits to the larvae in the nursery-pollination system *Silene latifolia/Hadena bicruris*. Using selection lines that varied in flower size (large- vs. small-flowered plants), we investigated the effects of variation in flower and fruit size and of a potential defense, fruit abortion, on larval performance. In this system, infested fruits are significantly more likely to be aborted than non-infested fruits, however it is unclear whether fruit abortion is effective as a defense. Larger flowers gave rise to larger fruits with more seeds, and larvae that were heavier at emergence. Fruit abortion was frequently observed (ca. 40% of the infested fruits). From aborted fruits, larvae emerged earlier and were substantially lighter than larvae emerging from non-aborted fruits. The lower mass at emergence of larvae from aborted fruits indicates that abortion is a resistance mechanism. Assuming that lower larval mass implies fewer resources invested in the frugivore, these results also suggests that abortion is likely to benefit the plant as a defense mechanism, by limiting both resources invested in attacked fruits, as well as the risk of secondary attack. This suggests that selective fruit abortion may contribute to the stability of mutualism also in this non-obligate system.

Key words: flower number, nursery pollination, pollination mutualism, pre-dispersal seed predation, *Silene alba*

Introduction

Plant-pollinator interactions are well-known examples of mutualism, but are not free of antagonism. Antagonistic interactions and defenses or counter-defenses are expected particularly in systems in which the adult pollinator lays its eggs in the flower and juveniles act as seed predators (nursery pollination; Dufaÿ & Anstett, 2003; Kephart *et al.*, 2006). For the plant, the outcome of this interaction will depend on the balance of pollination benefits vs. seed predation costs. Similarly, fitness of the seed predating and pollinating insect will likely be affected by the quantity and quality of food provided by the developing fruit to its larvae, but also crucially by the plant's ability to control damage (Bronstein, 1992; Holland *et al.*, 2004b). Control mechanisms by the plant are indeed likely to have a large impact on fitness of both the plant and the insect if they affect growth and survival, and thus demographic rates of the seed predator.

Fruit abortion may represent such a control mechanism, as indicated by its occurrence in several nursery-pollination systems, where the plant aborts the attacked seeds or fruits. Beyond the effect of limiting the costs to a given plant (by limiting the investment of resources in infested fruits and by lowering the risk of attack of more fruits on the same plant), both selective abortion of infested fruits, and random abortion of fruits (i.e., abortion irrespective of whether flowers or fruits have eggs or not) can limit the population size of the pollinating and seed predating insect (Holland & DeAngelis, 2001; Westerbergh & Westerbergh, 2001; Holland & DeAngelis, 2006). In the well-studied case of the yucca/yucca moth interaction, different Yucca species employ different strategies to control damage: abortion of flowers with high loads of eggs or larvae (Addicott, 1986; Pellmyr & Huth, 1994; Richter & Weis, 1995), reduction of seed number available to developing larvae by protecting some seeds from consumption (Ziv & Bronstein, 1996), or larval starvation caused by a physical barrier that prevents larvae from reaching the fertilized seeds (Bao & Addicott, 1998). In the senita cactus/senita moth interaction, reduction in damage is achieved by random fruit abortion, irrespective of egg/larval load (Holland *et al.*, 2004a).

Unlike the yucca/yucca moth and senita cactus/senita moth associations (obligate mutualism; Fleming & Holland, 1998), the interaction studied here between *S. latifolia* and *H. bicruris* is not obligate and is therefore considered to be a basal form of nursery pollination (Dufaÿ & Anstett, 2003; Bernasconi *et al.*, In press). However, there is evidence for potential specialization in this system, at least to some degree: the two species have similar geographic distributions, flowering and oviposition are synchronous (Biere & Honders, 1996; Wright & Meagher, 2003; Bopp & Gottsberger, 2004), moths respond to specific scent compounds emitted by the flowers (Dotterl *et al.*, 2006), and *H. bicruris* larvae grow better on *S. latifolia* than on other host species (Bopp & Gottsberger, 2004). In the *Silene latifolia/Hadena bicruris* interaction, experimental infestation of flowers with eggs of the seed predator leads to a significantly higher probability of fruit abortion compared to sham-manipulated fruits (Jolivet & Bernasconi, 2006). It is therefore important to investigate whether, also in this less specialized system, fruit abortion in response to infestation is an effective control mechanism. This implies to test whether fruit abortion can reduce damage to the plant (i.e. serve as a defense mechanisms by reducing costs of herbivory) and effectively lower larval performance in terms of growth or survival prospects (i.e. serve as resistance mechanisms by reducing herbivore performance). In addition, both a plant's propensity to abort its fruits (Stephenson, 1981), as well as larval performance on that plant, may depend on variation in flower size or in fruit size, and therefore variation in resource allocation per flower or per fruit should also be taken into account when studying the effect of fruit abortion on larval performance.

In this study, using artificial-selection lines that differ in flower size, we investigated the effects of (i) fruit abortion and of (ii) experimentally controlled variation in flower size (resulting in variation in fruit size) on larval performance in the *Silene latifolia/Hadena bicruris* system (i.e. resistance). The use of these selection lines provided us with the variation needed to ensure the statistical power to assess the impact of flower or fruit size. Assuming that abortion serves as a defense against moth attack, we predicted that fruit abortion would reduce the damage to the plant by reducing the amount of resources invested in attacked fruits, resulting in a cost to larvae (i.e., lower larval performance) on aborted compared to non-aborted fruits. We further expected that large fruits would provide better resources for the developing larvae, or differ potentially in their attractiveness to ovipositing females, and that large-flowered plants might have a different propensity to abort their fruits than small-flowered plants. Consistent with the idea that abortion acts as an effective control mechanism in this non-obligate system, our results indicate that fruit abortion reduces the benefits to the larva (i.e. resistance). Assuming that lower larval mass at emergence implies a smaller investment of resources by the plant, this also suggests that fruit abortion thereby reduces the costs that the plant suffers by limiting investment in attacked fruits.

Material and methods

Study system

The white campion *Silene latifolia* (Poiret) (= *Silene alba* (Miller) Krauss, = *Melandrium album* (Miller) Garcke; Caryophyllaceae) is a short-lived perennial, dioecious plant native to Europe and found in disturbed habitats (Goulson & Jerrim, 1997). *Silene latifolia* is dioecious and sexually dimorphic for several traits, including calyx width and floral display (Delph *et al.*, 2002). Male plants carry more, but smaller and shorter-lived flowers than females (Carroll & Delph, 1996; Meagher & Delph, 2001; Young & Gravitz, 2002). Fruits contain up to several hundreds of seeds (Jolivet & Bernasconi, 2007a). *Silene latifolia* flowers from April to October; its white flowers open and start emitting scent at dusk (Jurgens *et al.*, 1996). Nocturnal (moths) and diurnal (e.g. hoverflies) pollinators visit the plant (Shykoff & Bucheli, 1995; Jurgens *et al.*, 1996; Young, 2002; Van Putten *et al.*, 2003). The moth *Hadena bicruris* Hufnagel (Lepidoptera: Noctuidae) is considered to be the main pollinator in the European native range (Brantjes, 1976b; Jurgens *et al.*, 1996). This moth has a dual role as pollinator and seed predator (Fig. 1).

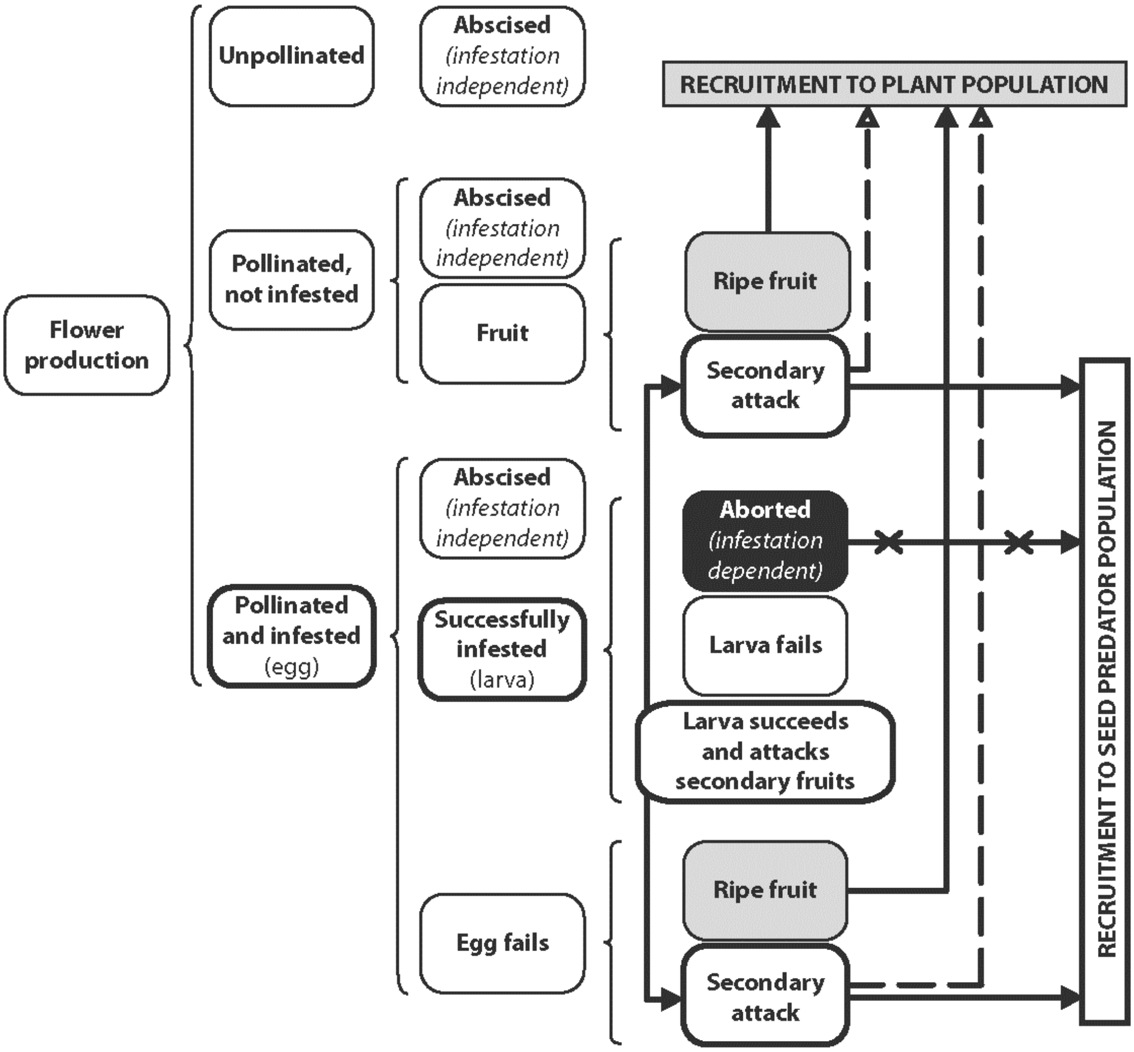

Figure 1. Schematic diagram of flower fates (pollination, infestation, infestation-independent abscission, infestation-dependent abortion, secondary attack) and their contribution to plant and seed predator populations, based on the example of the interaction between *Silene latifolia* and the associated pollinating and seed-predating insect *Hadena bicruris*. Although larvae in aborted fruits can emerge, it is likely that most of them will perish. Secondarily attacked fruits may also abort, if they are attacked early during development. *Dashed lines*: secondarily attacked fruits may occasionally ripen and disperse a few seeds. Under resource limitation, flower and fruit fates will feedback on flower production.

Adult females of this moth lay usually a single egg inside or on a female flower (Brantjes, 1976b). After hatching, the first instar larva enters the fruit (primarily infested fruit). After consuming the developing seeds, the larva (usually fourth or fifth instar) leaves this primarily infested fruit through the enlarged entrance hole and starts feeding on other fruits on the same plant (secondarily infested fruits). On secondarily infested fruits larvae are often larger than the fruit, and parts of their body are thus exposed to parasitoids (Biere *et al.*, 2002). Larval development from eclosion to pupation lasts around three to four weeks and larvae need several fruits (3-5) to complete development. The moth is present from May to October in most (over 90% in a recent survey; Wolfe, 2002) European populations, with two or more overlapping generations per year (Elzinga *et al.*, 2007c), and high prevalence (i.e., often 50% or more of the fruits are attacked; Biere & Honders, 1996; Wolfe, 2002; Elzinga *et al.*, 2005).

Plants respond to experimental egg infestation by increased flower or fruit abortion, which suggests that abortion may be a plant response to reduce damage (Jolivet & Bernasconi, 2006). Indeed flowers infested with an egg were significantly and substantially (six-fold probability) more likely to abscise than sham-manipulated flowers (i.e., in which we inserted the toothpick in the flower without placing an egg), the latter having a rate of abscission of 0 to 2.6% (Jolivet & Bernasconi, 2006). Also, among marked fruits of naturally pollinated plants that were dropped there was a significant excess of primary infestation compared to fruits which were not prematurely dropped (Elzinga & Bernasconi, In press). Because of this, we refer to *fruit abortion* if it is known that the flowers are infested (as after experimental infestation in our study) rather than fruit abscission (a term that we use for non-infested fruits, see Fig. 1, or for fruits of unknown infestation status). In this we do not follow Stephenson (1981), who uses abscission for damaged fruits and abortion as a response to resource limitation, because it is not straightforward to distinguish the roles of damage versus resource limitation in determining the premature shedding of developing fruits in plant species that frequently face seed predation and may therefore be selected to produce an excess of flowers. Abortion in this system acts at the level of the fruit rather than of seeds. Fruits contain only one larva because a single egg is deposited (Brantjes, 1976b) or, in the rare cases of multiple oviposition, because of larval competition and cannibalism. We use the term *larval emergence* to describe the stage when the larva exits the primary fruit, after which it will try to locate secondary fruits. Although under natural conditions many larvae in aborted fruits probably perish, some may still emerge, but their chances to survive will depend on their success in locating secondary fruits to complete their development.

Plant material and rearing conditions

To simultaneously investigate the roles of fruit abortion and of fruit size (and provisioning) on larval performance, and of fruit size on the propensity to abortion, we conducted our experiment using plants derived from seeds arising from two artificial-selection programs (Delph *et al.*, 2004b). Using two American source populations, two replicate “large flowered” (LF) lines and two replicate “small flowered” (SF) lines were created by selection on calyx width (Delph *et al.*, 2004b). Because of a flower size vs. flower number trade-off, LF plants produce fewer flowers compared to SF plants (Delph *et al.*, 2004b). LF and SF selection lines were crossed within line type and between replicates in the generation preceding our experiments to eliminate inbreeding effects. The advantage of using selection lines originating from a common genetic background are that it avoids confounding variables that cannot be excluded with natural variation in flower size, and it provides clear-cut phenotypic differences thereby increasing statistical power. However, the flower sizes in these lines are within the range observed for natural variation (Delph *et al.*, 2002). We germinated twenty seeds per family in Petri dishes filled with glass pearls and water, then transplanted the seedlings to Jiffy® peat pellets. After 30 days, we potted the plants individually (pots with 16 cm^3 of 1:3 sand: soil (Tref-De Baat BF4, GVZ-Bolltec AG, Zurich, Switzerland) mixture, and kept them in a pollinator-free greenhouse (16h light at 22°C and 8h dark at 18°C, 45% relative humidity, natural daylight was supplemented by lamps EYE Clean-Ace, 6500K, 400W, Iwasaki Electronics co., Japan).

Hand pollination and crosses

For crosses, we used eight LF full-sib families and eight SF full-sib families, and as far as possible used three females per family (2 lines x 8 families x 3 females = 48 females). Because some plants did not flower, we were able to cross 44 females (SF: (7 families x 3 females) + (1 family x 2 females) = 23 plants; LF: 7 families x 3 females = 21 females). For pollen donors, we used males from the same selection line as the female, but from a different family, and as far as possible males were used only once. We used 37 males to pollinate the 44 females (SF: one male used in four crosses; LF: four males used in two crosses; all other 32 males used only in one cross). We brushed three anthers on the stigmatic surface, which ensures full seed set (Burkhardt *et al.*, 2009b, thesis chapter 4). On each female, we pollinated three replicate flowers (2^{nd}, 3^{rd} and 4^{th} flower produced, for 132 flowers pollinated). The 1^{st} flower was left unpollinated because flower production shuts down very quickly if first flowers are pollinated, which suggests resource limitation of flower and seed production (Meagher & Delph, 2001).

Estimation of fruit quality in LF and SF selection lines

We used one control fruit per plant (resulting from the pollination of the 2^{nd} flower) to estimate variation between selection lines and among individual plants in fruit size and seed provisioning. These estimates were then entered as covariates in the analysis of larval performance (see below). When the control fruit was ripe, we measured maximum *fruit length* and *fruit width* with a caliper (precision: 0.1 mm), and estimated *fruit volume* assuming an ellipsoid (volume = 1/6*π*width^2*length). We measured the *total seed mass* per fruit (mg), and assessed *number of seeds* and *individual seed size* with a seed counter equipped with an optical sensor (Elmor C3, Elmor Angewandte Elektronik, Schwyz, Switzerland). For each fruit we calculated *mean seed size* (µm) and we quantified the *% carbon* and *% nitrogen* (CHNS-932 analyser, Leico Corp., St. Joseph, Michigan, USA) in a dried (24h at 80°C) and ground (Mixer Mill MM 300, Qiagen, Basel, Switzerland) subsample of the seeds (2.7 - 3.3 mg of ground powder, nearest µg; Mettler MT5, Greifensee, Switzerland). The *seed C/N* ratio was calculated as % carbon divided by % nitrogen for each fruit. *Total carbon and total nitrogen* were calculated as the % carbon or % nitrogen, respectively, multiplied by the total seed mass per fruit (mg). The rationale of measuring C/N content of seeds, along with the above variables, was to dissect how variation in flower size (calyx width), which was the target of artificial selection (see *Plant material*), affected different components of fruit size and seed provisioning, and whether such variation in allocation may be associated with larval performance and the propensity to abort.

Variation in the number of fertilized ovules per fruit and or fruit abortion may additionally depend on pollination quality. Although we used ample amounts of pollen in hand pollinations (see above), pollen of *S. latifolia* also varies in germination rates (Jolivet & Bernasconi, 2007a; Teixeira & Bernasconi, 2008). We preliminarily verified that there was no significant difference in the in-vitro pollen germination rate of the SF and LF males used for pollination (Table 1), so that selection line differences in seeds per fruit or in the propensity to abort cannot be ascribed to variation among pollen donors, but represent differences among SF and LF female plants.

Assessment of larval performance and fruit abortion

To measure larval performance and plant defenses, we infested the 3rd and 4th flowers produced on each female plant (hereafter 1st and 2nd infested fruit). Experimental infestation allowed us to examine the effect of flower size and fruit provisioning on larval performance and on the propensity to fruit abortion, whereas a study examining larval performance after natural oviposition could confound the effects of flowers size and fruit provisioning, given that moths may choose particular flowers (e.g. the most profitable ones) for oviposition (Milinski, 1997). Immediately after pollination we introduced a fertilized egg of H. bicruris in the corolla tube and carefully placed it close to the ovary using a wet toothpick, and bagged the flower to cage the larva when it emerged. Since the toothpick is much smaller that the inner diameter of the corolla tube, eggs could be placed very easily in both SF and LF flowers. We used eggs collected in a natural population near the campus of Lausanne University, Switzerland (46N31, 6E38). The eggs were stored 1-3 days on a moist filter paper at 4°C before use. Typically eggs hatch 3-4 days after having been laid (Elzinga *et al.*, 2002). This ensured that the eggs used for experimental infestation were fertilized, since fertilized eggs turn brown. Assignment of eggs to plants was randomized.

For each infested fruit (two fruits with larvae per plant, in eight cases only one larva developed) we recorded two components of larval performance: larval age at emergence (days since egg infestation), and larval mass at emergence (mg). We calculated larval mass gain as larval mass divided by larval age at emergence. We recorded fruit abortion, which can be recognized as a gradually extending abscission zone through the pedicel at the base of the fruit that ultimately causes the fruit to drop. When there was no sign of larval presence in the fruit ten days after infestation (no frass extruding from the fruit), we crossed and infested an additional flower on the same plant, and so on, until we obtained two successful infestations per plant (up to a maximum of nine attempts). We counted the number of attempts necessary to achieve the first and the second successful infestation per plant (henceforth, 1st and 2nd infestation).

