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Mechanisms of habitat adaptation in *Silene dioica* and *S. latifolia* (Caryophyllaceae)

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Summary

Habitat adaptation allows species or populations to survive and reproduce in certain habitats but not in others. The evolution of such ecological differentiation has been identified as a major driver of divergence processes that can lead to reproductive isolation and speciation. In this thesis I contribute to the understanding of ecological divergence using several related studies on the naturally hybridizing champions *Silene dioica* and *S. latifolia*.

First, we investigated habitat differentiation between and within sites of *Silene dioica* and *S. latifolia* using AFLP banding patterns and vegetation relevés around individual plants. Only three putative early-generation hybrids were detected at natural contact sites. *Silene dioica* was found in moister, colder and less disturbed sites than *S. latifolia*. However, asymmetric habitat overlap was evident with contact sites found in intermediate conditions that were more similar to *S. latifolia* sites. Within contact sites, however, the micro-habitats of the two species did not differ making habitat-mediated selection against intermediate phenotypes of hybrids unlikely and suggesting that other reproductive barriers contribute to the rarity of early-generation hybrids.

Secondly, we studied responses to shade and drought stress in crosses between and within *Silene dioica* and *S. latifolia* to further understand ecological differences between the species and their hybrids. Responses to drought stress did not differ between cross types. Shade stress, in contrast, led to a reduced flowering incidence in *Silene dioica* and the hybrids but not in *S. latifolia*. Rapid flowering under stress conditions in *S. latifolia* could be an adaptation to higher disturbance in its habitat, whereas a delay of reproduction might be adaptive in the more predictable environment of *S. dioica*. While hybrids did not show a generally reduced stress tolerance in comparison to the two species, our results do suggest that *S. dioica* and hybrids may be excluded from highly disturbed and stressful habitats of *S. latifolia* due to their delayed flowering.

In a third study, we investigated whether habitat adaptation, phenological divergence or low performance of first- and second generation hybrids could act as reproductive barriers between *S. dioica* and *S. latifolia*. For this purpose we

transplanted *Silene dioica*, *Silene latifolia* as well as two classes of hybrids (F_1 and F_2) into six sites, three within natural populations of each species and assessed cumulative fitness and flowering phenology. We found clear evidence for habitat adaptation: in each habitat, the resident species had the highest fitness. Such habitat adaptation may limit possibilities for contact between the two species. Furthermore, flowering times of the two species overlapped only partially further reducing possibilities for mating. While first-generation hybrids performed intermediate between the two species, second-generation hybrids had a generally low performance that may be due to a disruption of epistatic interactions and further limit gene flow between the species. A stronger fitness reduction of *S. latifolia* in *S. dioica* habitats than vice versa and a longer flowering overlap of hybrids with *S. latifolia* than with *S. dioica* further suggest that reproductive barriers between *S. dioica* and *S. latifolia* lead to preferential gene flow from *S. dioica* into *S. latifolia*.

In a fourth part, we investigated mechanisms of habitat adaptation in more detail and analyzed the relative importance of survival, flowering and the production of an overwintering rosette in *S. latifolia*, *S. dioica* and hybrids in our transplant experiment (see above). Differences in survival were the main restriction to the establishment of one species in the other species habitat. F_2 hybrids, however, had a generally lower flowering incidence than the other cross types. Production of an overwintering rosette was under positive selection within F_2 hybrids in the *S. dioica* habitat and might be a key trait limiting the establishment *S. latifolia* and hybrids within the *S. dioica* habitat.

This thesis adds to the understanding of ecologically-mediated reproductive isolation between two cross-fertile species and shows the importance of combining several approaches including field observations and experimental crosses, as well as greenhouse and field experiments.

Résumé

L'adaptation à l'habitat permet à des espèces ou à des populations de vivre et se reproduire dans certains habitats, mais pas dans d'autres. L'évolution d'une telle différenciation écologique a été identifiée comme élément majeur dans le processus de divergence, menant à l'isolation reproductrice et la spéciation. Dans cette thèse, je contribue à la compréhension de la divergence écologique de *Silene dioica* et *S. latifolia* grâce à plusieurs études complémentaires. Tout d'abord, il est important de savoir que ces deux espèces sont connues pour s'hybrider naturellement.

Premièrement, nous nous sommes intéressés à la différenciation de l'habitat à l'intérieur et entre les sites de *S. dioica* et *S. latifolia* en employant des AFLPs et des relevés de végétation autour de plantes focales. Seuls trois présumés hybrides de première génération ont été détectés sur l'ensemble des sites où les deux espèces se trouvent en contact. *Silene dioica* a été trouvé dans des milieux plus humides, plus froids et moins perturbés que *S. latifolia*. Cependant, les deux habitats se chevauchent de manière asymétrique, les sites de contact trouvés dans des conditions intermédiaires étant plus similaires à l'habitat de *S. latifolia*. Par ailleurs, à l'intérieur des sites de contact, les microhabitats des deux espèces ne différaient pas. De ce fait, la sélection exercée par l'habitat contre les phénotypes intermédiaires des hybrides est peu probable et suggère que d'autres barrières reproductives contribuent à la rareté des hybrides de première génération.

Deuxièmement, nous avons étudié la réponse au stress de sécheresse et d'ombre de *S. dioica*, *S. latifolia* et leurs hybrides afin de pouvoir élucider les différences écologiques dans le domaine de la tolérance au stress. Les réponses à la sécheresse des différents types de croisements étaient similaires. Néanmoins, *S. dioica* et les hybrides avaient un taux de floraison réduit lorsqu'ils étaient placés à l'ombre, ce qui n'était pas le cas de *S. latifolia*. Une floraison rapide dans des conditions de stress pourrait être une adaptation de *S. latifolia* à son milieu naturel plus perturbé, tandis qu'un report de la floraison à une année ultérieure pourrait être un signe d'adaptation à un milieu plus prévisible, comme c'est le cas pour *S. dioica*. Parce que la tolérance au stress des hybrides n'est pas généralement réduite en

comparaison avec les deux espèces, nos résultats suggèrent que *S. dioica* et les hybrides seraient exclus de l'habitat de *S. latifolia* à cause de leur floraison retardée.