Statistical analyses

We ran Generalized Linear Mixed Models (GLMM) with family as a random factor (to account for repeated measures within full-sib families) on fruit-quality traits (measured in the control fruit), larval performance (measured in the 1st and 2nd infested fruits) and on probability of abortion. Further, abortion and covariates (fruit quality, performance of 1st larva) were entered in more complete models where applicable and as described below. All initial models included the two-way interactions between fixed factors and the covariates. We applied a stepwise reduction procedure to GLMMs by deleting terms with $P>0.06$, and we present the minimal adequate models and Log-likelihood ratio tests (LLRT) in the Results. For all models, where applicable, we transformed the variables to improve homogeneity of variances and normality of errors, or we corrected for overdispersion.

To investigate whether selection lines differed in fruit quality, we ran univariate GLMMs with selection line as a fixed effect, family as a random effect and normal errors for each of the response variables: log(fruit volume), total seed mass, total carbon and nitrogen content; proportion of carbon and nitrogen (all angularly transformed), C/N ratio in the seeds (inverse transformed), mean seed size. We assumed quasi-Poisson errors for number of seeds per fruit, and binomial errors for in-vitro pollen germination. We ran a mixed–effects ANCOVA to test for the effect of selection line and in-vitro pollen germination (as a covariate) on the number of seeds per fruit.

To compare larval performance (mass and age at emergence) between selection lines and between plants that aborted or did not abort fruits, we ran univariate GLMM (using selection line and fruit abortion as a fixed factor, with family as a random factor) with covariates. Based on the examination of bivariate Pearson's correlations (Table 2) among fruit traits, we selected the following independent covariates: number of seeds per fruit, seed C/N, and the proportion of germinated pollen grains as control for pollen quality. The 1st and 2nd larva were treated separately in different models. In the GLMM for performance of the 2nd larva we additionally entered the performance of the 1st larva (mass, and age of 1st larva serving as a covariate for mass and age, of 2nd larva, respectively).

We ran univariate GLMMs with binomial errors to investigate the correlates of fruit abortion. For abortion of the 1st fruit, the initial model included selection line and family, plus the following covariates: number of seeds per fruit, seed C/N, proportion of germinated pollen grains, number of infestation attempts, and larval mass gain of the 1st larva, and all two-way interactions. For abortion of the 2nd fruit, the initial model contained selection line and fruit abortion of the 1st fruit as fixed factors, family as random factor, and the following covariates: seed C/N, larval mass gain of the 1st larva and total number of attempts to obtain both larvae, all two-way interactions between selection line and covariates, the interaction between selection line and total number of attempts, and that between selection line and abortion of the first fruit. Number of seeds per fruit and proportion of germinated pollen grains were not included as covariates in the second initial model because they did not significantly explain the variance in abortion of the first infested fruit. We analysed all data using the R software version 2.6.2 (R Development Core Team, 2006). Unless specified, data are given as mean ± SD.

Results

Differences between selection lines in fruit quality

LF plants produced fruits with approximately twice as many seeds, and double the fruit volume, total seed mass, total carbon and total nitrogen than SF plants (Table 1).

Table 1. Fruit and seed traits in small- (SF: $N = 23$ females) and large-flowered (LF: $N = 21$ females) selection lines of *Silene latifolia*. Since these traits may additionally depend on the quality of the pollen used, we also show *in vitro* pollen germination of the pollen donor used in hand-pollination (SF: $N = 20$ males, LF: $N = 17$ males). All analyses performed with GLMM models accounting for within-family repeated measures. Data are given as mean ± SD; SD was calculated on family means (N=15 families).

Plant trait	Selection line		Ratio of means#	Test statistic	*P*
	LF	SF			
Number of seeds	335.90 ± 91.64	184.91 ± 54.58	1.82	$X^2 = 11.22$	< 0.001
Fruit volume (mm^3)	869.69 ± 101.88	510.95 ± 124.18	1.70	$F = 33.06$	< 0.001
Total seed mass (mg)	257.22 ± 62.95	122.10 ± 43.26	2.11	$F = 17.06$	< 0.01
Total carbon (mg)	117.61 ± 28.91	55.97 ± 19.55	2.09	$F = 17.14$	< 0.01
Total nitrogen (mg)	7.18 ± 1.87	3.43 ± 1.15	2.10	$F = 17.06$	< 0.01
Mean seed size (µm)	1822 ± 22	1757 ± 32	1.04	$F = 5.15$	0.07
% carbon	45.69 ± 0.19	45.91 ± 0.31	1.00	$F = 2.56$	0.13
% nitrogen	2.80 ± 0.06	2.84 ± 0.11	0.99	$F = 0.52$	0.48
Seed C/N	16.35 ± 0.34	16.26 ± 0.57	1.01	$F = 0.28$	0.60
Pollen germination	19.24 ± 12.32	15.10 ± 9.39	1.27	$X^2 = 0.37$	0.54

[#] Ratio of means: LF/SF.

These variables were generally positively correlated (Table 2). LF plants tended to produce slightly (+4%) larger seeds than SF plants. Selection lines did not differ significantly in the provisioning of individual seeds, i.e., seeds of both lines contained the same amount of carbon and nitrogen, and in similar proportions (there was also no significant difference in C/N, Table 1). Thus LF plants produced larger fruits with more seeds, and differed from SF plants in food quantity rather than food quality.

Table 2. Correlations between fruit and seed traits in *Silene latifolia*. Given are Pearson's correlation coefficients (r). *Italic*: two-tailed error probability for the null hypothesis that r = 0. See Methods for variable definitions.

	Fruit volume	Number of seeds	Total seed mass	Total carbon	Total nitrogen	Seed C/N	Mean seed size
Fruit volume	1	0.582	0.662[#]	0.663[#]	0.652[#]	0.213	0.544
		0.023	*0.007*	*0.007*	*0.008*	*0.446*	*0.036*
Number of seeds		1	0.948[#]	0.949[#]	0.943[#]	0.189	0.106
			<0.001	*<0.001*	*<0.001*	*0.501*	*0.708*
Total seed mass			1	1[#]	0.999[#]	0.091	0.374
				<0.001	*<0.001*	*0.746*	*0.17*
Total carbon				1	0.999[#]	0.088	0.372
					<0.001	*0.756*	*0.172*
Total nitrogen					1	0.04	0.382
						0.888	*0.16*
Seed C/N						1	-0.265
							0.34
Mean seed size							1

All correlations are calculated using family means, *N* = 15 families.

[#] Significant after Bonferroni correction (nominal *a* = 0.017, i.e. 0.05/28 tests).

Effects of selection line, fruit abortion and fruit quality on larval performance

In agreement with the finding that LF plants produce larger fruits, larvae were significantly larger at emergence on LF than SF plants (Fig. 2). For both the 1st and 2nd larva, mass at emergence was significantly affected by selection line (Table 3).

Importantly, we found also clear evidence that fruit abortion significantly decreases larval performance. First, at emergence the larvae on aborted fruits had reached less than half the mass of larvae growing on non-aborted fruits (Fig. 2, Table 3). For the 2nd larva, both the abortion of the previous infested fruit on the same plant (abortion of the fruit with the 1st larva) and the abortion of the fruit on which the 2nd larva itself was growing had a significant effect on larval mass at emergence. If the 1st fruit had been aborted, the larva growing on the 2nd fruit was smaller. Further, the 2nd larva was significantly heavier, the higher the seed C/N ratio. Finally, fruit abortion also significantly affected larval age at emergence. Fruit abortion was the sole significant explanatory variable for larval age at emergence for both the 1st and the 2nd larva (Table 3). Larvae emerged significantly earlier (approximately 3 days, Fig. 2) from aborted compared to non-aborted fruits. The larval mass gain over time (mg/day) was significantly smaller in aborted fruits (1st: 6.28 ± 1.70; 2nd: 6.12 ± 2.58) than in non-aborted fruits (1st: 12.19 ± 3.74; 2nd: 9.47 ± 3.42), and in SF fruits (1st: 7.37 ± 1.21; 2nd: 5.00 ± 0.87) compared to LF fruits (1st: 13.13 ± 3.20; 2nd: 10.41 ±2.78).

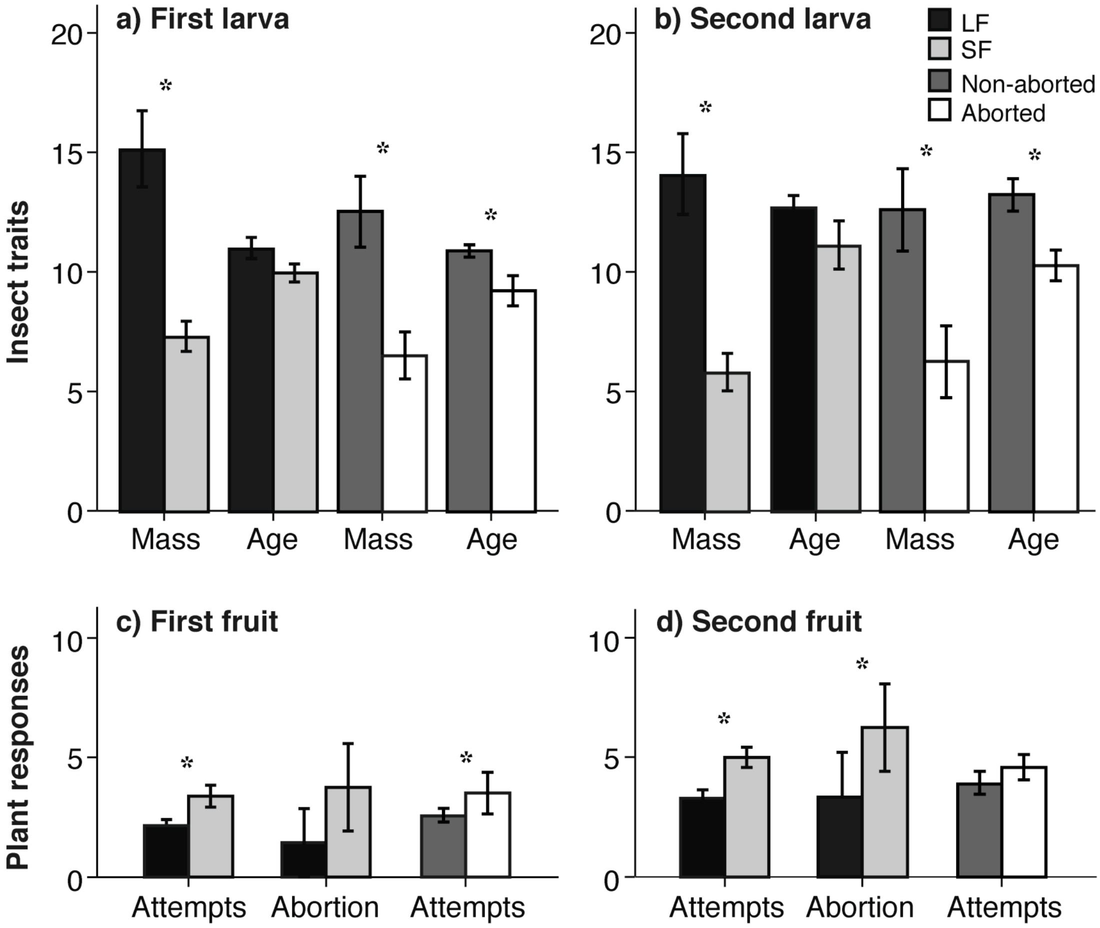

Figure 2. Performance of *Hadena bicruris* larvae by selection line (light bars = SF, small-flowered; black bars = LF, large-flowered *Silene latifolia*) and by fruit abortion (grey bars = aborted fruits; open bars = non-aborted fruits) for **a)** the first and **b)** second larva per plant. Plant responses by selection line and fruit abortion for **c)** the first and **d)** second infested fruit. Two fruits (1st and 2nd fruit) on each plant were infested (1st and 2nd larva). Performance was measured as: larval mass (mg; divided by 10 to improve readability of the graph) and larval age (days) at emergence from the primary fruit; plant responses as proportion of infested fruits that were aborted (multiplied by 10) and as the number of attempted infestations needed to obtain a successfully infested primary fruit (see Methods). Data are given as mean ± SE; SE was calculated on family means. * indicates significant (GLMM; *P*<0.05) differences between selection lines or between non-aborted and aborted fruits.

Table 3. GLMM models for the effects of selection line, fruit abortion and covariates (fruit provisioning traits) on mass and age at emergence of two *Hadena bicruris* larvae per plant. Given are minimal adequate models after stepwise reduction.

Trait	Larva	*N*		Minimal adequate model			
		plants	families	fixed effects	*d.f.*	*F*	*P*
Mass	1st	43	15	selection line (SF vs. LF)	1;13	13.15	0.003
				1st fruit (aborted vs. not aborted)	1;27	36.28	<0.001
	2nd	35	15	selection line (SF vs. LF)	1;13	9.36	0.009
				2nd fruit (aborted vs. not aborted)	1;17	11.14	0.004
				1st fruit (aborted vs. not aborted)	1;17	8.59	0.009
				Seed C/N	1;17	6.3	0.023
Age	1st	43	15	1st fruit (aborted vs. not aborted)	1;27	25.79	<0.001
	2nd	35	15	2nd fruit (aborted vs. not aborted)	1;19	28.8	<0.001

Effect of selection line on infestation failure and predictors of fruit abortion

For the establishment of the 1st larvae, significantly more infestation attempts were needed (i.e. more flowers had to be infested with an egg until we observed a fruit with extruding excrements, as a sign of successful infestation) in SF than LF plants (Fig. 2, LLRT: deviance difference between a model with selection line and the constant-only model = -2.92, $P(X^2_1>5.83) = 0.02$). In addition, for the 1st infested fruit, plants that aborted their fruit also needed more attempts until there was a successful infestation (Fig. 2, LLRT: deviance difference between a model with fruit abortion and the constant-only model = -5.33,

$P(X^2_1>10.66) = 0.001$). However, there was no significant difference in number of attempts between plants aborting vs. not aborting their 2nd infested fruit.

In complete GLMMs, abortion of the 1st fruit was significantly explained only by the larval mass gain (slowly-growing larvae in aborted fruits, fast-growing larvae in non-aborted fruits, either because fast-growing larvae were more likely to escape abortion, or because abortion reduces the intake of resources and slows down growth). The probability of abortion of the 1st fruit increased marginally with increasing C/N ratio in seeds (Table 4). Selection line and all other covariates (including pollen germination) did not significantly affect the probability of abortion of the 1st fruit and were eliminated during model simplification. Abortion of the 2nd fruit was significantly affected by the interaction between selection line and abortion of the 1st fruit (Table 4). When the analysis was repeated separately for LF and SF plants to dissect the interaction, in LF plants the 2nd fruit was significantly more likely to be aborted if the 1st fruit had been aborted (LLRT: deviance difference = -2.73, $P(X^2_1>5.46) = 0.019$), but not significantly so in SF plants (LLRT: deviance difference = -0.39, $P(X^2_1>0.78) = 0.38$).

Table 4. Minimal adequate GLMM models for the predictors of fruit abortion in response to infestation with *Hadena bicruris* eggs. Factor levels for which the estimates are given appear inside parentheses.

Response	*N*		Model comparisons						
	Plants	Families	Model	Fixed effects	Effect size	Deviance	*d.f.*	Deviance difference	Likelihood ratio test
abortion 1st fruit	43	15	minimal model (III)	mass gain 1st larva	-0.44			I-III: -10.56	$P(X^2_1>21.14)<0.001$
				seed C/N	0.62	-16.91	4	II-III: 1.87	$P(X^2_1>3.75)=0.052$
			model (II)	mass gain 1st larva	–	-18.78	3		
			model (I)	seed C/N	–	-27.47	3		
			null model (0)	constant only	–	-27.52	2	0-III: -10.61	$P(X^2_2>21.23)<0.001$
abortion 2nd fruit	35	15	minimal model (II)	selection line (SF)	1.85	-22.94	5		
				1st fruit (aborted)	2.69				
				line * 1st fruit (aborted)	-3.65			I-II: 2.59	$P(X^2_1>5.17)=0.023$
			model (I)	selection line (SF)	–	-20.35	4		
				1st fruit (aborted)	–				
			null model (0)	constant only	–	-24.13	2	0-II: -3.78	$P(X^2_3>7.56)=0.056$

Discussion

In nursery pollination, the pollinator acts as a seed predator during its larval stage (Dufaÿ & Anstett, 2003; Westerbergh, 2004; Kephart *et al.*, 2006). This should lead to plant defenses to reduce the costs imposed by seed predation. A potential defense is the ability to abort attacked fruits, as in yucca/yucca moth (Addicott, 1986; Pellmyr & Huth, 1994; Richter & Weis, 1995) or senita cactus/senita moth (Holland & Fleming, 1999; Holland *et al.*, 2004a). The *Silene latifolia/Hadena bicruris* interaction is not obligate and potentially less specialized than that between yucca/yucca moth and senita cactus/senita moth (Dufaÿ & Anstett, 2003). However, here too it was recently found that experimental infestation with seed predator eggs significantly increased the probability of fruit abortion (Jolivet & Bernasconi, 2006), and that under natural pollination and infestation, individually marked fruits which had been dropped by the plant were significantly more likely to have been infested compared to fruits of the same age that had not been dropped (Elzinga & Bernasconi, In press). These findings suggest that fruit abortion may also be an effective control mechanism in this non-obligate system. However, to be effective as a defense, abortion of infested fruits needs to reduce the costs of predation to the plants, and to lower the profitability to the larvae, so as to ultimately limit survival and reproductive rates of the seed predator (Shapiro & Addicott, 2004; Holland *et al.*, 2004b).

Our results clearly demonstrate that fruit abortion reduces larval mass and age at emergence, and thus strongly suggests that it is effective in imposing a reduction in fitness of the seed predator that is likely to benefit the plant. Enemy attack (e.g., predators, parasitoids) may be more likely (and thus larval survival lower) if the larva emerges at an earlier age from the primary fruit (where the egg was laid), because larvae that emerge at smaller size and earlier age likely need to locate a larger number of secondary fruits to complete development than larvae that can develop further in a non-aborted, primary fruit. In our study, larval mass at emergence from aborted fruits was less than half the mass of larvae emerging from non-aborted fruits. Leaving earlier and at smaller size from the primary fruit implies increased risks, since the primary fruit grants protection from parasitoids (Benrey & Denno, 1997; Awmack & Leather, 2002; Biere *et al.*, 2002). At least 14 parasitoid species have been described from *H. bicruris* larvae (Elzinga *et al.*, 2007c), most of which attack the larvae at instar L4 or L5 (Elzinga *et al.*, 2007c). Lower larval survival as a consequence of fruit abortion may be reinforced by the fact that aborted fruits are dropped away from the plant, thus reducing the probability of secondary attack for other fruits on the same plant. In this study we measured larval performance and did not directly address effects on plant fitness. However, it seems reasonable to assume that the plant has lost fewer resources in aborted, infested fruits (producing a larva with a smaller mass at emergence) than in non-aborted, infested fruits. A low mass at emergence may reduce larval survival but also adult fecundity. Indeed, larval growth rate is positively correlated with adult fecundity in several insect species (e.g., Honek, 1993; Kause *et al.*, 1999; Awmack & Leather, 2002). It would thus be very interesting in future studies to quantify how fruit abortion translates into reduced energetic costs and risk of secondary attack for the plant, and into lower moth survival and fecundity, and as a result lower abundance of the seed predator (Westerbergh & Westerbergh, 2001; Holland & DeAngelis, 2002; Holland *et al.*, 2004b).

We found that infesting larvae were influenced by previous abortion: if the 1st larva had been aborted, the 2nd larva was significantly smaller (compared to 2nd larvae on plants that did not abort their 1st larva). This suggests a difference in defense or allocation in plants that were previously exposed to attack and aborted their fruit, compared to plants that were exposed but did not abort the fruit containing the 1st larva. A previous investigation asked whether infestation with *H. bicruris* eggs induced plant responses for fruit-wall thickness, seed mass and C/N content (as measures of allocation), but no significant differences were found between induced and non-induced plants (Jolivet & Bernasconi, 2006). Thus the reduced larval growth observed in the present study on plants that previously aborted attacked fruits may be mediated by other mechanisms, including the production of chemical defenses that may be toxic, or a reduction in the digestibility of seeds (Mattson, 1980). Both resource allocation to developing fruits (Kliber & Eckert, 2004) and how it is modified by abortion in response to attack may change in the flowering sequence and with increasing levels of attack. In our study, we examined one non-infested control fruit and analysed larval performance in two additional successfully infested fruits per plant, which required attempted infestation of up to nine flowers per plant. However, during one season *S. latifolia* females will usually produce more than three fruits. In a study exposing plants from the same selection lines as in this experiment (see *Plant material*) to natural pollination and seed predation, each plant produced 4.5 ± 0.4 (mean ± s.e.) flowers per week in the LF line, and 6.9 ± 0.5 in the SF line. Of those flowers, a mean of 12.4% (i.e. roughly one flower per week) were primarily attacked in the LF, and 15.1% in the SF lines (difference between lines not significant, A. Burkhardt, L.D. Delph, G. Bernasconi, see thesis chapter 3). Thus, although the number of infested fruits we examined in our study is within the range of natural infestation levels, future work is required to investigate the effects of abortion on larval performance over the entire season or plant life cycle.