Dans une troisième étude, nous avons testé si l'adaptation à l'habitat, la phénologie ou la faible performance des hybrides de première et seconde générations pouvaient agir comme une barrière à la reproduction entre *S. dioica* et *S. latifolia*. Pour ce faire, nous avons transplanté ces deux espèces ainsi que deux classes d'hybrides (F_1 et F_2) dans six différents sites, trois populations naturelles de chacune des espèces. Nous avons calculé une valeur cumulative de fitness et observé la phénologie de floraison. Nous avons trouvé une claire indication de l'adaptation à l'habitat : dans chacun des deux habitats, l'espèce originellement résidente avait toujours le fitness le plus élevé. Une pareille adaptation à l'habitat pourrait limiter les chances de contact entre les deux espèces. De plus, les phénologies de floraison respectives des deux espèces ne se recouvraient que partiellement, réduisant encore la probabilité de fertilisation croisée. Alors que les hybrides de première génération ont obtenu un fitness intermédiaire entre *S. dioica* et *S. latifolia*, les hybrides de seconde génération avaient un fitness constamment bas, peut-être dû à l'alteration d'interactions épistatiques. A nouveau, ceci pourrait limiter le flux de gènes entre les espèces. Une plus forte réduction de fitness *Silene latifolia* dans l'habitat de *S. dioica* et un recouvrement plus long de la phénologie de floraison des hybrides avec *S. latifolia* pourraient suggérer que les barrières reproductives entre les deux espèces mèneraient à un flux de gènes de *S. dioica* à *S. latifolia*, préférentiellement.

Dans une quatrième partie, et sur la base de la même expérience de transplants (voir plus haut) nous nous sommes intéressés plus en détail aux mécanismes de l'adaptation à l'habitat en analysant l'importance relative de la survie, de la floraison et de la production de rosette hivernale pour *S. latifolia*, *S. dioica* et leurs hybrides. La différence de survie était la principale restriction à l'établissement d'une espèce dans l'habitat de l'autre espèce. Les hybrides F_2 , cependant, avaient un taux de floraison plus bas que les autres types de croisements. Dans cette même classe d'hybrides, la production de rosette hivernale était un trait phénotypique sous sélection positive dans l'habitat de *S. dioica*, et pourrait être une caractéristique limitant l'implantation de *S. latifolia* et des hybrides dans l'habitat de *S. dioica*.

Cette thèse contribue à la compréhension de l'isolation reproductrice engendrée par l'habitat entre deux espèces interfertiles et démontre l'importance d'une approche méthodologique combinée incluant des observations de terrain, de croisements expérimentaux, et d'expériences en serre et en milieu naturel.

General Introduction

Ecological speciation and habitat adaptation

In the process of ecological speciation, reproductive isolation may evolve between two groups of organisms that are under ecologically-based divergent selection (Schluter, 2000, 2001). This divergent selection stems from environmental differences in abiotic (for example climate, nutrient availability, soil characteristics) or biotic factors (the presence of competitors or predators) and can lead to the evolution of adaptive trait differences. Identifying these traits is necessary to understand how differences in habitat adaptation contribute to reproductive isolation and to ecological speciation (Rundle and Nosil, 2005; Schluter, 2001, 2009). Genetically-based trait correlations within species are a major difficulty in detecting such traits under selection (Jordan, 1991; Lande and Arnold, 1983; Lexer et al., 2003; Nagy, 1997). A promising method to investigate selection on individual traits is to generate recombinant hybrids between ecologically differentiated species (for example F₂ hybrids) and expose these to selection experiments (Lexer *et al.*, 2003). Thus, hybridizing species represent an opportunity to gain an insight in the process of habitat adaptation and ecological speciation.

Hybridization and reproductive isolation

Hybridization is a common phenomenon in nature (Arnold, 1997; Ellstrand et al., 1996; Rieseberg et al., 2006) and can have different outcomes. If the hybrids are not viable or sterile, hybridization will have no evolutionary consequences (Schemske, 2000). Given that the hybrids are fertile, hybridization between species might cause gene flow. If the gene flow is extensive, genetic assimilation can occur (Burgess and Husband, 2006; Levin et al., 1996; Rhymer and Simberloff, 1996). Alternatively, a limited gene flow can contribute to the spread of adaptive alleles without compromising the species integrity (Anderson, 1949; Arnold, 1997; Martin et al., 2006; Rieseberg, 1997; Rieseberg and Carney, 1998). The extent of gene flow is determined by the strength of reproductive barriers that can be complex (Ramsey et al., 2003) and act sequentially (Kay, 2006; Nosil et al., 2005; Ramsey et al., 2003; Schluter, 2001). Reproductive barriers are pre-zygotic or post-zygotic whether they

whether they occur prior to the zygote formation or after it (Coyne and Orr, 2004; Rieseberg and Willis, 2007; Templeton, 1989; Widmer et al., 2009). Cross-fertile species that are adapted to different habitats might show habitat-mediated reproductive barriers

In this thesis I was interested in the mechanisms of habitat adaptation that could contribute to the reproductive isolation between two cross fertile species.

Study Species

Silene dioica and *S. latifolia*

Silene dioica (L.) Clairv. and more particularly *S. latifolia* Poiret largely contributed to the recent re-emergence of the genus *Silene* (Caryophyllaceae) as a model system in the interrelated fields of ecology, genetics, evolution and developmental biology (Bernasconi et al., 2009). These two species are dioecious, short-lived perennials and closely related (Desfeux and Lejeune, 1996) but differ in many aspects. *Silene dioica* is a red- or pink-flowered, wintergreen rosette plant up to 80 cm high with a shallow root system. *Silene latifolia* grows up to 120 cm high, does not produce a rosette during winter, and possesses a thickened taproot. The two species partly share their pollinator guild, but because flowers of *S. latifolia* open at night and those of *S. dioica* open during the day, the former is pollinated primarily by sphingid and noctuid moths (Jurgens et al., 1996; Shykoff and Bucheli, 1995), whereas the latter is primarily pollinated by butterflies, bumblebees and muscid flies (Goulson and Jerrim, 1997; Westerbergh and Saura, 1994). The odor bouquet produced at flowering also differs between the two species (Jurgens, 2004; Jurgens et al., 2002; Waelti et al., 2008). The distribution range of both species is large and sympatric in most of Europe. However, *S. dioica* extends further north in Scandinavia and *S. latifolia* can be found further east in Asia (Friedrich, 1979). In the European Alps, our study area, both taxa are widely distributed with *S. dioica* being more common particularly at higher altitudes (Lauber and Wagner, 2007). The white and the red champions occur in different habitats (Goulson and Jerrim, 1997). *Silene dioica* habitat (forests, fat meadows, pastures) is moister, colder and less disturbed than

that of *S. latifolia* that occurs in more open habitats, such as hedgebanks, and as a weed in arable fields (Baker, 1947a, 1947b; Friedrich, 1979).