Plants more likely to abort fruits also exhibited significantly lower egg survival, as reflected in the fact that significantly more attempted infestations were needed to successfully infest the first fruit. This suggests that plants with a higher propensity to fruit abortion are also more likely to prevent eggs from developing, or very young larvae from establishing themselves. Since our experimental plants stemmed from controlled crosses under greenhouse conditions, it is possible that the association between the number of attempted infestations and fruit abortion may reflect at least some genetic variation in defense, however the mechanistic basis for this remains to be elucidated.

We found no significant difference between SF and LF lines in their propensity for fruit abortion. However, the difference was in the direction of abortion and also failed infestation being more likely, albeit not always significantly so, in the SF line, possibly reflecting the fact that abortion of the smaller SF fruits may lead to lower costs to the plant (Wright & Meagher, 2003).

We found strong evidence that LF plants, which produced fewer, larger fruits with twice as many seeds provided a better food source for larvae (larvae growing on LF fruits reached twice the mass at emergence compared to larvae growing on SF fruits). Since food quantity and quality affect larval growth (Mattson, 1980; Wheeler & Halpern, 1999) and adult fitness in insects (Awmack & Leather, 2002), moths may be selected to oviposit in plants that offer better resources to their offspring (Thompson & Pellmyr, 1991). Oviposition choice based on food quality or quantity has been shown in the Lepidoptera *Tyria jacobaeae* (Vandermeijden *et al.*, 1989) and *Euphydryas editha monoensis* (Singer *et al.*, 1988). In our study system, oviposition choice for more profitable flowers may be reinforced because a larva growing in a larger primary fruit may need fewer secondary fruits to complete its development. Indeed, oviposition was found to decrease with decreasing flower and ovary size in naturally occurring *S. latifolia* (Biere & Honders, 2006). Similarly, *Hadena compta* moths prefer to lay eggs in *Dianthus sylvestris* plants with large perfect flowers compared to plants with small pistillate flowers (Collin *et al.*, 2002). However, although such preferences may clearly be important under natural conditions, by using experimental infestation, our design specifically highlights the effects of fruit size and abortion on larval performance and avoids confounding by choice behaviour – i.e. if the insect would choose specific plants for oviposition (for instance those with low propensity to abort) this would mask the effects.

On the other hand, flower size may evolve in response to selection imposed by the behaviour of the seed predator, which deserves further investigation. In particular, flower size is known to trade off with flower number in *S. latifolia* (Delph *et al.*, 2004b). Large floral displays attract more pollinators, but they also bring in more pollinator-borne pathogens (Shykoff & Bucheli, 1995) and may increase the risk of receiving eggs (e.g.; Collin *et al.*, 2002; Biere & Honders, 2006). Hence, interactions with pollinators, pathogens, and seed predators, as well as the positive relationship between seed number and flower size, are likely to interact in terms of selecting for or against large or small flowers in females. Moreover, in dioecious species like *S. latifolia*, male and female plants may evolve sexually dimorphic responses to biotic interactions.

In conclusion, this study demonstrates that fruit size is an important determinant of larval growth, and that fruit abortion reduces the mass and age at emergence of juvenile seed predators. From the lower mass of larvae in aborted than non-aborted fruits we can infer that abortion likely reduces the amount of resources that the plant invests in attacked fruits and thus the costs it suffers. Since the smaller larvae emerging from aborted fruits are most likely to perish in the search for additional fruits to complete development, this strongly suggests that fruit abortion is effective both as resistance and defense in this system, and may contribute to the maintenance of balanced costs and benefits also in this non-obligate, less specialized association.

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CHAPTER 3 : The contribution of seed predation to selection on sexually dimorphic floral traits in *Silene latifolia*

Manuscript in preparation

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Primarily attacked fruit

Pedicel after fruit abortion

Abstract

In dioecious plants, pollinator selection can lead to the evolution of sexually dimorphic flower traits. Despite their potential impact on plant fitness, the role of antagonists as selective agent on dimorphic flower traits is unexplored. We used *Silene latifolia* selection lines varying in a dimorphic trait (flower number and size), exposed them in a common garden to the specialist pollinating-seed predator *Hadena bicruris,* and followed the fate of each fruit. We expected SF plants (many Small Flowers) to suffer from higher predation rate than LF plants (few Large Flowers), and that interaction with *H. bicruris* moths would favour smaller floral displays as a *pre*-oviposition « defense ». However the difference we observed between lines was more indirect and mediated by differential effects of selection against fruit abortion (i.e., by a *post*-oviposition response of the plant to infestation) presumably resulting from differences in fruit abortion costs. We found positive total selection but no significant moth selection on flower number. Total seed production, the number of eggs received, and the proportion of parasitized or aborted fruits did not differ significantly between lines. SF plants reared significantly more larvae, tended to have a larger parasite load, and lost more fruits due to predation and abortion than LF plants. Overall abortion was selected against, and moths contributed to the selection against abortion in the SF but not in the LF plants. Abortion was significantly more common in plants with a high parasite load. These results suggest that fitness of female *S. latifolia* may be maximized by producing fewer larger flowers that decrease the cost of rearing parasite larvae. Because male siring success can increase with the number of flowers, this may have led to the sexual dimorphism observed in this species.

Key words: pollination, mutualism, sexual dimorphism, host-parasite coevolution, phenotypic selection, selective source analysis, *Silene alba,* white campion, *Hadena bicruris.*

Introduction

Flowering plants exhibit a variety of reproductive systems (Barrett, 2002; Charlesworth, 2006). Dioecy, although relatively rare among plants (6 % of species, Renner & Ricklefs, 1995), is of particular interest because it allows sexes to respond differently to selection, and this can lead to the evolution of traits with different phenotypic optima in the two sexes (Meagher, 1992; Geber, 1999). Sexual dimorphism can be found in flower and inflorescence traits (Delph *et al.*, 1996; Eckhart, 1999), life history (Delph, 1999), vegetative morphology and physiology (Dawson & Geber, 1999), and ecological traits such as competitive ability and susceptibility to herbivores and pathogens (Ågren *et al.*, 1999).

For plants that depend on insect pollination, flower number and size are expected to be under strong selection. Indeed many studies have shown that plants with a large floral display attract more pollinators (Davis, 1981; Eckhart, 1991; Ohara & Higashi, 1994; Conner & Rush, 1996; Grindeland *et al.*, 2005; Sandring & Ågren, 2009). In dioecious plants, floral display is sexually dimorphic and males typically produce more flowers than females (Delph *et al.*, 1996). Past research shows that plants with large flowers – which is also a dimorphic trait in temperate regions (Delph *et al.*, 1996) – are generally more attractive to pollinators (Galen, 1996; Vaughton & Ramsey, 1998; Thompson, 2001). Clearly, because of the interactions between pollinators and plants, pollinators may generate potentially strong selection on floral traits (e.g.; Galen, 1996; Arista & Ortiz, 2007; Gomez *et al.*, 2008).

Despite their potentially large impact on plant fitness, the role of natural enemies as selective agents on flower traits has received less attention. For instance, enemies such as nectar thieves (Galen, 1999; Irwin, 2009), castrating fungi (Shykoff *et al.*, 1997) and seed predators (Brody & Mitchell, 1997) differentially affect plants with varying flower number or size. Opposing selection pressures on flower traits by pollinators and antagonists are widespread (e.g. Elzinga *et al.*, 2007a), but to our knowledge these studies do not explicitly measure what is the contribution of the antagonist to total selection. For example in *Polemonium viscosum* ants can select for narrower flowers than preferred by the major pollinator in populations where copollinators are frequent because narrow flowers are more likely to escape ant damage (Galen & Cuba, 2001). In *Silene dioica,* female flowers with short stigma lobes can escape infestation with fungal spores (Giles *et al.*, 2006) but suffer from reduced seed production when the fungus is absent (Olsson, 1995).

Of special interest for the evolution of sexually dimorphic traits are pollinating-seed predators. These insects usually visit both plant sexes, thus ensuring pollination, while only female plants pay the cost of rearing the insect offspring (nursery pollination). About 13 nursery pollination interactions have been described (Dufaÿ & Anstett, 2003), among them are the well-known examples of yucca/yucca moth (Pellmyr & Huth, 1994) and fig/fig wasp (Bronstein, 1992). These systems are particularly interesting for the analysis of selection on sexually dimorphic flower traits. Selection due to the rearing of the seed predator offspring can be separated from other types of selection using selective source analysis (SSA; Ridenhour, 2005; Nuismer & Ridenhour, 2008), a method derived from the Lande and Arnold approach (Lande & Arnold, 1983; Arnold & Wade, 1984).

Plants respond to attack by pollinating-seed predators with defense mechanisms that act before or after oviposition and may thus mitigate the effect of seed predation. For instance, plants can respond to egg or larval infestation with fruit abortion (Pellmyr & Huth, 1994; Richter & Weis, 1995). Abortion is expected to benefit the long-term plant fitness by decreasing the seed-predator population (Westerbergh & Westerbergh, 2001; Holland & DeAngelis, 2006) . Furthermore the benefits or costs of abortion to plant fitness may depend on the level of attack and the number or the size of fruits that are aborted. Consequently, seed predators may indirectly select for flower traits that minimize the costs of abortion and thereby contribute to selection on sexually dimorphic traits.

We investigated the role of a pollinating seed-predator on the evolution of sexual dimorphism, using the dioecious plant *Silene latifolia* and its pollinating-seed predator *Hadena bicruris*. This plant is sexually dimorphic for flower number and size (Carroll & Delph, 1996; Meagher & Delph, 2001; Delph *et al.*, 2002), aborts fruits attacked by the seed predator (Jolivet & Bernasconi, 2006; Burkhardt *et al.*, 2009a), and artificial selection experiments can provide plants that vary in flower number and size (Delph *et al.*, 2004b). In Europe, the insect is present in most populations and causes substantial damage to *S. latifolia* (Wolfe, 2002).

The aim of this study was to determine (i) the fitness effects of seed predation for *S. latifolia* plants exposed to naturally occurring seed predators in a common garden, (ii) whether interactions with seed predators lead to selection on specific *S. latifolia traits* and (iii) whether selective pressure is different between selection lines that differ for flower size and number, a sexually dimorphic trait in *S. latifolia.* Simply attributing selection to parasite visitation opens the possibility that reciprocal selection and coevolution may be occurring (Forde *et al.*, 2004; Thompson, 2005), but, more interestingly, differences in selection between selection lines address whether interactions with parasites contributes to the evolution or maintenance of sexually dimorphic floral traits and displays (Nuismer & Ridenhour, 2008).

Material and methods

Study system

The white campion *Silene latifolia* Poir. (= Silene alba (Miller) Krause, = Melandrium *album* (Miller) Garcke; Caryophyllaceae) is a short-lived perennial, dioecious plant native to Europe. *Silene latifolia* is sexually dimorphic for several traits, including floral display (Delph *et al.*, 2002). The dimorphism is evidenced by male plants carrying more flowers, but these flowers are, in comparison to flowers of female plants, smaller and shorter-lived (Carroll & Delph, 1996; Meagher & Delph, 2001; Young & Gravitz, 2002). In Europe *S. latifolia* is pollinated by noctuid moths and mainly by the specialist seed predator *Hadena bicruris* (Hufnagel, Noctuidae; Brantjes, 1976b; Jurgens *et al.*, 1996). The moth is present in most surveyed European populations and can attack more than 50% of the fruits in a population (Biere & Honders, 1996; Wolfe, 2002) or per plant (Biere & Honders, 1996). Typically female moths lay one egg per flower in female plants, where each larva consumes a *primary fruit* (i.e. the fruit of the flower in which it was laid) and additional *secondary fruits* before pupating (Elzinga *et al.*, 2005). Moths are able to discriminate between male and female plants, and avoid laying eggs in male plants (Brantjes, 1976b). Plants respond to experimental egg infestation by increased flower/fruit abortion, suggesting abortion may be a plant response to reduce damage (Jolivet & Bernasconi, 2006).

Plant material and rearing conditions

We raised plants from seeds originating from two artificial-selection programs (Delph *et al.*, 2004b). In natural populations, flowers on females have wider calyces than those on males (Delph *et al.*, 2002), and this difference is the basis of the imposed selection regimes. Using two American source populations, two replicate “large flower” (LF) lines were created by selecting males with large calyx widths, and two replicate “small flower” (SF) lines were created by selecting females with small calyx widths (as described in Delph *et al.*, 2004b). In addition, plants with large flowers (LF lines) also produce fewer flowers and thus smaller floral displays. In contrast, plants with small flowers (SF lines) produce more flowers and thus have larger floral displays. The differences in floral display found between LF and SF lines indicates a strong negative correlation between flower size and number (Delph *et al.*, 2004b). To eliminate inbreeding effects, plants arising from one replicate selection line were crossed with plants from the other replicate line but from the same selection regime (i.e. SF with SF, and LF with LF) in the generation preceding our experiments. In these experiments we used eight families from LF lines and eight from SF lines. After germination of seeds in Petri dishes, we transplanted seedlings to Jiffy® peat pellets and placed them in a greenhouse (22°C day/ 18°C night, 45% H) for a month before transplanting them to pots (16 cm^3) in an experimental garden.

Measurement of plant fitness under continuous exposure to seed predation

We recorded fruit predation by naturally-occurring *H. bicruris* on LF and SF plants, which differed in flower size but also floral display, by exposing them in a common garden near Lausanne, Switzerland (46°31'19"N, 6°34'49"E). Upon flowering we placed four females and two males of each family (8 LF and 8 SF families, i.e. a total of 64 females and 32 males; we added one additional male to the plot to alternate male and female plants) in a mown experimental plot. We placed plants in three rows of plants at 1m-interplant distance, one central row of 33 males and two lateral rows of 32 females. Along the rows, we alternated plants from each selection line type (LF or SF), so that each female was equidistant to two males (one LF and one SF). We measured each female once a week for eight weeks from onset of flowering, and each male during four weeks from onset of flowering.

Plants were continuously exposed to *H. bicruris* attack during this experiment, and their floral/fruit display was not manipulated (i.e. we did not remove flowers and we allowed natural fruit and larval development). On females, we marked each new flower or fruit with a numbered label and recorded: fruit development, attack by *H. bicruris*, fruit maturation or abortion, and presence of the aphid *Brachycaudus lychnidis* Linnaeus 1758 (Wolfe). *Brachycaudus lychnidis* is a specialist of the genus *Lychnis* and *Silene*, including *S. latifolia* (Heie, 1992; Blackman & Eastop, 2006). We recorded whether fruits attacked by *H. bicruris* were *primarily attacked* (i.e. had a hole and extruding frass at the fruit basis) or *secondarily attacked* (i.e. had a hole and missing teeth at the fruit top, Elzinga *et al.*, 2005). We calculated *total seed production* of each female as the number of healthy (non-attacked) fruits that matured multiplied by the number of seeds in one fruit. To estimate the number of seeds, we bagged one healthy fruit per plant, and counted the number of seeds with a seed counter (Elmor C3, Elmor Angewandte Elektronik, Schwyz, Switzerland).We calculated the per plant *primary-* or *secondary-attack rate* as the number of primarily or secondarily attacked fruits over the total number of fruits produced per female. *Primary abortion rate* was defined as the number of aborted fruits over the number of primarily attacked fruits. On male plants, we counted open flowers, dissected them, and counted *H. bicruris* eggs inside flowers in four consecutive weeks. As a result of artificial selection, we expected an increase in the probability that ovipositing moths would mistake a LF male (with “female-looking” flowers) for a female.

Measurements of floral traits

We germinated seeds from the same 16 families used for the common garden experiments, and grew them in the greenhouse in 10 cm diameter pots under the same conditions as described above until onset of flowering. For each family, we measured the following traits with a caliper (precision 0.1 mm) on number of individuals (between 6 and 13 per family for 148 plants in total): *corolla petal diameter*, *corolla tube inner diameter*, *corolla tube length*, *calyx width*, *calyx length*, and *ovary length* (Fig. 1). We calculated family means for each floral trait and used these means as factors in the Selective Source Analysis (see below).

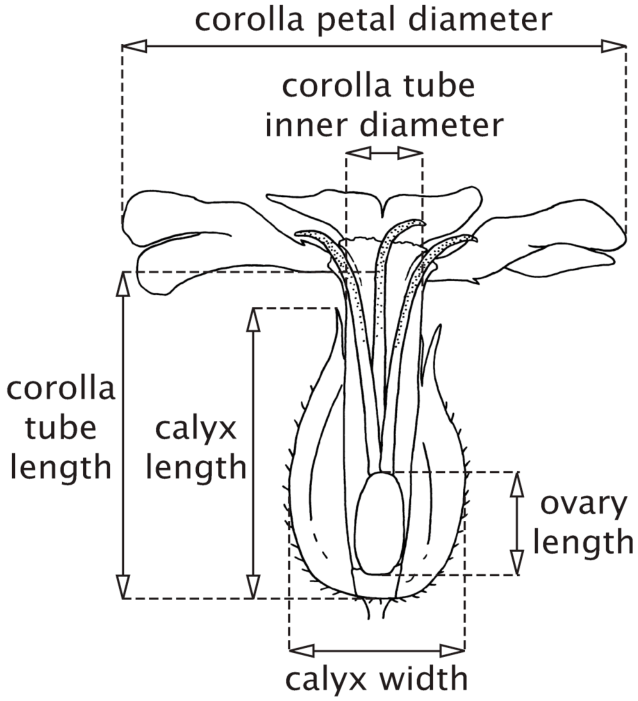

Figure 1. Schematic drawing a *Silene latifolia* female flower and definition of the measured floral traits (after Elzinga, 2005).

Control experiment: oviposition in female plants displaying only flowers

The presence of previously attacked fruits on a plant may modify the oviposition choice of the seed predator, and not all eggs will survive and establish as larvae in primarily attacked fruits. Therefore, to better isolate the effect of floral size *per se* on the risk of egg infestation, we additionally tested whether LF and SF plants differ in their per-flower risk of infestation when plants only display flowers but do not carry any fruit. Plants in this experiment were intermittently exposed to *H. bicruris* attack (on single nights) in a mown plot in the common garden, and the morning following exposure we removed all flowers so that no fruit nor larvae developed. We dissected the flowers and counted the eggs of *H. bicruris*. We placed female plants (4 plants per family x 8 families per selection line x 2 selection lines = 64 plants) at random positions on a 0.5 m x 0.5 m grid in a mesh cage of 5 m x 5 m x 1.2 m (height) and placed thirty-two male plants (2 males per family) near the cage to provide pollen. We opened the cage on one evening per week for five consecutive weeks and calculated the per-flower *egg infestation risk* as the number of flowers infested with *H. bicruris* eggs divided by the total number of flowers produced per female in the five weeks.

Statistical analyses

If not stated otherwise, we analysed all response variables using Generalized Linear Mixed Models (GLMM) with selection line as a fixed factor, family as a random factor, and corrected for over-dispersion where applicable. We used Log Likelihood Ratio Tests (LLRT) to judge the significance of the difference in deviance between models with and without the factor selection line. GLMMs for binary response variables and proportions used binomial error distribution and logit link function, and models for count data used Poisson error distribution and log(y) as a link function. All data analyses were performed using R version 2.6.2 (R Development Core Team, 2006).

We analysed the effect of selection line on rates (primary and secondary attack rates, primary abortion rate in continuously exposed plants, and per-flower egg infestation rate in intermittently exposed plants), and the effect of selection line on attack by aphids. In the model for aphid attack, we included as a covariate the mean number of fruits per plant over the eight weeks. To investigate further if fruit abortion was selective following attack by *H. bicruris* larvae, we analysed the effect of fruit attack status (primary, secondary, or unknown – i.e. not attacked or no external signs of attack) and selection line on the *rate of total fruit abortion* (i.e. the total number of aborted fruits over the total number of fruits produced).

Finally, we analysed the effect of selection line on the number of ripe fruits or total flower production per female; for total seed production we ran a GLMM with normal distribution.