Occurrence of hybrids

Silene dioica and *S. latifolia* are completely cross-fertile and can produce viable and fertile hybrid progeny under controlled conditions. In nature, hybrid zones exist and have been characterized using AFLP markers and morphological variation (Minder et al., 2007). Introgression between the two species occurs (Minder and Widmer, 2008), but intermediate hybrid phenotypes are extremely rare in nature (Minder et al., 2007).

Main methods

Ecological and genetic differentiation (Chapter I)

To identify environmental factors that differentiate the habitats of *S. latifolia* and *S. dioica* we analyzed Ellenberg indicator values from vegetation relevés around focal *Silene* plants in 6-8 natural populations of each species and in 5 contact sites. Ellenberg indicator values have been shown to correspond to measurements of abiotic parameters (Ertsen et al., 1998) and are thus very useful when long-term measurements are not available.

To investigate genetic differentiation and detect early generation hybrids between *Silene dioica* and *S. latifolia* we used amplified fragments length polymorphism (AFLPs, see Vos *et. al* (1995) for technical details). This method is advantageous because AFLPs are highly polymorphic and no prior sequence knowledge is required (Schlotterer, 2004). AFLPs are commonly used to evaluate species genetic differentiation, identify hybrids and detect introgression. In *Silene*, AFLPs were previously used to characterize two hybrid zones between *S. dioica* and *S. latifolia*: most of the hybrids were late generation backcrosses indicating that introgression occurred (Minder et al., 2007).

Stress reaction, a greenhouse experiment (Chapter II)

We used a greenhouse experiment to investigate the stress reaction of *Silene dioica*, *S. latifolia* and their reciprocal hybrids. To generate the experimental plants, we first of all grew plants of both species from seeds collected in six natural populations (three per species, see Table 1). Then, these parental plants were crossed in a full-sib-half-sib crossing design to obtain four cross types: within *Silene latifolia* and *S. dioica* crosses as well as reciprocal crosses between the two species (*S. dioica* female x *S. latifolia* male and *S. latifolia* female x *S. dioica* male). Because these *Silene* species are dioecious, we molecularly sexed a large number of young experimental plants prior to the experiment to insure a balanced sex ratio. We used a molecular method developed in our group (Hobza and Widmer, 2008).

The experiment was set up in a greenhouse under ambient temperatures at Zurich Höggerberg, Switzerland for two growing seasons (2006-2007) after which the plants were harvested. The experimental design was a strip-split-plot, composed of nine whole-plots (Cochran and Cox, 1968). Two stress factors were chosen from the conditions that differ between the species habitats, i.e. drought and shade. The experiment was composed of four treatments: benign, drought, shade and combined shade and drought stresses. We collected the following data: fitness proxies (survival, flowering incidence and total biomass), leaf traits (specific leaf area, leaf area and leaf dry weight) as well as root traits (root mass ratio and root cross sectional area).

Transplant experiment in natural habitats (Chapter III & IV)

To study reproductive isolation and the mechanisms of habitat adaptation in natural conditions, six study sites were established within typical natural habitats of *Silene latifolia* and *S. dioica*. Because environmental factors influencing reproductive isolation and habitat adaptation can vary across the distribution range of a species, we took account of this natural variation by replicating transplant sites (Kawecki and Ebert, 2004). There were three replicated sites for each species habitat located in Canton Valais (Swiss Alps) and regularly ranged from an elevation of 420m to 1800m.

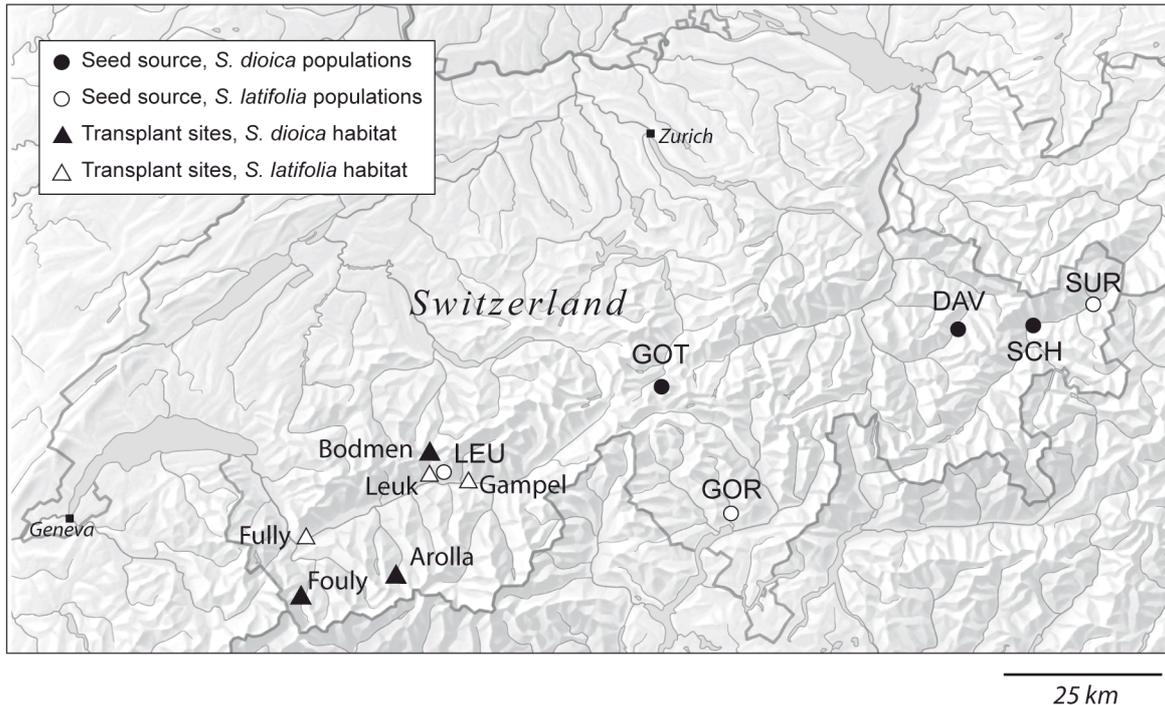


Fig 1 List of seed source populations used to perform crosses between *S. dioica* and *S. latifolia* and transplant sites within natural *Silene* populations

We transplanted five different cross types: within species crosses (*Silene latifolia* and *S. dioica*), reciprocal hybrid crosses (*S. dioica* female x *S. latifolia* male and *S. latifolia* female x *S. dioica* male) and second-generation hybrids (F_2). Experimental plants resulted from a second generation of crosses between numerous spare plants of the above greenhouse experiment. Before transplantation, a total of 6480 plants were pre-cultivated under greenhouse conditions (Mottiez Fleur, 1903 Collonges, Valais, Switzerland). In this experiment, we were interested in identifying traits under selection. Because genetically-based trait correlations within species are a major difficulty in detecting such traits (Jordan, 1991; Lande and Arnold, 1983; Lexer et al., 2003; Nagy, 1997), we included a large proportion (one third) of recombinant hybrids (F_2). This method generates phenotypic variation in traits that are additively controlled by several or many genes as is assumed for most ecologically relevant traits (Lander and Botstein, 1989; Wu et al., 2007). From fall 2007 to fall 2009, we collected data. Survival was scored twice a year (spring and fall) and flowering incidence each year. Shortly before winter 2007, we assessed the shape of the overwintering rosette and every year at the same time we measured the plant size. By visiting the sites every 7 to 10 days in 2008, we recorded flowering

phenology, the number of flowers and capsule produced by each plant. These data we used to compile a cumulative fitness measure for each plant. We used a mixed effect model to test whether cumulative fitness depended on the cross type, habitat and their interaction (fixed factors). Random factors were blocks, sites nested within habitat, lines nested within cross types and lines nested within sites. When the interaction was significant, the data were split by habitat and site.

Main objective

Understanding the mechanisms of habitat adaptation and how they contribute to the reproductive isolation between *S. latifolia* and *S. dioica*, was the main objective of this thesis. For this purpose, we first performed a habitat study to investigate the ecological differentiation of the habitat of the two species. The result were obtained by characterizing the microhabitat of focal plants using vegetation relevés and by attributing these plants to either *S. dioica*, *S. latifolia* or hybrids using AFLP markers (Chapter I). Then, we tested the reaction to stress factors of both species and their first generation reciprocal hybrids chosen from the conditions that differ between the species habitats, i.e. drought and shade (Chapter II). With this greenhouse experiment we aimed at contrasting the stress reaction of the two species and understanding whether a deficiency in stress tolerance in the hybrids might cause their rarity in nature. In a third step, we transplanted both species and two hybrid classes into natural habitats of *S. dioica* and *S. latifolia*. With this experiment, we aimed at understanding which reproductive barriers could contribute to the reproductive isolation between the species (Chapters III). In this third chapter, we studied the differences between the cross types with a cumulative fitness measure and flowering phenology. Finally, in the same transplant experiment, we were interested in the mechanisms underlying the observed advantage of *S. dioica* and *S. latifolia* in their respective habitats and analyzed selection on a candidate adaptive trait (Chapter IV).

The main research questions were:

- 1) Do the habitats of *S. dioica* and *S. latifolia* differ between sites and within contact sites, do the habitats overlap and are the species genetically differentiated between and within sites? (Chapter I)
- 2) How do *S. dioica*, *S. latifolia* and their reciprocal hybrids differ in their fitness reduction under drought and shade stress and does the plastic response in leaf morphology differ between the two species and their hybrids? (Chapter II)
- 3) Are *Silene latifolia* and *S. dioica* more fit in their respective habitat and how does the fitness of the different classes of hybrids compare with each other and with *S. dioica* and *S. latifolia*? Does flowering time of the species and their hybrids overlap? (Chapter III)
- 4) Do *S. latifolia*, *S. dioica* and their hybrids differ in their survival and/or flowering between the contrasting habitats and is the production of an overwintering rosette a trait under selection in either of these habitats? (Chapter IV)

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Chapter I

Genetic and ecological differentiation in the hybridizing champions

Silene dioica and *S. latifolia*

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Abstract

Ecological differentiation is a major contributor to reproductive isolation and speciation. We investigated habitat differentiation between and within sites in the fully cross-fertile and hybridizing *Silene dioica* and *S. latifolia* using AFLP profiles and corresponding vegetation relevés around individual plants. Nineteen study sites in the Swiss Alps included pure sites and contact sites (both taxa present within 30 m). In pure sites and at contact sites, the two taxa showed consistently differentiated AFLP banding patterns across regions but few discriminating bands. This indicates that although the two taxa are weakly differentiated, current introgression has not led to genome-wide admixture. Only three putative early-generation hybrids were detected at contact sites. The habitats of the two taxa differed between pure sites with *S. dioica* occurring in moister, colder and less disturbed sites than *S. latifolia*. However, asymmetric habitat overlap was evident with contact sites found in intermediate conditions that were more similar to *S. latifolia* sites. This situation might favor introgression from *S. dioica* into *S. latifolia*. Evidence for habitat-genotype associations within contact sites was weak making habitat-mediated selection against intermediate phenotypes of hybrids unlikely in the contact sites investigated. We suggest that other reproductive barriers together with dispersal limitation contribute to the rarity of early-generation hybrids.

Key words: adaptation, hybridization, ecological selection

Introduction

Ecological differentiation has been identified as one of the generally important factors preventing co-adapted gene pools from mixing with each other and is thus a major contributor to biological divergence (Funk et al. 2006; Nosil et al. 2005; Rieseberg and Carney 1998). Whether or not ecological differentiation is causal for the historical divergence of lineages is difficult to determine, but it is clear that ecological and reproductive differentiation are associated (Funk et al. 2006). In ecologically differentiated taxa with incomplete reproductive isolation, intermediate phenotypes that arise from hybridization can be at an ecological disadvantage in both parental habitats (Anderson 1948) preventing them from mating (e.g., ecologically-dependent post-zygotic isolation Rundle and Nosil 2005). If some hybrids survive to mate, however, mixing of the gene pools at adaptively neutral loci would be expected while the loci related to habitat adaptation would become associated with the respective habitats (Gavrilets and Vose 2005). Ecologically-dependent reproductive isolation also has to be dismissed when the ecological amplitudes of the taxa under consideration overlap and they are able to occur under the same, possibly intermediate habitat conditions (e.g., niche overlap, Gravel et al. 2006). Here, little ecological disadvantage of phenotypically intermediate individuals is to be expected and thus, in the absence of other reproductive barriers, free mixing between taxa should occur. This would generate hybrid swarms and potentially obliterate the differences between taxa (Rhymer and Simberloff 1996). In the case of habitat overlap, habitat-mediated selection could only occur after dispersal to the more extreme habitats of each taxon and it may be the nature of these dispersal processes that control the mixing or maintenance of the taxa (Metcalf and Pavard 2007).