To investigate whether the distribution of larvae among plants (estimated as the number of primarily attacked fruits) influenced the propensity of secondary attack or fruit abortion, we pooled the weekly observations with at least one primary attack per week for each of 32 females of each line (i.e. a maximum of 32 x 8 observations per line). Each week, we counted how many plants had one (*low parasite load*), or more than one larva growing simultaneously (*high parasite load*). Further, each week we monitored all other fruits on the same plant and recorded whether there was abortion (no abortion versus at least one aborted fruit) and secondary attack (no attack versus at least one attacked fruit). Because we censused all new flowers and fruits each week, we could record unambiguously each new instance of primary attack, secondary attack and fruit abortion. To estimate total attack and abortion independently of week-by-week variation in *H. bicruris* abundance, for each experimental plant, we thus summed these observations over the eight weeks and then summed the plant sums for each line. We analyzed the effect of selection line, parasite load and abortion on total secondary attack (i.e. sum over eight weeks). In a similar model, we analyzed the effect of selection line, parasite load and secondary attack on total abortion (i.e. the sum over eight weeks). We tested for differences between selection lines in parasite load using a Chi-squared test on total primary attack (i.e. sum over eight weeks).

Moreover, we analysed the difference between selection lines in the proportion of plants that were attacked or not over the whole experiment (experiment with continuous exposure to seed predation and natural fruit development) and in the proportion of plants I that received eggs or not (exposure to seed predators on discrete nights, only flowers displayed) with a Fisher's exact test. In the experiment with continuous exposure, we found eggs on male plants in three out of four weeks, and tested for difference between selection lines in the proportion of male plants receiving eggs over the whole experiment using a Chi-squared test. In the experiment addressing per-flower egg infestation risk (exposure to seed predators on discrete nights, only flowers displayed), we counted how many female plants had one (*low egg load*), or more than one egg (*high egg load*) deposited simultaneously on different flowers each week and summed the observations of infestations over the five weeks (i.e. a maximum of 32 x 5 observations per selection line) and tested for differences between selection lines in egg load with a Chi-squared test.

Selective Source Analysis

We analysed selective pressures on *S. latifolia* traits measured in this study for both the LF and SF lines separately. Specifically, our objective was to determine if interactions with parasites lead to selection on *S. latifolia* and if that selective pressure is different between the two selection lines. Differences in selection between the two lines may indicate that parasites could contribute to the evolution or maintenance of sexual dimorphism.

In order to differentiate selection related to parasite visitation from other types of selection, we used selective source analysis (SSA; Ridenhour, 2005; Nuismer & Ridenhour, 2008) rather than calculating “traditional” selection gradients (Lande & Arnold, 1983; Arnold & Wade, 1984). Selective source analysis allows researchers to partition selection gradients to specific “sources” – in our case, this source was attack by *H. bicruris*. Because we had relatively few individuals per line (32) and a large number of traits measured (13 traits including six floral traits), and because many floral traits are correlated with each other, we performed a principal component analysis to reduce the dimensions of our dataset. The first principal components explained 98.4% and 91.4% of the variance in plant floral display in the

SF and LF lines respectively. In both cases, total number of flowers produced over the experiment was the most predominant factor for the PC and composite flower trait thus was essentially a surrogate of total flower production per female.

To further reduce the size of selection model, we performed an initial (traditional) selection analysis to determine which traits to investigate via selective source analysis; this greatly reduces the chance of detecting false positives (Type I error). To this end, we performed a stepwise regression with entry/exclusion from the fitness model based on penalized likelihood scores (specifically, the Bayesian information criterion). The initial model contained total seed production as measurement of plant fitness, our composite flower trait (PC1), date of flowering onset, and fruit abortion as factors explaining plant fitness. The resulting model indicated that the composite flower trait in combination with the number of primarily aborted fruits per plant provided the best fitness model (i.e. there was evidence of significant selection on these two traits). We therefore excluded other factors from subsequent analyses.

Using the aforementioned two *S. latifolia* traits, we partitioned their respective selection gradients using SSA to detect parasite selection (Ridenhour, 2005). A preliminary fitness model was run to detect which parasites (aphids or *H. bicruris*) may be influencing plant fitness. Attack by aphids was not a significant predictor of plant fitness and was therefore excluded from the SSA analysis. Thus we only used the total number of attacked fruits per plant (i.e. primarily and secondarily attacked fruits) by *H. bicruris* as parasite traits in the analysis. In order to determine the significance of the resulting selection gradients, we performed 19,999 bootstrap analyses. Significance intervals were calculated using the BCa method (Efron & Tibshirani, 1993).

Results

Floral traits and flower, fruit and seed production in SF and LF lines

Flowers on LF female plants had significantly wider and longer corolla tubes, wider calyx and longer ovary than SF flowers (Table 1). In the common garden experiment, in which plants were under continuous exposure to seed predators and natural fruit development was allowed, SF females produced significantly more flowers (Table 2), initiated significantly more fruits (Fig. 2A, Table 2), and produced significantly more ripe i.e. healthy, non-attacked fruits (Table 2) over the observation period than LF females. Thus in absolute terms, SF plants lost about twice as many fruits due to seed predation or abortion than LF plants (Table 2). However, because LF fruits contained significantly more seeds than SF fruits (Table 2), total seed production did not differ significantly among selection lines (Table 2).

Table 1. Floral traits in large flowered (LF) and in small flowered (SF) selection lines of *Silene latifolia.* Shown are means ± s.e. of the family means (8 families per selection line). *F*= test of differences between selection lines in floral traits (univariate GLMMs at the family level).

	Selection line				
	LF	SF	Ratio[#]	*F*	*P*
Corolla petal diameter	25.6 ± 0.8	24.1 ± 0.8	1.06	2.99	0.10
Corolla tube inner diameter	3.0 ± 0.1	2.3 ± 0.1	1.30	69.39	<0.001
Corolla tube length	23.4 ± 0.9	20.4 ± 0.9	1.15	9.42	<0.01
Calyx width	12.4 ± 0.4	8.7 ± 0.4	1.43	72.08	<0.001
Calyx length	18.2 ± 0.8	16.7 ± 0.8	1.09	3.50	0.08
Ovary length	8.4 ± 0.3	6.8 ± 0.3	1.24	29.16	<0.001

[#] LF/SF means

Table 2. Number of flowers, fruits and seeds produced by plants in the large flowered (LF) and in small flowered (SF) selection lines of *Silene latifolia.* Shown are means ± s.e. of the family means (8 families per selection line). All differences between selection lines (univariate GLMMs at the family level) tested with X^2, except total seed production (*F* test).

	Selection line				
	LF	SF	Ratio[#]	Test	*P*
Number of flowers	26.9 ± 0.4	49.1 ± 0.4	0.55	12.20	<0.001
Number of initiated fruits	26.2 ± 4.7	47.0 ± 4.7	0.56	12.24	<0.001
Number of ripe fruits	20.8 ± 3.9	36.3 ± 3.9	0.57	11.19	<0.001
Number of lost fruits	5.2 ± 1.0	10.6 ± 1.8	0.49	7.33	<0.01
Number of seeds per fruit	388 ± 48	221 ± 48	1.76	11.63	<0.001
Total seed production	8011± 1020	7929 ± 825	1.01	<0.001	0.99

[#] LF/SF means

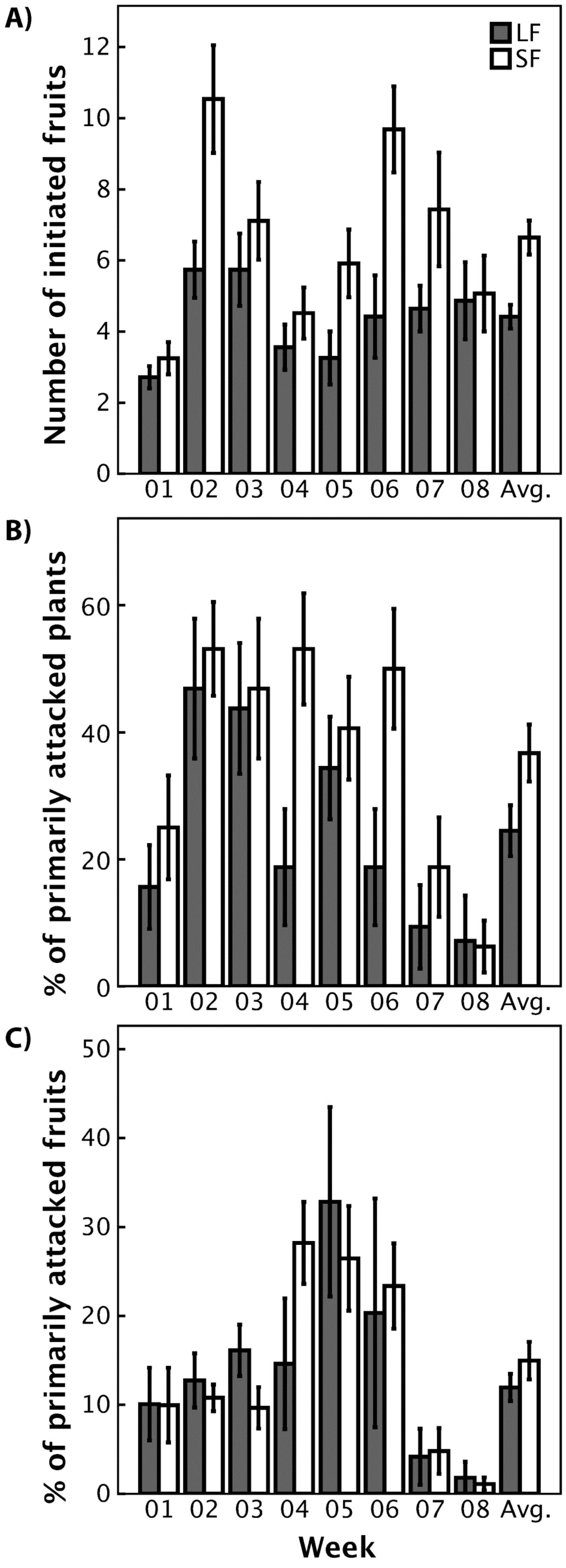

Figure 2. Fruit production and intensity of attack by *Hadena bicruris* on large-flowered (LF, grey bars) and small-flowered (SF, empty bars) *Silene latifolia* plants exposed continuously to natural pollination and seed predation in a common garden for eight weeks since onset of flowering. Panels show: **A)** the number of new fruits, **B)** the percentage of plants that were primarily attacked and **C)** the percentage of fruits on each plants that were primarily attacked. Error bars give s.e. (calculated on family means).

Fruit predation and abortion

Over the duration of the common garden experiment in which plants were continuously exposed to seed predators and natural fruit development was allowed, *H. bicruris* larvae attacked almost all plants, with a non-significant difference between selection lines in the proportion of attacked plants (31 out of 32 SF plants attacked, 29 out of 32 LF plants; Fisher exact test: $F = 0.54$, $P = 0.61$; Fig. 2B). Considering only eggs that survived to become established as larvae in primarily attacked fruits, in total there were significantly more larvae growing on SF females (226 out of 318 primarily attacked fruits, 71%) than on LF females (92 out of 318 fruits, 29 %, binomial test on proportions: $X^2_1 = 111.2$, $P < 0.0001$). The distribution of larvae (total primary attack) tended to differ between selection lines: it was more common in SF females to observe high parasite load (42 observations with one larva, 52 observations with more than one larva per plant) while in LF females low parasite load was more common (38 observations with one larva, 24 observations with more than one larva per plant; Fig. 3A, Chi-square test: $X^2_1 = 3.5$, $P = 0.06$). However, when accounting for differences among selection lines in flower number, attack rates did not differ significantly between SF (primary attack: median = 12% of fruits per plant, range = 0-47%; secondary attack: 1%, 0-38%) and LF females (11%, 0-38%; secondary attack: 1%, 0-26%; Table 3, Fig. 2C).

Neither abortion ($P(X^2_1 > 0.002) = 0.97$) nor selection line ($P(X^2_1 > 0.33) = 0.56$) had a significant effect on total secondary attack. However, as expected high parasite load resulted in significantly higher secondary attack. Indeed, total secondary attack was significantly more common in plants with a high primary attack (high parasite load; SF plants: 21 observations of secondary attack out of 52 observations with more than one primary attack, LF plants: 7

out of 24 observations; Fig 3A) than in plants with a low parasite load (SF: 7 observations of secondary attack out of 42 observations with one primary attack, LF: 7 out of 38 observations; Fig. 3A; $P(X^2_1 > 7.51) = 0.006$).

Table 3. GLMMs for the effects of selection line (SF versus LF) on rates of primary attack of *Silene latifolia* fruits by *Hadena bicruris* larvae, secondary attack of subsequent fruits, and abortion of primarily attacked fruits. Given are the initial and final models after model simplification.

Response	*N*		Model comparisons						
	Plants	Families	Model	Fixed effects	Effect size	Deviance	*d.f.*	Deviance difference	Likelihood ratio test
Primary attack rate	64	16	initial model	selection line (SF)	0.24	-104.64	3	-0.61	$P(X^2_1>1.22)=0.27$
			null model	constant	–	-105.25	2		
Secondary attack rate	64	16	initial model	selection line (SF)	0.1	-83.37	3	-0.03	$P(X^2_1>0.06)=0.81$
			null model	constant	–	-83.4	2		
Primary abortion rate	60	16	initial model	selection line (SF)	0.64	-48.92	3	-1.1	$P(X^2_1>2.21)=0.14$
			null model	constant	–	-50.02	2		

Abortion rates of primarily attacked fruits varied greatly (range = 0-100%), but did not differ significantly between SF (median = 24%), and LF females (median = 0%, Table 3). However, fruit abortion tended to occur more often in SF females (43 observations of abortion out of 94 observations with primary attack) than in LF females (17 out of 62 observations; Fig. 3; $P(X^2_1 > 3.71 = 0.054)$. Interestingly, parasite load had a strong and significant effect on the frequency of total fruit abortion. At high parasite load, we observed fruit abortion significantly more often (SF: 30 observations of abortion out of 52 observations with more than one primary attack, LF: 8 out of 24 observations) than at low parasite load (SF: 13 observations of abortion out of 42 observations with one primary attack, LF: 9 out of 38 observations; Fig. 3A; $P(X^2_1 > 6.71 = 0.01)$. Selection lines did not differ significantly in total abortion rate (i.e. which proportion of fruits per plant were aborted, no matter if fruits were attacked or not; LLRT: $P(X^2_1 > 0.85) = 0.36$). Post-hoc tests showed that primarily attacked fruits were more likely to be aborted than secondarily attacked fruits or fruits of unknown attack status (i.e. not attacked or no external signs of attack; LLRT: $P(X^2_1 > 141.41) < 0.001$; Fig. 4).

SF females were significantly more likely to be infested by the specialist aphid *Brachycaudus lychnidis* (17 out of 32 plants, 53%) than LF females (11 out of 32 plants, 34%; LLRT: $P(X^2_1 > 6.04) = 0.014$; effect size (SF) = 2.09), even after accounting for the significant effect of mean number of fruits per plant (LLRT: $P(X^2_1 > 5.73) = 0.017$; effect size (SF) = -0.44). We occasionally found some *H. bicruris* eggs in male flowers. Four out of 16 SF males (25%) received at least one egg compared to nine out of 18 LF males (50 %), but this difference was not significant (Chi-squared test: $X^2_1 = 2.29$, $P = 0.13$).

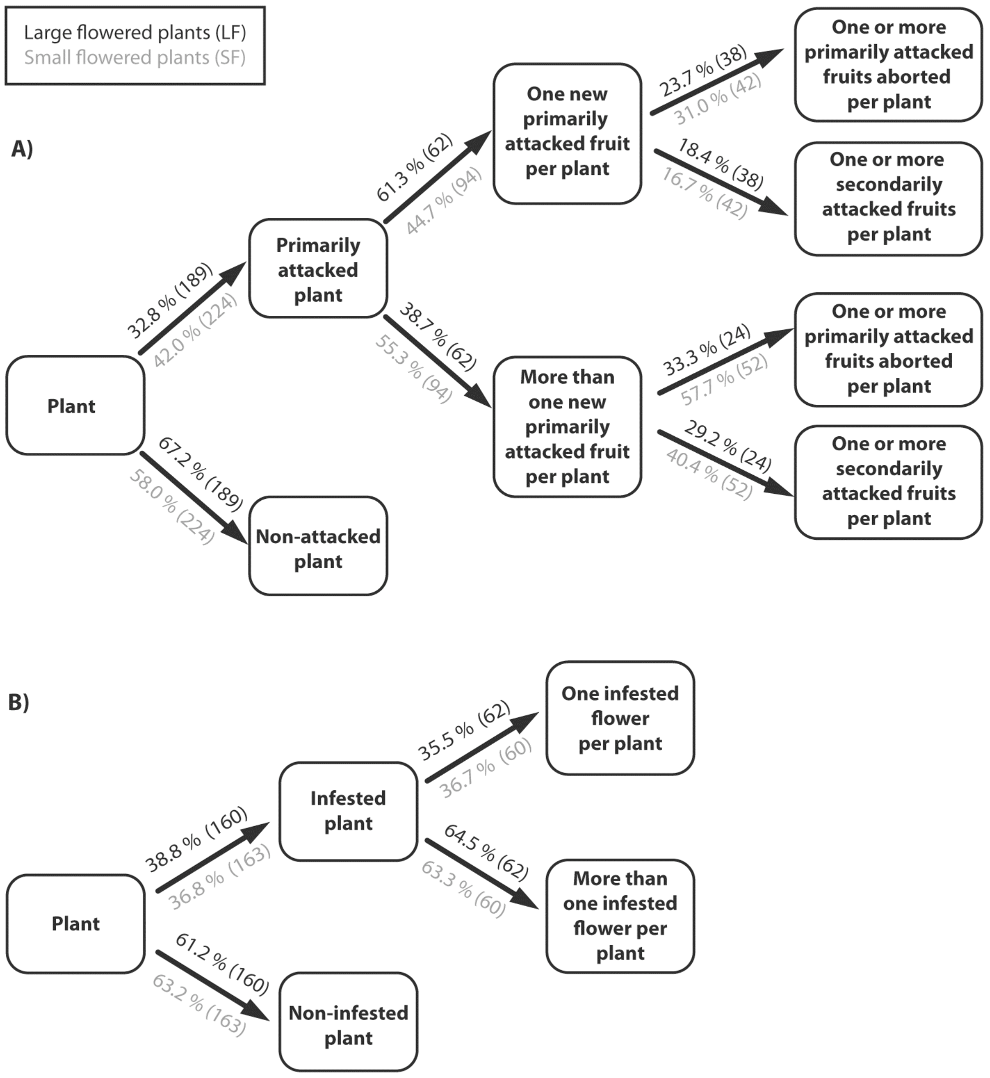

Figure 3. A) Parasite load for LF and SF plants continuously exposed to *Hadena bicruris* during eight weeks. Plants were censused weekly for new flowers or fruits and for presence of parasite larvae. Each new fruit was marked and monitored individually during its development. Flowers receiving an egg can develop into primarily attacked fruits, and larvae emerging from these fruits subsequently attack secondary fruits on the same plant. However, attacked fruits can be aborted. Percentages of total fruit abortion or total secondary attack over the total number of observations of primary attack are presented separately for plants presenting one (low parasite load) *versus* more than one new primary attack per week (high parasite load). **B)** Oviposition by *Hadena bicruris* on plants presenting large (LF) versus small (SF) flowers, summed over five discrete exposure nights. All flowers were removed in the morning following exposure and examined for egg presence, hence no fruit developed on these plants. On average, LF plants had 6.2 ± 1.3 open flowers and SF plants 9.5 ± 2.0 flowers (mean ± s.e., n = 5 nights). Numbers in brackets give the denominator for percentages, and sum instances of infestation, attack or abortion for all plants per line.