Investigating habitat-genotype interactions depends critically on the definition of the habitats, yet few modern studies in plants investigate the habitats. Rather, habitat differentiation is inferred from geographic patterns (e.g. Ramsey et al. 2003). In order to identify environmental factors that differentiate taxa it is necessary to conduct field studies that span a range of different sites of each taxon. This way, the outcome of the interplay of various abiotic factors (soil, climate) and biotic interactions (competition, herbivory) can be assessed. However, historical or current dispersal processes also influence habitat-genotype associations observed in natural

populations. Most taxa can occupy a range of optimal to marginal habitats and marginal habitats can be a bridge to gene flow in hybridizing taxa (e.g., Choler et al. 2004). Sites where both taxa can survive (contact sites) can also be created by natural or artificial disturbance (Anderson 1948; Baker 1948; Bleeker and Hurka 2001; Lamont et al. 2003).

The best studied examples of habitat differentiation in hybridizing organisms with information on both the within-site and the between-site scale come from small, mobile animals, including *Gryllus* crickets, *Bombina* toads, and *Chorthippus* grasshoppers. Studies on *Gryllus* crickets gave evidence for strong habitat-genotype associations by soil type at both the between-sites scale and the within-site scale suggesting that these differences in habitat adaptation maintain species integrity (Rand and Harrison 1989; Ross and Harrison 2002). In *Bombina* toads, strong habitat differentiation by pond type was observed only at the between-sites scale, whereas strength of within-site habitat differentiation varied between study areas (MacCallum et al. 1998; Vines et al. 2003; Yanchukov et al. 2006). In contrast, only weak habitat differentiation by vegetation type was observed at the between-sites scale in *Chorthippus* grasshoppers and, within sites, habitat differentiation was entirely absent suggesting that other mechanisms, such as extinction-colonisation dynamics and historical range expansions contributed to the structure of the hybrid zone (Bridle et al. 2001; Bridle et al. 2002). In plants, work on *Iris* has shown very fine environmental gradients separating two species and backcross hybrids within a site (Cruzan and Arnold 1993; Johnston et al. 2001) and for hybridizing species of *Aquilegia*, strong altitudinal and habitat differentiation between sites was demonstrated (Hodges and Arnold 1994).

The hybridizing taxa *S. dioica* and *S. latifolia*, native to Europe, present a puzzling case in that they show full cross-fertility (Goulson and Jerrim 1997) but unambiguous early-generation hybrids are rarely found in natural hybrid zones thus far (Minder et al. 2007). However, in the two hybrid zones of *S. dioica* and *S. latifolia* studied by Minder et al. (2007) only one contact site with plants resembling both taxa genetically and morphologically was included raising the possibility that other such contact sites contain a higher number of intermediate forms. On the other hand, a lack of intermediates could be due to strong habitat differentiation, as suggested by

Goulson & Jerrim (1997). Within-site habitat differentiation would exert selection against intermediate phenotypes arising from hybridization and favor individuals that are similar to either taxon (Anderson 1948; Nosil et al. 2005). The two taxa are easily crossed and pollen transfer between them is common, although *S. latifolia* has white flowers that open at night and *S. dioica* has red or pink flowers that open mainly during the day (Baker 1947a; Baker 1947b; Friedrich 1979; Goulson and Jerrim 1997). *Silene dioica* is described from meadows, pastures and moist forests while *S. latifolia* is described to grow in more open habitats, such as hedgebanks, and as a weed in arable fields (Baker 1947a; Baker 1947b; Friedrich 1979).

In this study, we investigate genetic and ecological differentiation between *S. latifolia* and *S. dioica* at the between-sites and the within-sites scale (contact sites: both taxa present within 30 m) using AFLP profiles and corresponding vegetation relevés around individual plants. Specifically, we ask (1) Are the two taxa separated genetically between sites and within sites? (2) Do contact sites differ from other sites of the two taxa? (3) Is there habitat overlap between *S. dioica* and *S. latifolia*, within or between sites?

Material and methods

Study taxa

Silene dioica (L.) Clairv. (synonym: *Melandrium dioicum* (L.) Simonkai) and *S. latifolia* Poiret (synonyms: *Silene alba* [Miller] Krause, *Silene pratensis* [Rafn.] Godron & Gren., *Melandrium album* [Miller] Garcke) are dioecious, short-lived, perennial herbs of the Caryophyllaceae family that are closely related (Desfeux and Lejeune 1996). *Silene dioica* is a red- or pink-flowered, wintergreen rosette plant up to 80 cm high with a shallow root system; its natural range covers mainly Central, Northern, and Western Europe (Friedrich 1979). *Silene latifolia* grows up to 120 cm high, does not produce a rosette during winter and possesses a thickened taproot. This taxon has an extensive range and occurs in most of Europe, as well as in Middle Asia and the Steppe area of South Siberia (Friedrich 1979). In the study area, Southern Switzerland, both taxa are widely distributed, however, *S. dioica* is more common particularly at higher altitudes (Lauber and Wagner 2001)

Study sites and field work

Study sites were distributed throughout the Swiss Alps (Table 1, Fig. 1) including eight sites with populations of red- or pink-flowering *S. dioica*-like phenotypes, six sites with white-flowering *S. latifolia*-like phenotypes and five contact sites with individuals of both and sometimes intermediate phenotypes. Unfortunately, it is impossible to identify the hybrid status morphologically using floral traits (Minder et al. 2007).