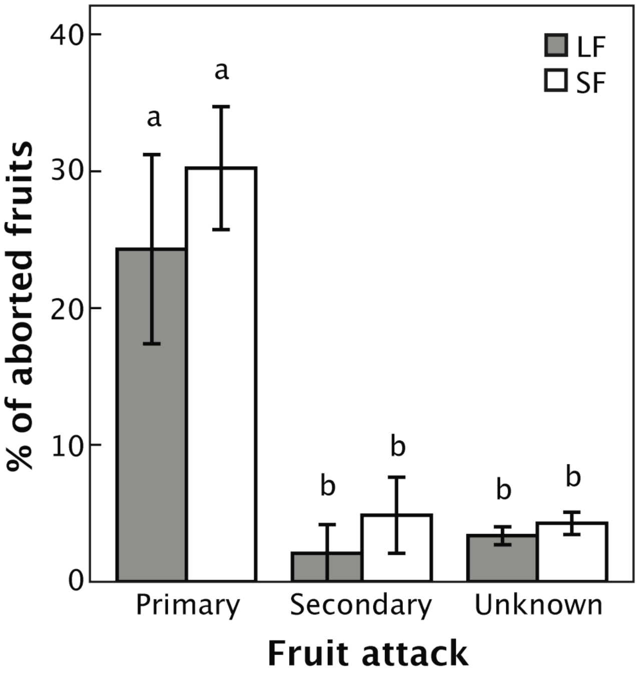

Figure 4. Percentage of *Silene latifolia* fruits that aborted after primary attack, secondary attack, or unknown attack status in plants from large flowered (LF, grey bars) and small flowered (SF, empty bars) selection lines. Bars give means ± s.e. (calculated on family means), and different letters above bars show significant differences.

Selective Source Analysis

Selective source analysis (SSA) partitioned selection gradients for our composite flower trait (PC1 from a principle components analysis) and abortion of primarily attacked fruits (Table 4). Specifically, these selection gradients were partitioned into two portions: that associated with attack by *H. bicruris* attack and an unexplained portion. This partitioning was done for both the LF and SF lines. Total selection was similar in the two selection lines: plants were selected to produce more flowers (positive selection for PC1) and to abort less of the primarily attacked fruits (Table 4). Moth attack exerted a significant negative selection on fruit abortion, but exerted no significant selection on flower production. Interestingly, selection due to moth attack on fruit abortion was marginally significantly different between the SF and LF lines ($P = 0.09$, 0.07 for the difference in selection gradient and differential, respectively). Also notable was the fact that moth attack explained a large part (~50%) of selection on primary abortion in the SF plants but very little of the selection on primary abortion (~6%) in LF plants.

Table 4. Results of selective source analysis for selection gradients (β) and coefficients (s) partitioned to identify selection due to attack by the seed predator *Hadena bicruris* on composite flower trait (a good surrogate of total number of open flowers produced by plants over 8 weeks and a measure of floral display) and abortion of fruit which had been visibly infested by an egg of *H. bicruris* (primary attack) in small flower (SF) and large flower (LF) selection lines. Due to a flower size/number trade-off, SF plants have large floral displays, and LF plants a small floral display. Interpretation of the sign of the difference (= SF – LF) is analogous to a two-sample *t*-test. If selection gradients in both lines have the same sign, the difference reflects whether the magnitude of selection was larger on SF (thus giving a positive difference, if the selection was positive in both lines, and a negative difference, if the selection was negative in both lines). Significance was determined by bootstrapping the data 19,999 times (*= $P < 0.05$; **= $P < 0.01$).

		β / s	
Trait	SF Line	LF Line	Difference (SF-LF)
Composite Flower Trait			
Total Selection	0.375** / 0.283**	0.321** / 0.323**	0.054 / -0.041
Moth Selection	0.005 / -0.037	-0.009 / -0.009	0.015 / -0.027
Unexplained Selection	0.370** / 0.320**	0.331** / 0.333**	0.039 / -0.013
Primary Fruit Abortion			
Total Selection	-0.261** / -0.136*	-0.169* / -0.170*	-0.092 / 0.035
Moth Selection	-0.127* / -0.125*	-0.013 / -0.013	-0.114 / -0.112
Unexplained Selection	-0.134* / -0.010	-0.156* / -0.157*	0.022 / 0.147

Control experiment: oviposition in female plants displaying only flowers

In the experiment testing for risk of egg infestation for plants displaying only flowers (and exposed to seed predators on single nights rather than continuously), *H. bicruris* moths laid eggs on almost all plants, with no significant difference between selection lines in the proportion of infested plants (32 out of 32 LF plants attacked, 29 out of 32 SF plants; Fisher's exact test: $F = 0.24$, $P = 0.24$). LF plants received an average of 1.14 ± 0.51 eggs per plant (201 eggs in total) and SF plants 1.18 ± 0.69 eggs per plant per night (195 eggs in total; n=5 nights). When accounting for variation in flower number, individual flowers on female LF plants were thus significantly more likely to receive an egg when exposed to moths (17% ± 17% of flowers infested) compared to flowers on SF plants (10% ± 12%; LLRT: $P(X^2_1 > 7.36) = 0.007$, effect size (SF) = -0.53). This model accounts for differences between selection lines in floral display, thus the above-reported infestation represents the per-flower risk of egg infestation when only flowers are displayed. The higher risk of per-flower infestation in LF plants was generally consistent across the five observation nights (Fig. 5). Out of the plants that received eggs, SF and LF females were equally likely to receive several eggs simultaneously (i.e. more than one flower infested) per night and per plant (LF: 22 observations with one egg per plant, 40 observations with more than one egg; SF: 22 observations with one egg per plant, 38 observations with more than one egg; Chi-squared test, $X^2_1 = 0.003$, $P=0.96$; Fig. 3B).

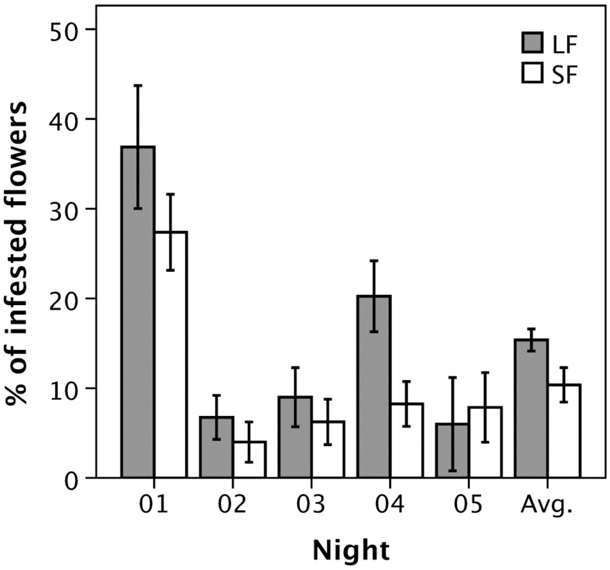

Figure 5. Per-flower risk of infestation by *Hadena bicruris* eggs on large-flowered (LF, grey bars) and small-flowered (SF, empty bars) *Silene latifolia* plants exposed to natural pollination and oviposition on single nights (five replicate nights over five consecutive weeks). All plants carried only flowers to avoid variation arising from the presence of healthy fruits or in attack rate. Bars give means ± s.e. (calculated on family means).

Discussion

In dioecious species, differences in reproductive success can select for different phenotypic optima in male and female plants, and lead to the evolution of sexually dimorphic traits (Geber, 1999). A typical dimorphism in temperate regions are larger flowers in male compared to female plants (Delph *et al.*, 1996). This is expected based on Bateman's principle that predicts stronger selection on the sex whose reproductive success depends more strongly on mating success (Bateman, 1948). *Silene latifolia* males presumably achieve a higher reproductive success by producing many small flowers (Delph *et al.*, 2004b). Because of positive between-sex genetic correlations for flower size (Meagher, 1992), females would be expected to produce many small flowers too. However this is not what is observed in nature. So what prevents females from making many, small flowers ? In this study we examined a yet unexplored hypothesis for the evolution of sexual dimorphism in this species, i.e. whether a specialist pollinator and seed-predatory insect that ensures pollination by visiting both plant sexes (A.M. Labouche and G. Bernasconi, unpublished results) but lays its eggs only in female plants (Brantjes, 1976b; Biere *et al.*, 2002; Wolfe, 2002; Jolivet & Bernasconi, 2006) may contribute to selection on female plants to produce fewer, larger flowers. Indeed, traits that increase attractiveness to pollinators to the benefit of pollen-donating male plants, may also directly, or through correlated traits, increase attractiveness to antagonists such as seed predators, to the detriment of female plant fitness (Shykoff & Bucheli, 1995; Biere & Antonovics, 1996; Biere & Honders, 1996).

To address the contribution of seed predation to selection on sexually dimorphic floral size and number, we used small-flowered and large-flowered selection lines and compared the female fitness in a common garden where plants were subjected to natural levels of seed predation by the specialist pollinator and seed predator, *Hadena bicruris*. Our results indicate a contribution of seed predation to selection on fruit abortion that differs between plants with many small flowers (where it had a significant impact) versus plants with few large flowers (where it was non-significant), and thus suggest that the seed-predator might have contributed to the evolution of large-flowered *S. latifolia* females from small-flowered females if ancestral plants reared many larvae and aborted many fruits.

Small-flowered plants (SF) produced smaller and more numerous flowers with fewer seeds per flower than large-flowered plants (LF), as expected from the flower size-flower number trade-off known in this species (Delph *et al.*, 2004b). While such trade-off is widespread among angiosperm families (Sargent *et al.*, 2007), it is not always found at the species level (reviewed in: Worley & Barrett, 2000; Ashman & Majetic, 2006). The two selection lines did not differ in total seed production. Seed production and thus fitness was influenced by floral traits, as shown by the total positive selection on flower number (composite flower trait). This agrees with the evidence that plants with large floral displays are more attractive to pollinators and that pollinators generally select for increased number of flowers (Benitez-Vieyra *et al.*, 2006; Hodgins & Barrett, 2008). In contrast with this, in other species pollinators exerted directional selection for larger flower size (Campbell *et al.*, 1991; Galen, 1996; Fenster *et al.*, 2006). Due to the negative genetic correlation between flower size and number in *S. latifolia* (Delph *et al.*, 2004b), selection for increased number of flowers that was found in our study also implies indirect selection for smaller flowers. Another study of *S. latifolia* found disruptive selection on corolla diameter in female plants, favouring females with very small or very large corollas over females with intermediate corolla sizes but this was not consistent across study years and populations (Wright & Meagher, 2004). Thus variation in the community of pollinators or seed predators among years or populations may lead to inconsistent selection and maintain the genetic variation in floral traits.

We expected SF plants to suffer from higher predation rate and that interaction with *H. bicruris* moths would favour smaller floral displays as a pre-oviposition « defense ». However the difference we observed between lines was more indirect and mediated by differential effects of selection against fruit abortion (i.e., by a post-oviposition response of the plant to infestation) presumably resulting from differences in fruit abortion costs. Selective source analysis was used in a hermaphroditic plant/seed predator system and a significant positive contribution of the seed predating moth to selection on flower number was detected when averaging several years of selection, but not within years (Nuismer & Ridenhour, 2008). The non-significant moth selection on flower number in our study may arise due to similar variation between years, or it may also be that the role of the seed predator in the selection process is weak. Alternatively, selection by the seed predator may be detectable only when plants suffer from a certain parasite load, and variance in parasite load may result from female moths ovipositing differentially among plants. Related to this, lack of detection may be due to overall increased levels of attractiveness in plants originating from the North American distribution range, where the seed predator is absent (Elzinga & Bernasconi, In press), because these plants produce more flowers than European plants (Blair & Wolfe, 2004).

Small– and large-flowered plants received similar numbers of *H. bicruris* eggs. This agrees with past research in other species showing no significant effect of the number of open flowers on the probability of oviposition by seed predators (Brody, 1992; Zimmerman & Brody, 1998; Arvanitis *et al.*, 2008). In contrast, flower size had a significant effect on oviposition; eggs were laid more often or in larger number in plants with large flowers than in plants with small flowers (Hemborg & Despres, 1999; Collin *et al.*, 2002; Despres *et al.*, 2007). In our study, this resulted in LF plants having a significantly larger proportion of their flowers oviposited on, but not in a higher proportion of fruits successfully parasitized (primary attacks). This may be due to a lower survival of eggs in the LF plants, although in a greenhouse experiment with the same lines we found that experimental egg infestation was more succesful in the LF plants (Burkhardt *et al.*, 2009a). However environmental conditions in a garden with natural pollination and seed predation may lead to different results.

Under continuous exposure to seed predators, SF plants reared significantly more larvae and suffered more simultaneous attacks (high parasite load) than LF plants. Several other studies found that seed predators are more likely to infest plants that display more flowers (Davis, 1981; Ehrlen, 1996; Brody & Mitchell, 1997; Fenner *et al.*, 2002; Leimu *et al.*, 2002; Vanhoenacker *et al.*, 2009). Importantly, although our lines did not differ in the proportion of predated fruits per plant, the number of larvae growing simultaneously on a plant (parasite load) influenced significantly both the frequency of secondary attack and fruit abortion.

Fruit abortion is a likely plant defense against the seed predator, which is known to occur also in other plant/ pollinating-seed predator systems, such as yuccas and yucca moths (Pellmyr & Huth, 1994; Richter & Weis, 1995) or senita cacti and senita moths (Holland *et al.*, 2004a). In *S. latifolia*, abortion is induced by *H. bicruris* infestation (Jolivet & Bernasconi, 2006; Elzinga & Bernasconi, In press) and is detrimental to larval growth and development (Burkhardt *et al.*, 2009a). In agreement with this, our study clearly shows that fruit abortion is a response to infestation by *H. bicruris* larvae, fruit abortion was not random but targeted against fruits infested with larvae. Even though SF and LF plants did not differ in the proportion of aborted fruits, SF plants tended to suffer a higher parasite load (simultaneous primary attack), and plants with high parasite load aborted their fruit significantly more often and suffered significantly more often secondary attack. Importantly, fruit abortion was costly to plants, as shown by the total negative selection on fruit abortion. The moth contribution to selection against fruit abortion was significant in the SF plants, but not in LF plants. This suggests that the cost of fruit abortion is larger in the SF than in the LF plants, and that these costs are evident already during the first flowering season of this short-lived perennial plant. This difference in costs may be due to the fact that SF plants lost twice as many fruits due to parasite attack (both primary and secondary) and to fruit abortion, possibly leading to stronger compensation costs. Models predict that fruit abortion can reduce the population of the seed predator and increase the plant fruit production (Westerbergh & Westerbergh, 2001; Holland *et al.*, 2004b). However, if seed predators are very mobile among plants, fruit abortion may not be effective at reducing the insect population (Holland & DeAngelis, 2002); thus if fruit abortion is an ineffective defense, then it merely represents an added fitness cost. *Hadena bicruris* is capable of long-range dispersal (Elzinga *et al.*, 2007b) possibly because it is attracted by *S. latifolia* scents (Dotterl *et al.*, 2006). Also, in *S. latifolia* plants produce fewer flowers after they start carrying fruits, suggesting that fruit production is costly (Delph & Meagher, 1995). Finally, if fitness depends on seed output, the per-unit costs of fruit production may be higher for small fruits, each of them requiring expensive tissues (e.g. fruit wall; Blair & Wolfe, 2004; Jolivet & Bernasconi, 2006), and the significant higher presence of phloem-feeding aphids in SF plants might increase these costs. Therefore the energetic cost inflicted to plants aborting a large number of fruits such as the SF plants in our study may be larger than that inflicted to the LF plants. It would be interesting to explore whether this discrepancy in costs affects winter survival or increases in the following flowering seasons.

In conclusion we show that female plants of *S. latifolia* selected to produce many small flowers do not differ from plants with few large flowers in the probability of being attacked by larvae of *H. bicruris*, in the proportion of attacked or aborted fruits, and in seed production during one reproductive season. The contribution to selection on flower number and size through pollinator attractiveness may explain why both type of plants are selected to increase flower number, while the overall contribution of seed predator avoidance to selection on floral display may be weak, not detectable in the populations we studied, or variable among years. Although fruit abortion was targeted specifically against predated fruits, total selection favored plants that aborted fewer fruits, suggesting that fruit abortion is costly and particularly costly when *H. bicruris* is present. Interestingly, costs of abortion appeared to be higher in plants selected to produce many, small flowers (as in male plants), rather than in plants selected to produce few, larger fruits (as in standard female plants), as indicated by the fact that moth contribution to selection against abortion was significant in small- but not in large-flowered plants.

Moderate fruit abortion may still benefit the plant if it reduces the seed-predator population in the following season (not explored here). Therfore, longer-term studies are needed to quantify exactly the costs of parasite load and fruit abortion on plant fitness, and to address specifically whether these costs are correlated to plant traits that are under sexually antagonistic selection. If such studies confirmed the results presented here, then the costs inflicted by *H. bicruris* on *S. latifolia* females may have selected them to maximize seed production while minimizing the costs due to fruit predation and abortion by producing fewer larger flowers, while fertility or sexual selection may have selected males to maximize pollen export by producing many small flowers, leading to the sexual dimorphism that is observed in nature.

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CHAPTER 4 : Effects of pollination timing on seed paternity and seed mass in *Silene latifolia* (Caryophyllaceae)

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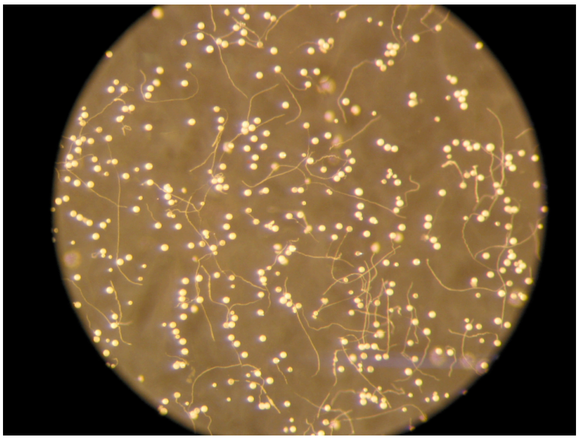

Germinating pollen tubes

Developing fruit

Abstract

Competition among genetically different pollen donors within one recipient flower may play an important role in plant populations, increasing offspring genetic diversity and vigour. However, under field conditions stochastic pollen arrival times may result in disproportionate fertilization success of the first-arriving pollen, even to the detriment of the recipient plant's and offspring fitness. It is therefore critical to evaluate the relative importance of arrival times of pollen from different donors in determining siring success. We used hand pollinations and genetic markers to experimentally investigate the effect of pollination timing on seed paternity, seed mass, and stigmatic wilting in the the dioecious plant *Silene latifolia*. In this species, high prevalence of multiply-sired fruits in natural populations suggests that competition among different donors may often take place (at fertilization or during seed development), however the role of variation due to pollen arrival times is not known. First-arriving pollen sired significantly more seeds than later-arriving pollen. This advantage was expressed already before the first pollen tubes could reach the ovary. Simultaneously with pollen tube growth, the stigmatic papillae wilted visibly. Individual seeds were heavier in fruits where one donor sired most seeds than in fruits where both donors had more even paternity shares. In field populations of *S. latifolia*, fruits are often multiply-sired. Because later-arriving pollen had decreased chances of fertilizing the ovules, this implies that open pollinated flowers often benefit from pollen carry-over or pollinator visits within short time intervals, which may contribute to increase offspring genetic diversity and fitness.

Key words: reproduction; reproductive success; pollen; siring success; microsatellite DNA; paternity; pollen tube growth; seed mass; *Silene alba*; stigma wilting.

Introduction

Variation in pollen donor reproductive success could be a selective force in plants, especially for species in which multiple paternity within fruits frequently occurs (Marshall & Ellstrand, 1985; Campbell, 1998; Bernasconi, 2003). Indeed, how many donors « compete » - i.e. have an effective chance of being in the pool of pollen that can contribute to fertilization - and the relative shares of paternity will determine reproductive success via the male function. In addition, this will also determine the degree of genetic heterogeneity among seeds within the same fruit, and the opportunity for pre- or post-zygotic selection (e.g. Teixeira *et al.*, 2009). However, the degree to which variation in pollen donor reproductive success is important in natural populations remains controversial, because both predictable, heritable factors and stochastic factors may contribute to it (Mulcahy, 1979). Predictable determinants of fertilization success of competing donors may include attractiveness to pollinators (e.g.; Mitchell & Waser, 1992; Kudo & Harder, 2005), pollen competitive ability (e.g. pollen germination, longevity and tube growth rate; Snow & Spira, 1991; Stephenson *et al.*, 1992; Walsh & Charlesworth, 1992; Snow & Spira, 1996; Arthur *et al.*, 2003; Jolivet & Bernasconi, 2007a; Teixeira & Bernasconi, 2008), or traits affecting the outcome of post-pollination selection, either among pollen tubes before fertilization (Marshall, 1988; Cruzan & Barrett, 1993; Snow, 1994; Stanton, 1994; Skogsmyr & Lankinen, 2000) or among embryos after fertilization (Marshall & Ellstrand, 1988). In the field, however, pollen vectors introduce a stochastic component, unpredictably determining the timing, quantity and genetic diversity of pollen deposited on the stigma (Spira *et al.*, 1992; Skogsmyr & Lankinen, 1999; Mitchell *et al.*, 2005; Karron *et al.*, 2006).

To understand the determinants of variation in male reproductive success in animal-pollinated plant, it is therefore important to evaluate the relative magnitude of stochastic vs. predictable effects (e.g. Skogsmyr & Lankinen, 1999). To disentangle the relative roles of stochastic vs. predictable effects in explaining field paternity, we investigated the influence of the timing between deposition of pollen from competing donors on paternity shares in the dioecious plant *Silene latifolia.* In this species within-fruit multiple paternity is frequent in natural populations (Teixeira & Bernasconi, 2007). On the other hand, it is known that pollen deposition induces rapid wilting of female flowers in several species (e.g.; Lankinen *et al.*, 2006; Abdala-Roberts *et al.*, 2007; Castro *et al.*, 2008) including *S. latifolia* (Young & Gravitz, 2002). It is therefore relevant to explore which pollen arrival times (compared to the timing of post-pollination wilting and pollen tube growth) are consistent with the observed levels of multiple paternity.

We experimentally applied the pollen of two males, either immediately after each other, or at increasing time intervals on female plants of the same population. We then analysed paternity with genetic markers, monitored pollen tube growth rates and stigmatic wilting, and examined seed mass to address the following questions: (i) Does the first-arriving pollen sire more seeds than the later-arriving pollen? (ii) For which time interval does first-male advantage become significant? (iii) How does this time delay compare to the time needed by the pollen tube to reach the ovary, and to the timing of stigmatic wilting? Additionally, to explore effects on offspring provisioning (Bañuelos & Obeso, 2003), we also asked: (iv) Is individual seed mass correlated with unequal paternity shares within fruits? We expected that for longer time intervals between pollinations, the proportion of seeds sired by the first male would increase. Also, if competition among seeds from different fathers affects seed provisioning we expected a correlation between differences in paternity shares and individual seed mass. Depending on the mechanism, this correlation could be either positive (“complementarity”; Bernasconi *et al.*, 2003) or negative (sibling rivalry; Bañuelos & Obeso, 2003). Finally, we asked whether the observed effects of pollen arrival time on paternity can shed light on the mechanisms leading to frequent multiple paternity within open-pollinated fruits and for the maintenance of genetic variability for pollen competitive ability in this species.

Material and methods

Study species, field collection and rearing

The white campion, *Silene latifolia* Poir., is a dioecious, short-lived perennial plant native to Europe and parts of Asia (Prentice, 1979) and introduced in North America (Baker, 1948). In Europe, it flowers from May to October and is pollinated mainly by nocturnal moths (Jurgens *et al.*, 1996). Males and females are dimorphic for several traits: male plants produce more numerous, smaller, and shorter-lived flowers than female plants (Primack, 1985; Carroll & Delph, 1996; Young & Gravitz, 2002; Burkhardt *et al.*, 2009a), and start flowering slightly later (Jolivet & Bernasconi, 2007b). Female flowers usually have five stigmatic lobes (Teixeira *et al.*, 2008), covered with papillae receptive to pollen germination (Lassere *et al.*, 1996). Each flower produces a fruit with around 200 seeds (Young, 2002; Jolivet & Bernasconi, 2007a).

In 2003, we collected one fruit of each of 50 plants (inter-plant distance ≥2 m) in a natural *S. latifolia* population in Sesto Calende, Northern Italy (45°44'08" N/8°37'00" E). We germinated 20 seeds/fruit in Petri dishes (ø90 mm) lined with cotton and filter paper, moistened with 1mM gibberellic acid solution (16h day/8h night, 21°C, 70% RH). We planted the seedlings in pots (ø10 cm) with 420 ml of 1:2 mixture of sand and soil (Tref-De Baat BF4, GVZ-Bolltec AG, Zurich, Switzerland), and grew them in a growth chamber under the same conditions.

Hand pollination with pollen from two males at varying time intervals

To investigate how the timing of sequential pollen deposition affects paternity by competing males and individual seed mass, we hand-pollinated one flower on each of n=30 females with pollen from two males (n=60 males; i.e. 30 two-donor *crosses*). All plants were derived from 14 field-collected seed families, whereby we avoided crossing plants from the same fruit. To experimentally manipulate *time between pollinations*, the pollen from the second male was applied at different time intervals after pollination with pollen from the first male, i.e. after 0, 2, 4, 8 or 24 hours. For each of the five time intervals, we pollinated six different females (6 crosses per time interval (replicates) x 5 intervals (treatments) = 30 crosses). We brushed one anther of each male uniformly over the entire stigmatic surface (i.e. all five lobes). We preliminarily tested whether one anther (pollen load of the first male) resulted in full seed set (see below). All female flowers were of the same age (36h old). After the second pollination we bagged each flower. We collected the seeds when ripe and weighed each seed individually to the nearest µg (*individual seed mass*, Mettler Toledo MT5) for a subsample of 20 seeds per cross (n=30 fruits from 30 two-donor crosses). We recorded *germination success* of these seeds on an agar substrate (16h day/ 8h night, 21°C, 70% RH). Finally we transplanted the seedlings in 2.5 x 16 cm tubular pots (Cone-tainers™, Stuewe and Sons, Corvallis, Oregon, USA) with 1:2 sand:soil mixture (Tref-De Baat BF4, GVZ-Bolltec AG, Zurich, Switzerland) for subsequent paternity analysis.

Preliminary experiment: pollen dose effects on seed set

To address pollen dose effects on seed set and ensure that the pollen load of the first donor was not merely preempting stigmatic surface, we examined seed set following pollination of 15 females with three unrelated males with variable pollen dose (1, 2, 3, 4, and 5 anthers). Five females were randomly assigned to each male and to a different pollen dose treatment, and one flower per female was hand-pollinated with the relevant number of anthers. We found a marginally significant effect of pollen dose on seed set (ANOVA, $F_{4,14}$= 2.2, p=0.1). However the mean seed set indicates that after applying pollen from the first pollen donor (one anther), the female has not yet received enough pollen for full seed set (mean ± s.e. seed set following pollination with: one anther: 57 ± 25; two anthers: 142 ± 29; three anthers: 185 ± 52; four anthers: 186 ± 40; five anthers: 181 ± 30).

Paternity analysis

We genotyped parents and offspring using four microsatellite DNA marker loci (Sl6, Sl8, Sl14, and Sl15; Teixeira & Bernasconi, 2007). We extracted DNA from parents from dried leaves using Macherey-Nagel Nucleospin® Plant Kit (Düren, Germany) and from 20 offspring/cross from freeze-dried leaves, using the CTAB method (Doyle & Doyle, 1988). The final sample of offspring genotyped was n=438 (i.e. 14.73 ± 0.45 offspring per fruit, *n* = 30 fruits) due to failures at germination, DNA extraction or amplification. We followed the amplification conditions described in Teixeira & Bernasconi (2007). We scored the amplified fragments using GeneMapper® v3.7 (Applied Biosystems, Foster City, California, USA). *Paternity* was assigned to the first or the second male by comparing the microsatellite patterns from each offspring with those of its mother, and of both putative fathers.

Pollen tube growth and post-pollination changes in stigmatic papillae

Since paternity shares may depend on pollen tube growth speed, we estimated how fast pollen tubes grow through the pistil after pollen deposition, we hand-pollinated 16 flowers, four on each of four different plants using pollen from an unrelated donor. At 2, 4, 8 or 24 hours after pollination, we fixed three stigma-style lobes per flower in formalin acetic acid (FAA) following the protocol described in Bernasconi *et al.* (2007). After 24h, we placed them in 70% ethanol, then softened them in NaOH (4N, 1.5-2h), and stained them during 2h using 1%-aniline blue solution in phosphate buffer (pH=7.8; after Martin, 1959). We placed each stigma lobe on a microscope slide with two drops of 1% aniline blue solution and we measured the *length of the longest pollen tube* for each lobe and the *length of the stigma lobe* to the nearest 0.5 mm in an epi-fluorescence microscope (Axioskop 2 Mot Zeiss; HBO 50W burner; excitation filter BP362/150, dichroic mirror FT395, barrier filter LP397; Plan Neofluar/Fluar objective; 10 x 10 magnification). Since the ovary is at the lower end of the stigma lobes, we could thus determine how close to the ovary the pollen tubes had arrived at 2,4, 8 and 24h after pollination. This estimates the minimum time needed for pollen tubes to reach the site of fertilization (ovary) under our experimental conditions.

To monitor changes in the stigmatic papillae after pollen deposition and during pollen tube growth qualitatively, we examined in the scanning electron microscope (SEM) the stigma lobes of another set of flowers for the same time intervals. To this end, we hand-pollinated four female flowers (36h old) on four different plants by brushing the pollen of all anthers of one male per female plant uniformly over all stigma lobes. Each of the stigma lobes was allocated to a different treatment and cut after 0, 2, 4, 8 or 24h since pollination. We fixed the stigma lobes in FAA for two days, stored them in 70% acetone for 3-4 days, transferred them to 100% acetone, desiccated them and covered them with gold following standard procedures for SEM examination.

Statistical analysis

We analyzed the effect of *time between pollinations* on the *number of seeds sired by the first male* (i.e. seed paternity; n = 6 replicates, i.e independent female plants, per time interval x 5 intervals = 30 crosses, involving 30 independent female plants and 60 independent male plants) in a linear model with the number of seeds genotyped per cross as a covariable. Since the residuals violated the assumptions of normality and homoscedasticity, and also showed overdispersion when using binomial errors, we tested for significance in the linear model with permutation tests on the mean squares (Manly, 1997). We estimated the effects of time between pollinations and of the number of seeds sired by the first male by permutating the levels of these factors separately. We calculated *P*-values for each factor as the proportion of permutated mean-square estimates (out of 1000) that were larger than or equal to the observed mean-square (Manly, 1997). Further, we conducted *post-hoc* tests to compare among each other the different time intervals. Finally, for each time interval between pollinations, we tested whether the proportion of seeds sired by the first male differed from equal paternity (i.e. expected mean of 0.5; separate Wilcoxon tests for each time interval).

To analyze the effect of *time between pollinations* on *individual seed mass* we ran Generalized Linear Mixed Models (GLMMs) with *time between pollinations* as fixed factor, and *cross* as a random factor (n= 30 crosses) to account for non-independence among seeds arising from the same cross. In a second step we restricted the analysis to the subset of crosses where *both* males sired at least one of offspring (n= 15 crosses; i.e. we excluded the crosses in which all genotyped seeds stemmed from the same father) to better investigate whether genetic variation within seed families was associated with variation in individual seed mass. We performed GLMMs with *individual seed mass* as response variable, *cross* as random factor and K_e (the effective number of donors, calculated as $K_e = 1 / \sum p_i^2$, where p_i = proportion of seeds in a fruit sired by the *i*-th male; Bernasconi, 2003) as an explanatory variable. K_e is a useful measure of the evenness in the proportion of offspring sired by several pollen donors, and a single value of K_e is calculated for each cross. In a two-donor cross, a value of K_e close to one means that the majority of the offspring were sired by one male; a value of K_e close or equal to two means that each male sired approximately half of the offspring, i.e. more even paternity.

To quantify pollen tube growth over time, we calculated the *distance to the ovary* for each pollen tube as the length of the stigma lobe minus the length of the longest pollen tube. We determined the earliest time interval when the distance to the ovary was not significantly different from zero, and consider this to be a minimum time for fertilization to occur. For each time interval since pollination (except for 24h when all pollen tubes had reached the base of the stigma lobe) we tested whether the distance to the ovary differed from 0 using one-sample t-tests.

Data were analysed with the R 2.6.2 or SPSS 13. Unless specified, data are given as mean ± s.d.

Results

Seed paternity

The proportion of crosses with double paternity (i.e. with offspring sired by both males) decreased significantly with increasing time between pollinations (0h: 6/6, 2h: 5/6, 4h: 1/6, 8h: 2/6, 24h: 1/6; Fisher's exact test: $P = 0.004$). Accordingly, time between pollinations significantly affected seed paternity (linear model with permutation tests, df = 1, MS = 50.45, $P = 0.046$, Fig. 1): the longer the time between pollinations, the higher the proportion of offspring sired by the first male, from 0.61 ± 0.15 when pollen of the second male was applied immediately after pollen from the first male (0h delay), to 0.94 ± 0.06 with a 24h delay between pollinations (n = 6 replicates per time interval). *Post-hoc* tests comparing pairwise each time interval revealed that seed paternity after 8h (intervals of 8h and 24h) was significantly more biased towards the first male compared to 0h delay between pollen depositions (0h, Fig.1). Seed paternity was not significantly different from equal paternity when the pollen from the second male was applied immediately after the pollen from the first male (0h interval, Wilcoxon test, V = 12.5, $P = 0.75$), but significantly greater than 50% in all other crosses in which the pollen from the second male was applied with a delay (Wilcoxon tests, all $V \geq 20$, all $P < 0.05$, n = 6 crosses per time interval, Fig.1).

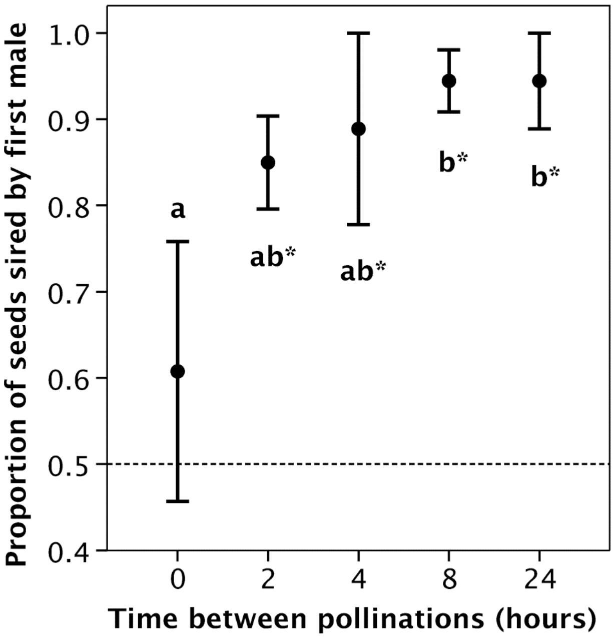

Figure 1. Paternity in two-donor crosses (mean ± s.e. proportion of offspring sired by the first male) as a function of time between pollinations in *Silene latifolia*. Six female plants were hand-pollinated for each time interval. Asterisks (*) denote significant ($P < 0.05$) deviations from equal paternity in Wilcoxon signed rank test (dashed line= equal paternity). Different letters (*a* vs. *b*) denote time intervals that differed significantly ($P < 0.05$) in pairwise *post-hoc* tests (see *Methods*).

Pollen tube growth and post-pollination changes in stigmatic papillae

Pollen tubes reached the style/ovary junction 8 hours after applying pollen to the stigma, as indicated by the fact that for this time the distance between the tip of the longest pollen tube and the style-to-ovary junction was no longer significantly different from zero (0.5 ± 1.1 mm, one-sample t-test, $t = 1.0$, df = 3, $P = 0.39$). From this we infer that fertilization had to occur later than 8 hours after pollen deposition under our experimental conditions. By contrast, for shorter times after pollen deposition the mean distance to the style-to-ovary junction was significantly greater than zero (2 hours after pollen deposition: 9.9 ± 1 mm, $t = 19.2$ df = 3, $P < 0.001$; 4 hours: 3.9 ± 1.5 mm, $t = 5.2$ df = 3, $P = 0.01$). In parallel, we observed changes in turgidity of the stigmatic papillae (Fig. 2).

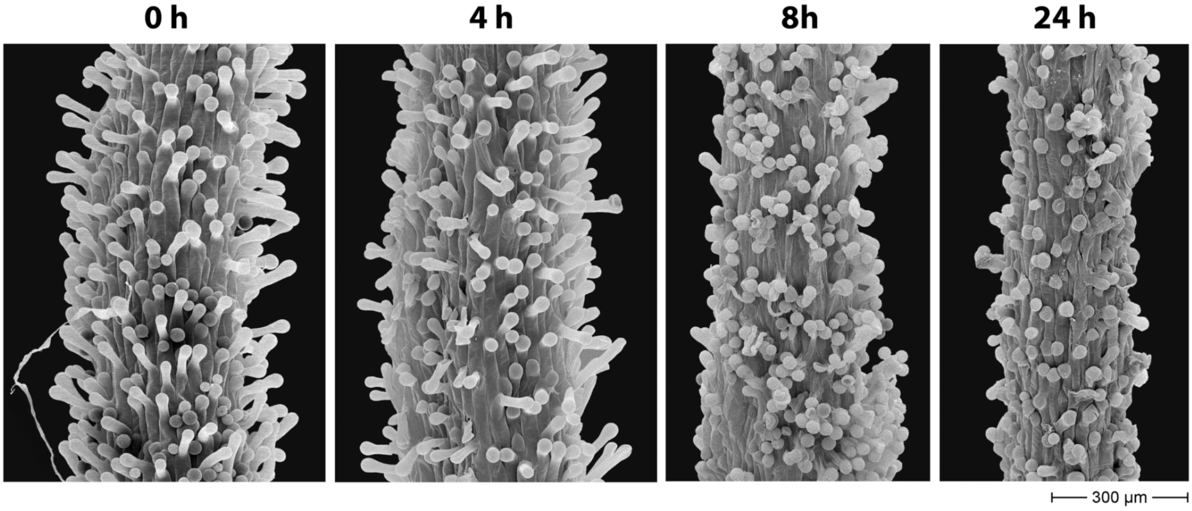

Figure 2. Scanning electron micrographs showing post-pollination loss of turgidity of the stigmatic papillae in *Silene latifolia* flowers at different time intervals since pollen deposition.

Individual seed mass

Time between pollinations did not affect significantly the mass of individual seeds (GLMM, $F_{4,25} = 0.57$, $P = 0.69$; n = 30 crosses; mass (µg), 0h: 624 ± 84, 2h: 625 ± 129, 4h: 528 ± 228, 8h: 602 ± 96, 24h: 574 ± 151) nor the total number of seeds (ANOVA, $F_{4,25} = 0.49$, $P = 0.74$; number of seeds/fruit : 0h: 297 ± 91, 2h: 278 ± 72, 4h: 235 ± 105, 8h: 285 ± 88, 24h: 245 ± 108), nor the total seed mass (ANOVA, $F_{4,25} = 0.94$, $P = 0.46$; total seed mass/fruit (mg): 0h: 185 ± 58, 2h: 177 ± 73, 4h: 123 ± 58, 8h: 177 ± 64, 24h: 139 ± 86). Interestingly, in the 15 crosses with offspring from both males (i.e. fruits with half sibs), we found that individual seed mass increased significantly as the proportions of offspring sired by the two competing males became more unequal (i.e. for decreasing K_e, GLMM, $F_{1,13} = 24.2$, $P < 0.001$; Fig. 3).

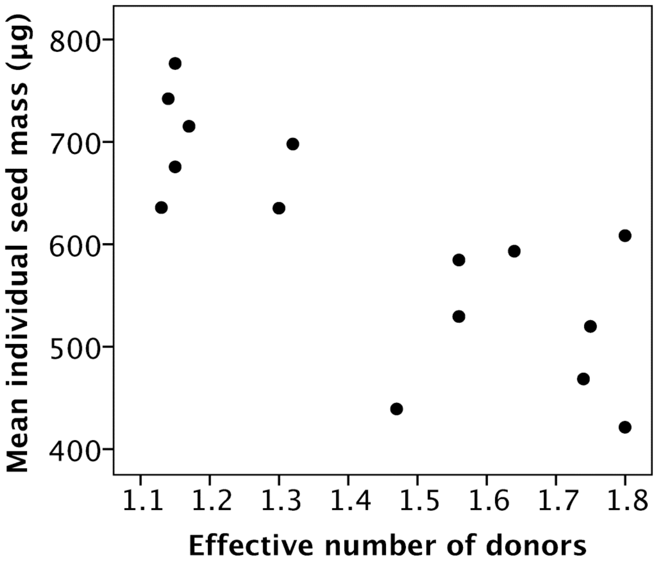

Figure 3. Mean individual seed mass (µg) as a function of the effective number of donors (K_e) in crosses with double paternity (estimated regression line: individual seed mass= 1086 (±100) µg – 334 (± 68) * K_e). Each dot is the mean seed mass of one cross (n=15 crosses). If K_e is close to 1, most offspring have been sired by one male, when K_e is close to 2, both males each sired approximately half of the offspring.

Discussion

In animal-pollinated plants, male reproductive success and the occurrence and outcome of competition among pollen donors are likely to depend on several factors, including inflorescence, floral and pollen traits. However to what extent competition among pollen donors will impose selection on plant traits depends on the relative role of unpredictable determinants of variation in male reproductive success, among which an important factor is the timing of pollinator visits.