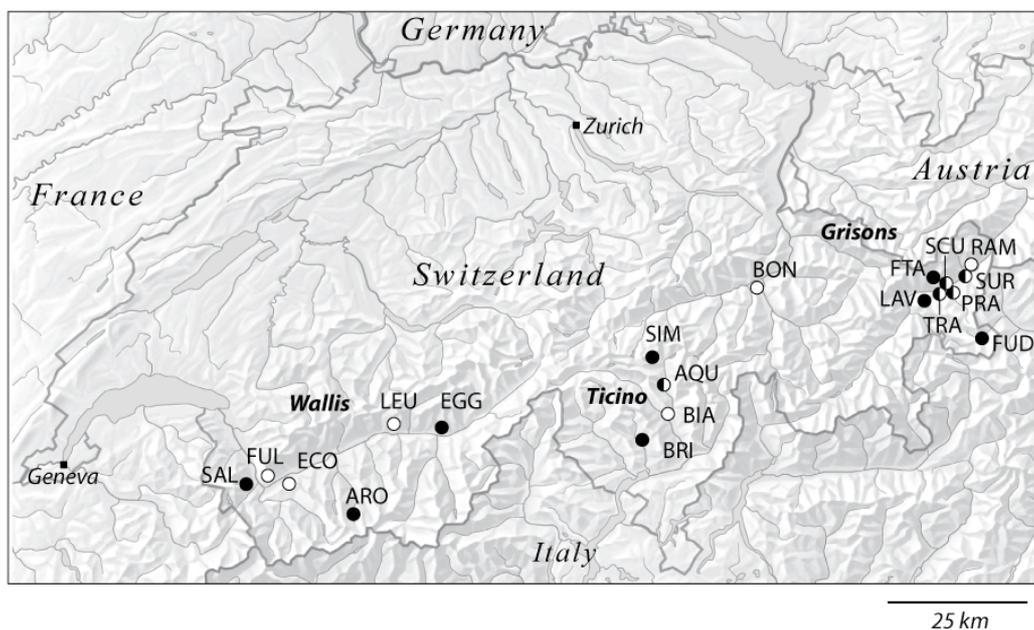


Figure 1 Map of Switzerland with study sites. Full circles: *Silene dioica*, empty circles, *S. latifolia*, half full circles: both taxa and intermediates in some cases (AQU and PRA).

From crossing studies we know that white-flowering individuals are likely to be *S. latifolia*, but red- or pink-flowering individuals can be either hybrids or *S. dioica* due to the large variability of flower color within *S. dioica* among and within populations as well as within individuals (unpublished results). Contact sites were defined here as sites where red- or pink-flowered hybrid or *S. dioica*-like individuals and white-flowered *S. latifolia*-like individuals occurred within 30 m of each other. Four of the five contact sites were situated in the region of Grisons (populations PRA, SCU, SUR, TRA), and the fifth in the region Ticino (population AQU). In the third study region, Valais, no contact sites were found despite intensive search.

In spring and summer 2006, ten *Silene* individuals were selected at random in each study site. We determined sampling points by laying one or several transects through the site. At regular intervals (5–50 m depending on site and population structure) we took pre-determined left or right turns of further 0–10 m and chose the closest *Silene* individual in reproductive stage, i.e. either bolting, flowering, fruiting or with dry reproductive stems. In contact sites, individuals of each phenotype, i.e. red/pink (hybrid or *S. dioica*) and white (*S. latifolia*) were included using this procedure.

To characterize the microhabitat of each sampled individual, 1 m² vegetation relevés around the focal plant were taken with abundance scores on the Londo scale (Dierssen 1990). Apart from the species-abundance list, total vegetation cover was estimated. Nomenclature follows Lauber and Wagner (2001).

Molecular analysis and genotyping

From each study plant, a leaf sample was taken and immediately placed into silica gel. DNA was extracted following Mogg and Bond (2003) with small modifications. DNA concentration was controlled using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, Delaware USA). Amplified fragment length polymorphisms (AFLP) were produced according to Vos et al. (1995) with modification detailed in Bratteler et al. (2006). EcoRI/TaqI restriction enzymes were used with the three different combinations primer extensions: EcoRI-AGC/TaqI-GCT, Eco R1-ACC/TaqI-CCC, and EcoRI-AGC/TaqI-ATG, the latter two correspond to BT6 and ET4 used in Minder et al. (2007). Fragments were separated on a capillary sequencer (ABI 3130x, Applied Biosystems, Foster City, California, USA) and scored as dominant markers using GeneMapper version 4.0 (Applied Biosystems); loci were scored independently of work done by Minder et al. (2007). Further analysis was restricted to polymorphic AFLP bands.

To estimate repeatability of AFLP scoring (Bonin et al. 2004), 16 individuals from 16 different sites covering all rows and 16 of 24 columns of the two 96-well extraction plates were randomly selected for re-scoring at all previously identified bands. Error rates per locus in 220 initially scored bands varied between 0 and 9

non-identical scores out of 14–16 individuals (some wells did not produce results in some primer pairs). 41 AFLP bands with error rates equal to or exceeding 25 percent of the cases were removed from the dataset, reducing the dataset to 179 AFLP bands with a mean error rate of 8.2 percent. For six individuals, amplification problems for one of the three primer pairs could not be resolved. However, with the data from the remaining two primer pairs (115 polymorphic AFLP bands), these individuals could be assigned to either *S. dioica* or *S. latifolia* unambiguously. For further five individuals clean peaks could not be produced for two primer pairs despite repeated amplification. These individuals were removed from the dataset reducing the number of individuals to 185.

Statistical analysis

The AFLP scores were treated as a binary (presence/absence) matrix and genetic distances were calculated as simple mismatch coefficients (Kosman and Leonard 2005; Meudt and Clarke 2007), Nei's genetic distance (=Dice coefficient) and the Jaccard index were also calculated. Groups of genetically similar individuals were identified graphically with principal coordinates analysis of the distance matrix using the *ade4* package for R (Chessel et al. 2004; R Development Core Team 2006). To investigate whether differences between taxa are due to specific AFLP bands or are also present in multivariate combinations of AFLP bands, this analysis was repeated without bands that had greater than 70 percent frequency differences between the taxa in pure sites. The significance of groups observed in the PCO analysis was assessed by subjecting the genetic distance matrix to hierarchical cluster analysis (complete linkage) with bootstrap re-sampling using the *pvclust* package for R with 1000 bootstrap replicates (Suzuki and Shimodaira 2006). Ordinary bootstrap *P*-values (BP-values) are reported instead of approximately unbiased bootstrap values (AU-values) because a possible breakdown of asymptotic theory was indicated by z-value by \sqrt{r} diagnostic plots for higher-level nodes (Suzuki and Shimodaira 2006). Furthermore, genetic differentiation between species at pure sites and at contact sites was quantified with AMOVA and significance of Φ_{st} -values was assessed by permutation tests with 999 permutations. This analysis was conducted using GenAlex version 6 (Peakall and Smouse 2006).