Our study shows that in *Silene latifolia* the pollen arrival times significantly affect siring success of competing pollen donors. Following our expectations, later arriving pollen sired a decreasing proportion of seeds with increasing time. The paternity success of the second male was in fact significantly lower than 50% after a 2h delay, significantly lower for

8h delay compared to 0h delay, and close to zero 24h after applying pollen from the first male. Studies in several other species showed similar effects of pollen arrival times on fertilization success of pollen from competing donors (Marshall & Ellstrand, 1985; Mitchell & Marshall, 1995; Spira *et al.*, 1996; Snow *et al.*, 2000). For example, in *Hibiscus moscheutos* pollen from later visits could compete with pollen from earlier visits, but the success of late-arriving pollen declined steeply after arrival of the first pollen (Spira *et al.*, 1996). In *Raphanus sativus* (Marshall & Ellstrand, 1985), a delay of 15 minutes between pollinations already resulted in increased paternity of the first male. In *S. latifolia* multiple paternity within fruits is frequent in natural populations (> 80 flowering individuals, 60-100% of individuals having more than one father; Teixeira & Bernasconi, 2007). Therefore, field data (Teixeira & Bernasconi, 2007) in combination with the results of hand pollination in the present study indicate that there is only a short time window after deposition of the first pollen for the later-arriving pollen to participate in fertilization. This suggests that multiple paternity within fruits is likely to arise from pollen loads which contain pollen from several males (pollen carry-over; e.g. Morris *et al.*, 1994; Campbell, 1998) or from pollinator visits that occur within a short time interval. Pollen carry-over is consistent with observations of the behaviour of *Hadena bicruris*, the main pollinator of *S. latifolia* in Europe (A.-M. Labouche and G. Bernasconi, University of Neuchâtel, Switzerland, unpublished results). Thus, pollen arrival times under natural pollination apparently do not entirely impair competition among pollen donors and its potential benefits for female and offspring fitness (Teixeira *et al.*, 2009). Consistent with this, there is variation among males in siring success in *S. latifolia* (Wright & Meagher, 2004).

The extent to which the timing of pollen deposition results in one father siring most of the seeds can be influenced by several pre-fertilization mechanisms, such as depletion of resources within the style, rapid growth and head-start by the first arriving pollen tubes

(Dickinson, 1965; Tadege & Kuhlemeier, 1997), stigma clogging by pollen of the first male (Cowan *et al.*, 2000), or post-pollination wilting of the stigma (Lankinen *et al.*, 2006; Lankinen & Kiboi, 2007). Our experiment was not designed to discriminate among these hypotheses, and future work is needed to investigate the involved mechanism. However, stigma clogging seems unlikely given that in our preliminary experiment we found that the pollen load used for the first donor was not saturating. Arrival of the first pollen modifies the residual longevity of the female flowers of *S. latifolia* (Young & Gravitz, 2002), and we observed changes in the turgidity of stigmatic papillae following pollination (Fig. 2). However, we do not know whether germination of later arriving pollen was compromised by the changes of the stigma/style tissue following pollen germination from the previous male, as has been suggested (Lankinen *et al.*, 2006). It would be interesting in future work to investigate whether female traits such as responses to post-pollination wilting can explain variation in paternity, as suggested by similar paternity responses of competing donors across related females (Teixeira *et al.*, 2008).

Paternity shares within fruits are not only important for male fitness but may also influence offspring genetic diversity, number and quality (Mazer *et al.*, 1986; Quesada *et al.*, 1996; Bernasconi *et al.*, 2003; Bernasconi *et al.*, 2004; Aizen & Harder, 2007) and the intensity of sib competition (Bañuelos & Obeso, 2003). However, the correlation between paternity shares and individual seed mass could be either positive or negative depending on the mechanism (“complementarity” (Bernasconi *et al.*, 2003) or sibling rivalry (Bañuelos & Obeso, 2003), respectively). Consistent with an effect of genetic diversity on competition among developing seeds, we found that the more unequal the paternity shares (i.e. in fruits consisting mostly of full sibs), the higher the individual seed mass. Seed mass can correlate to several traits expressed later in life. For instance, in some species heavier seeds have increased emergence probability (Gross, 1984; Lehtila & Ehrlen, 2005), or are more likely to establish in shaded or crowded habitats (Venable & Brown, 1988; Westoby *et al.*, 1992). However further study is needed to determine the causes and consequences of variation in seed mass in response to average within-fruit relatedness in this species.

In conclusion, we found that the first-arriving pollen sired more seeds than later-arriving pollen in *S. latifolia*. This advantage was expressed already for intervals between arrival of pollen from the first and second donors of 2 hours, i.e. intervals that were shorter than the time needed for pollen tubes to reach the ovary. This suggests that there is a narrow window of opportunity for pollen to initiate development once pollination occurs in this species. In the field, multiple paternity within fruits is frequent. Taken together, experimental and field results suggest that open pollinated flowers must often benefit from pollen carry-over or pollinator visits within short time intervals. Thus, « stochastic » effects such as pollen arrival times are not entirely preventing multiple within-fruit paternity or competition among genetically diverse donors over fertilization of the same set of ovules.

Acknowledgments

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CHAPTER 5 : Genetic variation among females affects paternity in a dioecious plant

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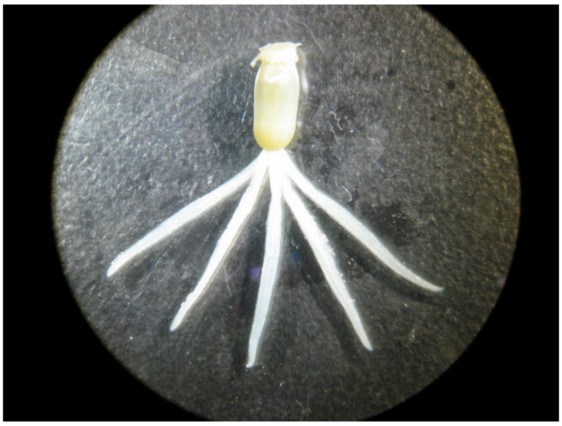

Ovary and five stigma lobes
of a dissected female flower

Male flowers

Abstract

Flowering plants rely on vectors for pollen transfer, and cannot choose their mates. Although recipient plants are unable to choose which pollen they receive, post-pollination selection (acting pre- or post-zygotically) may modify the outcome of pollination. Here we show that genetic variation among pollen recipients can predict the outcome of pollen competition (seed paternity) in the dioecious white campion. To investigate whether genetic variation among pollen recipients affects paternity, we applied the same pollen mixture from two males to three females, two of which full sisters and the third one chosen at random (unrelated). To control for maternal environmental effects, the plants used for these crosses were greenhouse-reared F1. We replicated this in two populations, for a total of 51 crosses, and genotyped a total of 772 offspring to assign paternity. If genetic variation affects paternity, we expected greater similarity of paternity success of the focal male with the sisters, compared to the unrelated female. Paternity of the focal male was significantly more repeatable over sisters, compared to repeatability over the mean of sisters and the unrelated females. When populations were analyzed separately, this was significant in one of the two populations. Paternity was not significantly correlated with stigma size. This provides evidence that in at least one population, genetic variation among individual plants influences the donors' paternity success, as assessed through genetic analysis of the seedling. Since due to gravity-dispersed seeds natural patches may often consist of related plants, the observed effect may contribute to variation in male reproductive success.

Key words: pollination; pollen competition; paternity; male reproductive success

Introduction

Flowering plants rely on vectors to transfer pollen and cannot choose with whom they mate. Nevertheless, seed paternity (i.e. the proportion of offspring sired by competing pollen donors) may be non-random. Pollen/pistil interactions and gene expression in pollen (Becker *et al.*, 2003) may facilitate pre-zygotic selection. This may increase female and offspring fitness if post-pollination selection can favour competitively superior, genetically more diverse or less closely related pollen (Aizen *et al.*, 1990; Arthur *et al.*, 2003; Bernasconi *et al.*, 2004). In addition, also several post-zygotic mechanisms (seed abortion, early-acting inbreeding depression due to the sharing of deleterious recessive alleles between mates, competition among developing seeds sired by different fathers) may contribute to variation in siring success of different males and influence number and quality of the resulting offspring.

In plant populations, seeds within one fruit often result from multiple-donor pollination (Ellstrand, 1984; Meagher, 1986; Campbell, 1998; Teixeira & Bernasconi, 2007). When different pollen donors compete, fertilization may favour given donors over others, through variation in pollen traits, or female influence (Marshall & Diggle, 2001). Pollen competition may shape traits influencing the probability of receiving and donating pollen, and traits controlling fertilization. Selection on traits affecting siring success may be antagonistic in donors and recipients (Lankinen *et al.*, 2006). It is therefore of interest to know whether genetic variation among pollen donors and among pollen recipients influences paternity shares (i.e., the proportion of the offspring sired by each of the competing pollen donors) in natural populations of plants. Pollen competition experiments found significant variation among donors in siring success (Pasonen *et al.*, 1999; Steiner & Gregorius, 1999; Skogsmyr & Lankinen, 2000). However, there is considerable residual variance in paternity, which may be due, among others, to variation among recipient plants affecting siring success (Marshall & Ellstrand, 1986).

Here, we ask whether recipient genotype affects success of competing pollen donors. We compare siring success of a focal male and a competitor across females that are related vs. unrelated to each other, and infer paternity with molecular markers. If there is a genetic influence of the recipient plant on the relative success of competing donors, paternity shares will be more similar amongst closely–related females than amongst distantly–related ones. To control for maternal environmental effects, we crossed greenhouse-reared F1 plants.

Moreover, we measured floral traits (stigmatic surface, ovary width and length, number of stigmatic lobes) in a control flower for each female to address whether variation in stigma-style morphology explains some of the variation in paternity, since stigma-style length may magnify differences among donors in pollen competitive ability and thus in siring success. This is suggested by several experimental pollination studies in species with elongated stigmatic surfaces (e.g. *Silene latifolia*: (Purrington, 1993); *Dianthus chinensis*: (McKenna & Mulcahy, 1983)), where pollen was applied either at the tip or at the base of the stigma-style. Placing pollen at the tip can intensify pollen competition by magnifying differences in pollen tube growth rates (see Delph & Havens, 1998 for a review also of analogous studies of distylous species).

Material and methods

Study species, field collection and plant rearing

The white campion, Silene latifolia (Miller) Krause, is a diploid, short-lived perennial, dioecious species (Baker, 1947). Dioecy and frequent multiple paternity in the field (Teixeira & Bernasconi, 2007) make it a suitable model to investigate female influence on paternity.

In 2003, we collected one fruit (=seed family) from 15 different plants in each of two populations (Cottendart: 46°58'30"N; 6°50'50"E; Millingerwaard: 51°52'45"N; 6°00'55"E). We germinated 600 seeds (20 seeds/15 fruits/2 populations) and after 15 days, repotted the seedlings and placed them randomly within the greenhouse as described in (Jolivet & Bernasconi, 2007a; Jolivet & Bernasconi, 2007b)To limit maternal environmental effects and thus better highlight genetic variation, we used F1 plants for our experiment, obtained by crossing one female from each field-collected seed family (F0) with a randomly assigned male from the same population but a different seed family (Fig. 1, *N*=15 F1 families per population).

Experimental crosses

We reared 20 seeds in each of the F1 seed families. At flowering, we randomly chose males and females, repotted them (ø 18 cm pots), and assigned them to experimental crosses: for each focal male, we randomly attributed a competitor (from the same population but another family), and crossed simultaneously these two males with three different females. The three females in each replicate included two sisters (from the same maternal family, i.e., full-sisters) and one randomly–chosen (henceforth unrelated) female. Sisters stem from the same fruit. Within replicates, females all stemmed from different fruits than the males. All plants in a replicate stemmed from the same population (Fig. 1).

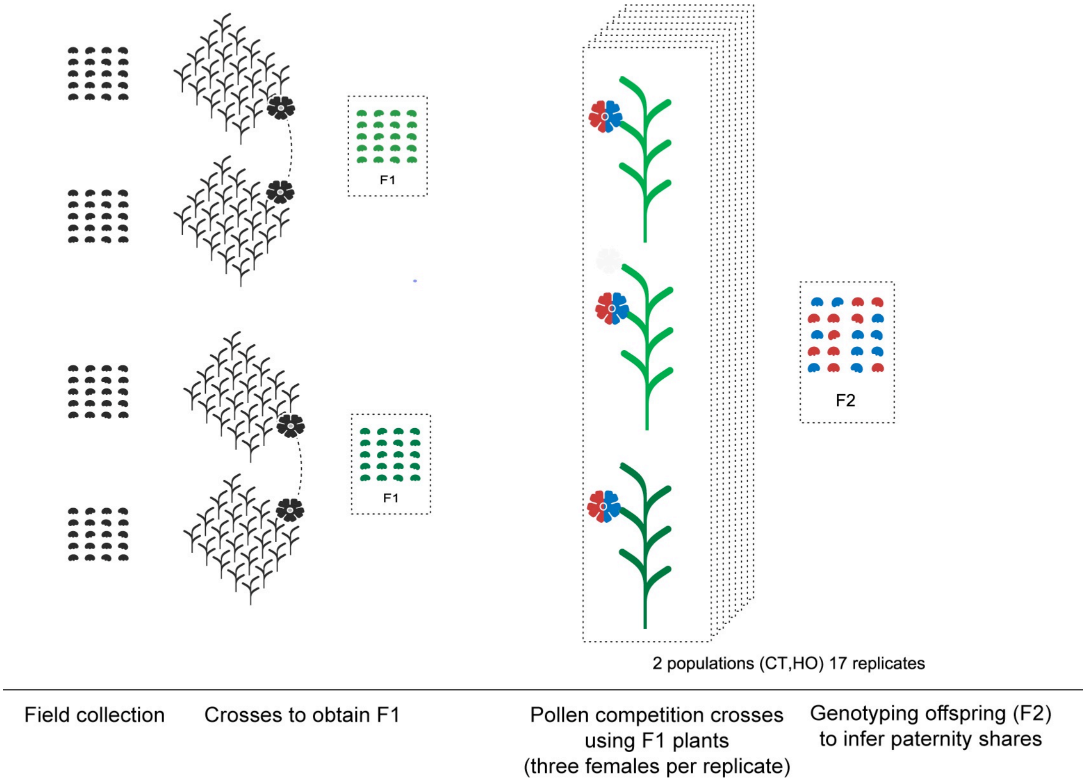

Figure 1. Design to test female genotype effects on pollen competition by estimating the intra-class correlation coefficient among related females (sisters) compared to an unrelated female in *Silene latifolia*. In each replicate (here one replicate is shown), two related females (sisters, *light green*) and one randomly chosen female (*dark green*) were pollinated with a mixture of pollen from two donors (*red*: focal male; *blue*: competitor). Paternity was assessed with genetic markers. To control for environmental variation, crosses were performed on greenhouse-reared *F1* plants.

To isolate female genotype effects, our design relies on the insight that if a trait is heritable, relatives (comparison between sisters) should resemble each other more than two randomly–selected individuals (comparison with the unrelated female), provided that competing males are the same within a replicate. We therefore address whether there is a significant correlation (intra-class correlation coefficient, Falconer & Mackay, 1996) between the paternity response of the two sisters, vs. of the unrelated female. To ensure that relevant genetic variability among males was also represented, different males were used in each different replicate. For pollination, we simultaneously brushed two anthers per male uniformly on the stigma, which ensures a saturating pollen load (Jolivet & Bernasconi, 2007a). The time sequence of pollinations (sister1, sister2 and unrelated female) was randomized.

Some families had low germination or flowering rates or only offspring of one sex. The final number of complete replicates was 17 (ten replicates, i.e. 30 females/20 males from Millingerwaard and seven replicates, i.e. 21 females/14 males from Cottendart) for a total of 51 crosses. For each cross with three successfully formed fruits, we recorded seeds/fruit, total seed mass (mg), and germination success (20 seeds/cross; N=1020 seeds). Moreover, for each female, we preserved one unpollinated control flower in formaldehyde-acetic acid solution (Bernasconi *et al.*, 2007) and used it to measure floral traits (stigmatic surface, ovary width and length, number of stigmatic lobes) using image analysis (Canon Power Shot S40; Leica stereomicroscope with 10x0.63 magnification; ImageJ software and Photoshop®CS2).

Paternity analysis

We extracted DNA from leaves of parents and seedlings. We inferred paternal genotypes using two autosomal microsatellite DNA loci (Sl6, Sl1; Teixeira & Bernasconi, 2007). We scored maternal and offspring genotypes to identify maternal alleles in the offspring. The remaining non-maternal alleles present were used to identify the father of each seed, by comparison with the genotype of the two competing males. After applying the restriction to include only replicates with five or more successfully scored offspring in every cross, and due to germination, growth or PCR failures, the final sample included 772 offspring in 14 replicates; per replicate (male pair), the median (min-max) number of offspring scored was 50 (29-57); per female the median ranged between 13 and 18.5 (Table. 1).

Data analysis

To characterize the influence of female genotype on paternity with the focus female, we conducted univariate ANCOVA, with the response value for one of the sisters as a dependent variable, and population, covariable, population*covariable as explanatory variables. The covariable was the response measured for the other sister. Which sister was used as a focal female, and which sister one as a covariable, was randomized. If female genotype affects a response variable, we expect a positive, significant correlation between the response in the focus female and the response in her sister (i.e. a significant effect of the covariable). To characterize the influence of the identity of the male pair on response variables, we also included in the initial model the trait measured for the unrelated female as an additional covariable. If the genotype (or combination) of the focal and competing male affected a given trait, we expected a positive, significant correlation between the response in a randomly selected sister (focus female) and the same response in the unrelated female (entered as a covariable). Further, we calculated repeatability (intra-class correlation coefficients r_I, Lessells & Boag, 1987), once comparing the values obtained for the two sisters and once comparing the mean value for the sisters to that for the unrelated female, and if significantly different from zero, compared them using z-tests (Sokal & Rohlf, 2003). If female genotype affects response variables, the between-sister repeatability should be higher than the repeatability for unrelated females. Proportions were angularly transformed before analysis. Data were analyzed with SPSS 14.0 and are given as mean ± s.e. unless specified.

Table 1. Female (flower/fruit) traits and paternity in experimental crosses in *Silene latifolia*, involving three females (two sisters, one unrelated female) in each replicate. Shown are means (SD) for continuous variables, and medians (range) for count data (i.e. *number of offspring scored,* and *number of stigma branches*).

Population	Millingerwaard (HO)				Cottendart (CT)			
	Focal	Sister	Unrelated	Total	Focal	Sister	Unrelated	Total
Trait								
Seeds/fruit	251.90(99.2)	176.80(75.5)	224.40(92.0)	217.70(91.9)	209.71(64.8)	175.14(27.9)	140.57(68.4)	175.14(61.1)
Total seed mass^1	194.92(109.4)	138.29(54.5)	177.71(69.3)	170.31(81.9)	151.72(77.7)	137.96(36.2)	120.26(77.5)	136.65(64.7)
Germination	0.81(0.3)	0.82(0.2)	0.95(0.05)	0.86(0.2)	0.71(0.3)	0.83(0.2)	0.86(0.1)	0.80(0.2)
Paternity	0.42(0.3)	0.45(0.3)	0.45(0.4)	0.44(0.3)	0.30(0.3)	0.31(0.3)	0.39(0.2)	0.33(0.3)
Offspring scored	17(15)	16.5(14)	18.5(7)	17(16)	13(19)	17(14)	16(9)	16(19)
Offspring scored^2	17.5(9)	16.5(14)	18.5(7)	17(15)	14(7)	17(14)	16(9)	16.5(14)
Ovary length^3	5.82(0.7)	5.60(0.8)	5.75(0.5)	5.72(0.6)	5.11(0.7)	5.61(0.8)	5.16(0.8)	5.29(0.8)
Ovary width^3	3.35(0.4)	3.17(0.4)	3.20(0.2)	3.24(0.3)	2.96(0.4)	2.84(0.3)	2.76(0.3)	2.85(0.3)
Ovary volume^4	52.98(16.8)	45.64(16.8)	46.44(7.7)	48.42(14.5)	36.66(16.2)	36.35(10.5)	31.37(10.6)	34.91(12.31)
Stigmatic surface^5	62.34(12.6)	57.89(9.3)	57.63(5.1)	59.35(9.6)	44.6(15.1)	47.9(8.7)	44.8(10.2)	45.75(11.2)
Stigma branches	5(1)	5(1)	5(0)	5(1)	5(2)	5(0)	5(1)	5(3)

(1) mg; (2) excluding three cases with <5 offspring scored; (3) mm; (4) mm^3, calculated assuming an ellipsoid; (5) mm^2.