From each relevé, mean Ellenberg indicator values (Ellenberg et al. 1992) for requirements of moisture, temperature, nitrogen, soil pH, and light, were calculated using presence/absence data. Ellenberg indicator values are available for almost all Central European plant species and are widely used. They have been shown to correspond to measurements of abiotic conditions (e.g. Ertsen et al. 1998). Especially when long-term measurements of abiotic conditions are unavailable or impractical, mean Ellenberg indicator values of plant communities serve as an integrated measure of site conditions. The species list of each relevé was further used to determine the percentage of annual plants (Ellenberg et al. 1992). To identify differentiating habitat factors between pure sites and contact sites and between the two taxa within contact sites, linear mixed model analyses were conducted. Ecological parameters were compared between *Silene latifolia* sites, *S. dioica* sites and contact sites using site type as a fixed factor and sites (random factor) as the error term. Where appropriate, this test was followed by multiple comparisons between the three site types with false discovery rate control (Venables and Ripley 2002; Verhoeven et al. 2005). Habitat differentiation within contact sites was analyzed using a mixed model with taxa (fixed) and sites (random); the residuals were used as the error term. We believe this is appropriate because we did not expect site-by-taxon interactions, and neither did we find significant interactions when tested in a fixed-effects model (Newman et al. 1997). Because only few hybrids were detected (see results), hybrids were excluded from the within contact sites analysis but not from the between sites analysis. Two habitat variables on the percent scale (vegetation cover and annuals) were transformed using arcsin ($Y^{-0.5}$). For all models, normality of residuals and model fit was ensured by inspection of diagnostic plots (Venables and Ripley 2002). Transformations were retained in all further analyses and where means are given these were back-transformed to the original scale. To aid the interpretation of the results, Pearson correlations of altitude with site means of all habitat variables were calculated. False discovery rate control (Verhoeven et al. 2005) with an overall significance level of 0.05 was applied to fixed effects P-values in all three sets of tests starting with an adjusted significance level of $0.05/7=0.0071$ for the lowest P-value, because seven variables that were taken on the same relevés were analyzed.

To further understand the relationship within and between sites, habitat variables and floristic composition were ordinated separately using the *ade4* package for R (Chessel et al. 2004; R Development Core Team 2006). Habitat variables were ordinated using principal components analysis with centering by the mean. Floristic composition was analyzed using the same approach as for the AFLP data with a principal coordinates analysis conducted on a distance matrix, in this case using Jaccard indices that were calculated from presence/absence data (Dierssen 1990). Relevés were analyzed as binary data after initial analyses using cover values had shown excessive variation within similar relevés. All statistical analyses, except AMOVA (see above), were performed in R (R Development Core Team 2006).

Results

Genetic differentiation

Of the 179 AFLP bands analyzed, only 18 had frequency differences between the two taxa (pure sites) greater than 70 percent (supplemental Fig. S1). Of these, 14 were more common in *S. dioica* and four were more common in *S. latifolia*. Principal coordinate analysis on simple mismatch coefficients of AFLP genotypes produced nearly identical ordinations whether or not these discriminating AFLP bands were included (Figs. 2 and S2): along the first axis two clearly separated groups were found that correspond to *S. dioica* (left side of Fig. 2A and S2A, black symbols) and to *S. latifolia* (right side of Fig. 2A and S2A, grey symbols). Along the second axis, a strong separation of *S. dioica* populations is evident, while *S. latifolia* populations were much more similar to each other. *Silene dioica* populations appear to be differentiated by regions with Valais populations on top, Grisons populations intermediate and Ticino populations on the bottom of Figs. 2A and S2A. The third and fourth axes did not reveal any additional patterns (not shown). The five contact sites (Figs. 2B and S2B, full symbols) almost exclusively contained individuals that clearly grouped with one of the two taxa. Only three genetically intermediate individuals, two in the population AQU in the region Ticino and one in population PRA in the region Grisons were identified (grey full symbols with black margin, Figs. 2 and S2). Without the discriminating AFLP bands, only one individual (PRA) was clearly genetically intermediate (Fig. S2). In this individual, discriminating AFLP bands of

both taxa were detected (two *S. latifolia* bands and ten *S. dioica* bands). The other two putative hybrids (AQU) were closer to the *S. latifolia* cluster when the discriminating AFLP bands were omitted (Fig. S2B); these individuals exhibited about half of the discriminating AFLP bands for *S. dioica* (six and seven AFLP bands) but none of the four discriminating AFLP bands for *S. latifolia*. Identical conclusions were reached when using Nei's genetic similarity index (= Dice coefficient) or the Jaccard coefficient (data not shown).