Results

Paternity by the focal male with a randomly–selected sister (focal female) was significantly explained by the paternity share he obtained with the other sister (Table 2). This indicates an influence of female genotype on paternity. Neither inclusion of paternity with the unrelated female ($F_{1,9}$=0.234, P=0.81) nor its interaction with population ($F_{1,8}$=0.225, P=0.65) significantly improved the model. The strength of this effect depended on the population (Table 2), with resemblance of paternity across sisters being stronger in Cottendart (Fig. 2). In agreement with this, for Cottendart paternity of the focal male was highly repeatable between sisters (repeatability r_I=0.86; ANOVA: $F_{5,11}$= 13.8, P<0.05). This value is considerable, however it must be noted that the confidence interval is also expected to be wide due to the relatively small sample size. By contrast, paternity shares were not significantly repeatable when comparing the mean value for the sisters and the value for the unrelated female (r_I=-0.09; $F_{5,11}$= 0.84, P=0.57). Repeatability for the sister replicates was significantly higher than between the mean of the sisters and the unrelated female (z-test: z=1.7, P<0.05). In Millingerwaard, paternity was not significantly repeatable across females (sisters: r_I=0.27, $F_{7,15}$= 1.8, P= 0.22; mean of sisters - unrelated female: r_I=0.39, $F_{7,15}$=2.3, P= 0.13).

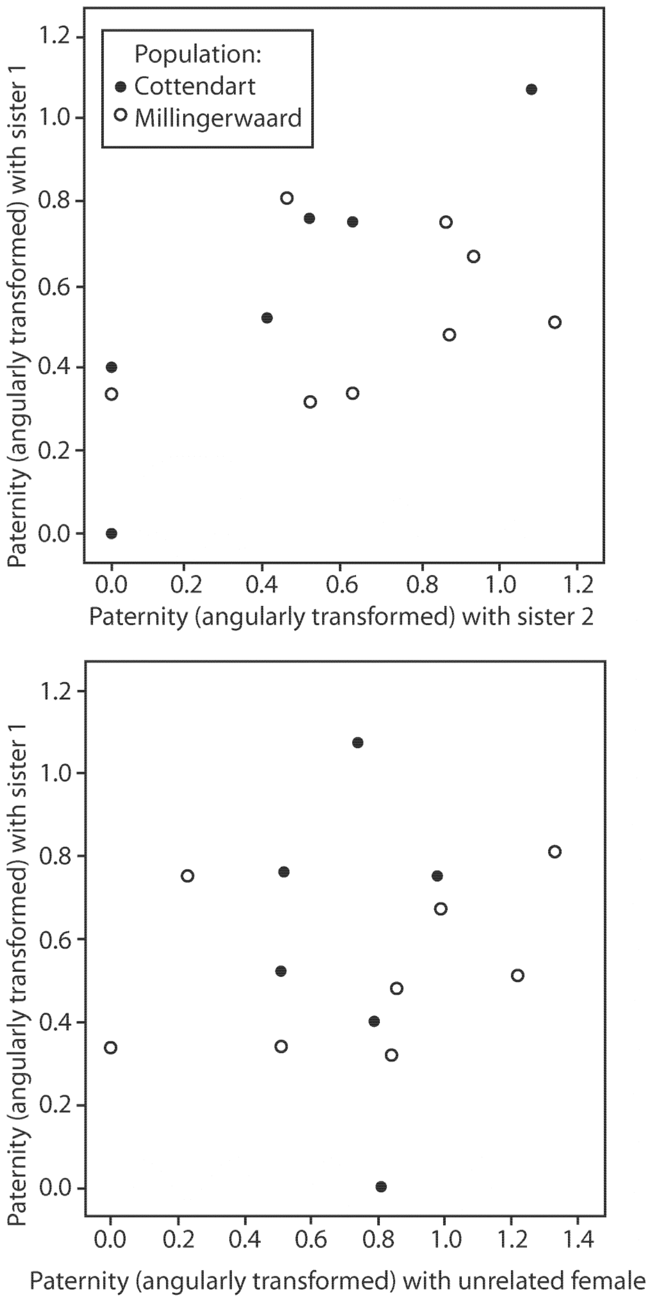

Figure 2. Scattergram for paternity shares (angularly transformed) of the focus male (proportion offspring sired by the focal male, angularly-transformed) against a competing male in replicated hand-pollinations with three females (two sisters and one unrelated female). *Top*: Paternity obtained with two females related to each other; Y-axis: sister(y), X-axis: sister(x). *Bottom*: Y-axis: Mean paternity obtained in crosses with two sisters; X-axis: paternity obtained in crosses with one unrelated female.

Table 2. ANCOVA for the effect of population and paternity with one sister, on paternity with the focal female. We included in this analysis only replicates with three crosses for which ≥5 offspring were scored. All proportions were angularly-transformed prior to analyis. Response variable: Paternity (angularly transformed) with the focal female.

Source	Sum of squares	*df*	Mean squares	*F*	*P*
Population	0.386	2	0.193	5.713	0.022
Sister	0.445	1	0.445	13.187	0.005
Population*Sister	0.177	1	0.177	5.250	0.045
Error	0.338	10	0.034		
Total	5.216	14			

Table 3. Spearman's rank correlation coefficients among female traits and paternity (*N*=21 females in CT=Cottendart, *N*=30 females in HO=Millingerwaard). Italic: error probabilities (for the null hypothesis that correlation is equal to zero). Bold: significant correlations (15 tests, α=0.0033 after Bonferroni correction).

		Seed mass	Germination success	Paternity	Ovary volume	Stigmatic surface
HO	Seed number	**0.790**	0.053	0.051	0.223	0.164
		<0.001	*0.780*	*0.790*	*0.246*	*0.394*
	Seed mass		0.236	-0.081	0.002	0.060
			0.210	*0.671*	*0.992*	*0.757*
	Germination success			-0.249	-0.331	-0.295
				0.185	*0.079*	*0.120*
	Paternity				0.299	0.293
					0.114	*0.123*
	Ovary volume					**0.524**
						0.004
CT	Seed number	**0.923**	-0.211	-0.174	-0.012	0.137
		<0.001	*0.358*	*0.451*	*0.960*	*0.554*
	Seed mass		0.003	-0.174	-0.029	0.220
			0.991	*0.451*	*0.902*	*0.339*
	Germination success			-0.069	-0.368	-0.279
				0.767	*0.101*	*0.220*
	Paternity				-0.080	-0.147
					0.732	*0.525*
	Ovary volume					**0.453**
						0.039

Paternity by the focal male was not significantly correlated with seed set, or the proportion of germinated seeds within populations (Table 3). Accordingly, when we included seed set by the focal female, and germination rate (angularly transformed) in the model of Table 3 these effects were not significant (both $P>0.30$). Flowers from Millingerwaard had a significantly larger stigma ($F=15.2$, *d.f.*=1, 77, $P<10^{-4}$), and a longer ($F=6.9$, *d.f.*=1, 77, $P=0.01$) and wider ($F=21.3$, *d.f.*=1, 77, $P<10^{-4}$) ovary compared to flowers from Cottendart (Table 1). Since we reared these F1 plants in a greenhouse, our results reveal genetic variation among populations in these traits. The number of stigmatic lobes varied between four and seven, but most flowers had five stigmatic lobes (Table 1). In both populations, stigmatic surface was significantly correlated with ovary volume, yet with no other trait, including paternity (Table 3).

Discussion

Seed paternity, i.e. the proportion of offspring in one fruit sired by competing pollen donors, is a complex trait that may depend on the specific combination of female and male genotypes (Wolf, 2003) and on different mechanisms, including variation among males in pollen germination and pollen tube growth rates and among females in the morphology of the receptive structures (Delph & Havens, 1998). We experimentally investigated whether genetic and morphological variation among F1 females of the white campion, Silene latifolia, can explain variation in male siring success at pollen competition. Female genotype plays an important role in determining paternity, at least in one of the study populations investigated here. In one population paternity success of focal males was significantly more repeatable between females, which were full sisters, than when comparing paternity success of the same males with a female unrelated to the previous ones. Since we used F1 plants, these effects are most likely due to genetic variability, rather than maternal environmental effects. In agreement with this, other studies revealed maternal identity effects on siring success of competing donors. For instance, in Lobelia cardinalis, siring success of pollen donors changed with different pollen recipients (Johnston, 1993). Female genotype effects on paternity have also been documented for animals (Wilson *et al.*, 1997; Clark & Begun, 1998; Bernasconi *et al.*, 2004).

Such an influence of female genotype on pollen competition success may arise if genetically different individual plants differ in their preference between pollen of different donors or in the morphology of receptive structures, if pollen performance differs on genetically different recipient plants, or if post-zygotic mechanisms (such as seed abortion, early-acting inbreeding depression or competition among developing seeds sired by different fathers) depend on the female genotype. There was no evidence that early-acting mortality generates variation in paternity, in that seed set or germination rate did not contribute significantly to explain paternity. However, lack of evidence does not necessarily imply lack of effect, and to distinguish between pre- and post-zygotic mechanisms, future studies are needed, for instance monitoring similarity of pollen tube growth between related females or investigating the effect of relatedness between mates. Since we observed paternity of a fixed male pair over three females per replicate, our study also does not address whether the effects depend solely on genetic variation among females, or on its combination with specific males. The effect was stronger and significant in one out of two populations investigated, and not significant in the other one. This may have several reasons. For instance, if female influence reflects inbreeding avoidance, both the differences among individual females, and differences among pollen, may vary with genetic variability of the population.

We determined paternity after controlled hand pollinations. Under conditions of natural pollination, pollen donor's success is dependent on many factors, including pollinator behaviour, some of which will introduce substantial stochastic variation. However, genetic variation for determinants of net siring success as found in our study may play a role in the field, since S. latifolia (formerly, S. alba) often occurs in small patches. Due to gravity-dispersed seeds (McCauley, 1997; Moyle, 2006), small patches may often consist of relatives that pollinators (including the specialist noctuid moth *Hadena bicruris*, Blair & Wolfe, 2004; Bopp & Gottsberger, 2004) may visit in sequence. The finding that seed paternity of related females is similar in at least one population suggests that the involved mechanisms (either pre- or post-zygotic) may favour or disfavour given pollen donors at this spatial scale. A previous study in S. latifolia found that gene flow was higher into experimental full-sib patches than into patches consisting of unrelated individuals (Richards, 2000). In that study, female plants could be pollinated from a brother at 0.5 m distance or an unrelated male at 20 m distance. Paternity analysis revealed more fertilizations by the unrelated male than expected at random. In the control groups, both male plants (at 0.5 m and at 20 m distance) were unrelated to the female, and paternity did not deviate from the random expectation. This suggests that the underlying mechanisms (pre-or post-zygotic) by which genetic variation influences paternity at this scale may depend on relatedness among parents. The net effect on male reproductive success will depend on the relative importance compared to other factors, such as plant traits (e.g. floral display, flowering phenology), patch characteristics (fine-scale genetic structure) and on the patterns of pollinator visitation, all of which require further investigation.

We found significant differences between populations in total stigmatic surface and ovary size, adding to the known evidence for quantitative variation in reproductive traits among S. latifolia populations (Delph *et al.*, 2002; Jolivet & Bernasconi, 2007b). However, there was no significant correlation of these traits with paternity shares in hand pollinations (nor with deviation of paternity from equality, data not shown). Thus apparently total stigmatic surface played no role in magnifying competition among pollen, although this was found previously in other species (see Delph & Havens, 1998 for a review). However, also in other cases natural variation in style length is unlikely to influence variation in siring success (*Clarkia unguiculata*, Travers & Shea, 2001). Even if variation in stigmatic surface is not as effective in modifying the relative success of competing pollen donors, it would be interesting to know whether it increases the chances and efficiency of pollen capture under natural pollination (by analogy to stigmatic receptivity schedules, Galen *et al.*, 1986).

In conclusion, we found that genetic variation among females significantly influenced pollen competition success in one of the two study populations of the white campion. This strongly suggests the existence of genetically–based mechanisms affecting differential pollen performance or embryo/seed survival on individual females in this species.

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CHAPTER 6 : General conclusions

Plants in the common garden

Experimental cage

The evolution of floral traits in dioecious plants is particularly interesting because each sex can be subjected to different selection pressures, leading to sexual dimorphism. For insect-pollinated dioecious plants, flower attractiveness is very important. However traits that make flowers attractive to pollinators may also render them attractive to natural enemies. When these enemies inflict costs only to females by consuming seeds, females may be selected for traits that reduce the costs. The use of defences such as abortion of infested fruits may help to reduce the cost of predation. However defences can be costly themselves, and defence costs may contribute to selection on floral traits. Thus in my thesis I investigate whether a plant defence against seed predation could mediate selection on floral traits (chapter 3) and what is the effect of this defence and of fruit size on the predator fitness (chapter 2). Moreover, I investigate how variation in floral number and size affect female fitness and the role of pollinators as selective agents on floral traits (chapter 3), and because plant fitness also depends on the dynamics of pollen deposition and germination, I study how traits at the flower level influence seed paternity (chapters 4 and 5).

In *Silene latifolia*, *Hadena bicruris* can reduce plant seed production by half (Wolfe, 2002). Therefore, I expected the insect to be a selective agent on floral display, but seed predation did not contribute to the total selection for larger floral display. On the other hand, fruit abortion emerged as being an important mechanism in this system. I showed that fruit abortion is costly for insect growth, thus likely affecting its survival in the field. While this may be beneficial for the plant on the long term if it reduces the population of the seed predator (Holland & DeAngelis, 2002), on the short term it is costly to plant fitness, as shown by the significant total negative selection on this trait. Interestingly, the two selection lines differed in the cost of abortion. In the SF plants, which floral display and flower size is more similar to males in the field, seed predation contributed significantly to the selection against abortion, while in the LF plants, which are more female-like, this contribution was not significant. Thus seed predation may indirectly contribute to selection on sexually dimorphic traits in *S. latifolia* through the cost of plant defence. My results suggest that SF plants pay a larger cost to abortion than LF plants because they lose more numerous fruits and probably more resources. The study of the cost of abortion in this plant/ insect system seems a very promising field.

In other nursery pollination systems fruit abortion is known in yucca/ yucca moth (Pellmyr & Huth, 1994), senita cactus/ senita moth (Holland *et al.*, 2004a), and *Lithophragma parviflorum*/ *Greya politella* (Thompson & Cunningham, 2002), but these plants are hermaphrodite. Among the dioecious species, the majority of seed predators lay their eggs on male plants (Dufaÿ & Anstett, 2003), but to my knowledge these plants are not sexually dimorphic for floral traits (Tang, 1987; Feil, 1992; Anstett, 1999). Sexual dimorphism is found in *Silene dioica,* but the larvae of *Perizoma affinitatum* consume only a small proportion of seeds, and no fruit abortion is known (Westerbergh, 2004). Because costs for this plant seem to be low, I would expect the contribution of seed predation to selection on floral traits to be negligible in this species. Such contribution seem more likely in the gynodioecious plant *Dianthus sylvestris*, where *Hadena compta* prefers to lay eggs on perfect flowers, that are larger than female flowers (Collin *et al.*, 2002). *Ficcus carica* is sexually dimorphic for floral scent (Grison-Pige *et al.*, 2001), and male plants are preferred as oviposition sites in experimental choice tests (Anstett *et al.*, 1998), but asynchronous flowering in male and female fig trees (Kjellberg *et al.*, 1987) is likely to reduce the opportunity for wasp selection on floral scent. Thus in nursery pollination systems, *S. latifolia* seem to be the first species where a plant defence plays a role in the selection on floral traits.

Evidence for pollinator selection on floral traits is common in angiosperms, and thus I was not surprised to find total positive selection on flower number. Seed predator contribution to selection was not significant, suggesting that selection was due to pollination by non-ovipositing *H. bicruris* and/ or co-pollinators. In other nursery-pollination systems, large floral display and flower size are known to increase attractiveness to pollinators. For example, in the dioecious palm *Chamaerops humilis*, more weevils were found on male and female plants that bore more inflorescences (Dufaÿ & Anstett, 2004). In the hermaphroditic senita cactus, large floral display increased attractiveness to senita moth and pollen export, but not seed set (Holland *et al.*, 2004a). *Trollius europeus* with a large flower reared more predator larvae, but still produced more seeds than plants with a small flower (Despres *et al.*, 2007). Thus pollinator selection for large floral display or flower size can benefit male and/ or female function, and is likely to be common in nursery-pollination systems.

Selection is unlikely to affect floral traits independently in *S. latifolia* because many traits are genetically correlated (Meagher, 1992; Delph *et al.*, 2004a). The use of selection lines (Delph *et al.*, 2004b) has the advantage that flower phenotype results from selection on all correlated floral traits, and differences in phenotypes are genetic since the environment is kept constant. Thus pollinator or seed predator selection on one floral trait will lead to indirect selection on other traits. Since flower number and size are negatively genetically correlated in *S. latifolia* (Delph *et al.*, 2004b), LF females may compensate for their reduced attractiveness due to their smaller display by producing larger flowers than SF plants. Both strategies (SF or LF) seem to be equally good for the female plant in terms of seed production, while males benefit more from the SF strategy because it enables them to produce more pollen (Delph *et al.*, 2004b). My results suggest that the LF strategy is more favourable to females because it lowers the costs of abortion. Simultaneously it benefits the seed predator by increasing the establishment probability of larvae and is likely to protect them longer against their own enemies (Biere *et al.*, 2002). Also this strategy may decrease the probability that females get infected by a pollinator transmitted venereal disease. Indeed large floral display increases the probability of infection in males (Thrall & Jarosz, 1994). Thus the evolution of floral traits in *S. latifolia* is likely to be the result of genetic correlations between traits and different selection pressures on male and female plants by pollinators and antagonists. Plant pollination and defences have long been studied separately, but together with other recent studies (e.g.; Galen & Geib, 2007; Nuismer & Ridenhour, 2008; Hanley *et al.*, 2009; Irwin, 2009) I show that their combination is important to understand the evolution of floral traits.

Plant fitness does not depend only on pollinator attraction and enemy avoidance but also on successful ovule fertilization. This depends on the dynamics of the interaction between pollen and the female stigma. As in other plant species (e.g.; Spira *et al.*, 1996; Karron *et al.*, 2006), I found that arrival time of pollen is important for male siring success. Male and female function may however have conflicting interest for paternity (Bernasconi *et al.*, 2004). While the first arriving male will increase its fitness by siring more offspring, the female may benefit from sampling more genetic diversity in the population (Bernasconi *et al.*, 2003). I found that increasing genetic diversity among offspring decreased individual seed mass, which may play a role in seedling establishment success. For a species like *S. latifolia*, which seeds fall close to the maternal plant, siblings are likely to compete for establishment. A recent study in *Lupinus angustifolius* showed that plants had a relatively higher fitness when competing with genetically distant individuals than with siblings (Milla *et al.*, 2009). Moreover, the stigma/ style may be an important arena for the conflict over paternity between male and female function (Lankinen *et al.*, 2006; Lankinen & Kiboi, 2007). The female function may benefit from a large stigmatic surface if it increases pollen capture (Cruden & Millerward, 1981; Jousselin *et al.*, 2004), competition among pollen tubes (Delph *et al.*, 1998), or the genetic diversity of sampled pollen. However, I found that paternity shares of two donors were not significantly affected by stigmatic surface. To limit competition with pollen from other donors, the male function may benefit from accelerating the wilting of the stigma. Forward wilting may be costly for the female function if it lowers the chance of obtaining larger quantity or diversity of pollen. In the presence of natural enemies, stigma wilting may also serve the female function by reducing the probability of infection by venereal diseases, or by rendering the flower unattractive to seed predator. My results show that wilting seem to be simultaneous with pollen tube growth, but the costs and benefits of wilting for each sex are still to be discovered.

In conclusion, my thesis corroborates that in insect-pollinated plants, floral traits are likely to evolve in response to multiple selection pressures (Strauss & Whittall, 2006). Male and female functions need to attract pollinators, but may be subjected to different selection pressures by natural enemies. Sexual dimorphism may be the result of these sex-specific selection pressures and its evolution will be constrained by genetic correlations among traits. Genetic variation in sexual dimorphism will be maintained by differences in selection among populations and years in pollinator and antagonist prevalence. Moreover, pollinators will affect the genetic diversity of pollen that is deposited on the stigma of flowers, and the result of inter-sexual conflict for seed paternity may affect floral traits, that in turn might change plant attractiveness to pollinators and enemies.

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