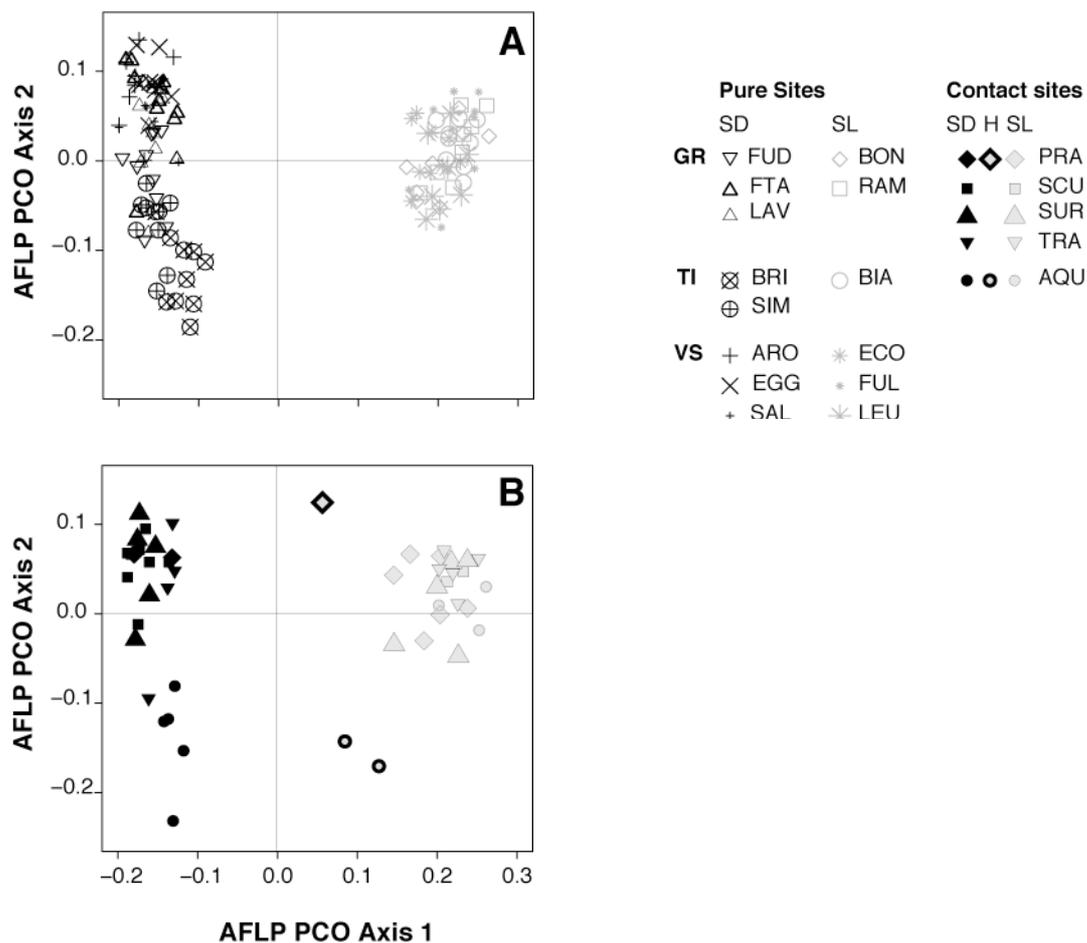


Figure 2 Principal coordinates analysis (PCO) of simple mismatch coefficients of 179 polymorphic AFLP bands in pure populations (**A**) of *Silene dioica* (SD) and *S. latifolia* (SL), as well as in contact sites (**B**) with both taxa and sometimes hybrids (H). Both figures are from the same PCO analysis but site types were separated for clarity (Axis 1: $\lambda=0.032$, 24% of variation explained, Axis 2: $\lambda=0.007$, 6% of variation explained).

Cluster analysis of the distance matrix (simple mismatch coefficients, full dataset) further supported the findings from the PCO: The two main clusters with 99 percent bootstrap support correspond to *S. dioica* and *S. latifolia* (Fig. S3). The three hybrid individuals clustered together and were included in the *S. latifolia* cluster, however, this clustering was highly unstable as would be expected of hybrids (15 percent bootstrap support for the hybrid cluster, 14 percent for the remaining *S. latifolia* cluster). All other individuals from contact sites grouped with individuals of pure sites. Clusters indicating the substructure within *S. dioica* or *S. latifolia* populations generally had low support (<60 percent) as did all other lower-level clusters, except for the cluster of the two putative hybrid individuals in population AQU (93 percent bootstrap support). AMOVA further indicated significant and large differentiation between species both at pure sites and at contact sites with Φ_{st} -values of 0.39 for pure sites and 0.40 at contact sites (both P-values were 0.001). Differentiation between populations within species was much smaller but also significant with Φ -values of 0.09 (P-value: 0.001) and 0.06 (P-value: 0.02) for pure sites and contact sites, respectively.

Table 2 Between sites analysis of habitat variables (Ellenberg indicator values [IV], % annuals and % vegetation) in *Silene dioica* sites, *S. latifolia* site and contact sites in the Swiss Alps (using site identity as the error term) and Pearson correlations of site means of habitat variables. P-values that were significant after false discovery rate control for multiple testing of seven variables are indicated in bold type.

Habitat variable	Site type effect df=2, error df =16		Correlation with altitude	
	F-value	P-value	r	P-value
Moisture IV	11.30	0.0009	0.529	0.019
Soil pH IV	3.24	0.066	-0.223	0.360
Light IV	2.10	0.154	-0.032	0.894
Temperature IV	15.23	0.0002	-0.766	0.0001
Nitrogen IV	2.28	0.134	0.359	0.131
% annuals*	6.77	0.007	-0.412	0.079
%Vegetation cover*	1.41	0.272	0.430	0.066

*Transformation before analysis: $\arcsin(Y^{-0.5})$

Habitat differentiation between sites

Altogether, 314 species were detected, with an average number of 45 species per site (range 31–71, Supplementary Table 1). Three environmental variables were significantly different between *S. dioica* and *S. latifolia* and contact sites: these are indicator values for moisture and temperature requirement and percent annuals (Table 2). *Silene dioica* sites had significantly higher indicator values for moisture requirement, significantly lower indicator values for temperature requirement and significantly lower percent annuals than did *S. latifolia* sites (Table 3). Contact sites had intermediate values for all three variables but were more similar to *S. latifolia* sites; indicator values for temperature requirement were significantly different from both types of pure sites, while indicator values for moisture requirement and percent annuals were significantly different only from values in *S. dioica* sites (Table 3). Of the seven habitat variables analyzed, only site means of indicator values for temperature requirement were significantly correlated with altitude (Table 2). Study sites above 1500 m contained only *S. dioica* and sites below 700 m only *S. latifolia*, but for the largest part of the altitudinal range studied here, sites of both taxa and contact sites were found (Table 1).

Table 3. Means of site means with 1 SE for seven habitat variables (Ellenberg indicator values [IV], % annuals, % vegetation cover) in *Silene dioica* sites, *S. latifolia* sites and contact sites in the Swiss Alps. Means that do not share a letter are significantly different. No letters are given when the site type was not to significant overall (compare Table 2).

	Site type		
	<i>S. dioica</i>	Contact site	<i>S. latifolia</i>
Moisture IV	5.33 ± 0.14 ^a	4.82 ± 0.09 ^a	4.42 ± 0.15 ^b
Soil pH IV	6.40 ± 0.08	6.73 ± 0.05	6.76 ± 0.20
Light IV	6.59 ± 0.23	6.89 ± 0.14	7.15 ± 0.15
Temperature IV	4.63 ± 0.23 ^a	5.60 ± 0.06 ^b	5.89 ± 0.08 ^c
Nitrogen IV	5.83 ± 0.27	5.87 ± 0.11	5.12 ± 0.25
% annuals ^a	1.6 ± 0.01 ^a	10.4 ± 0.05 ^b	11.5 ± 0.04 ^b
%Vegetation cover [*]	79.7 ± 0.10	78.1 ± 0.04	79.7 ± 0.07

^{*}back-transformed from arcsin (Y^{-0.5})-transformation

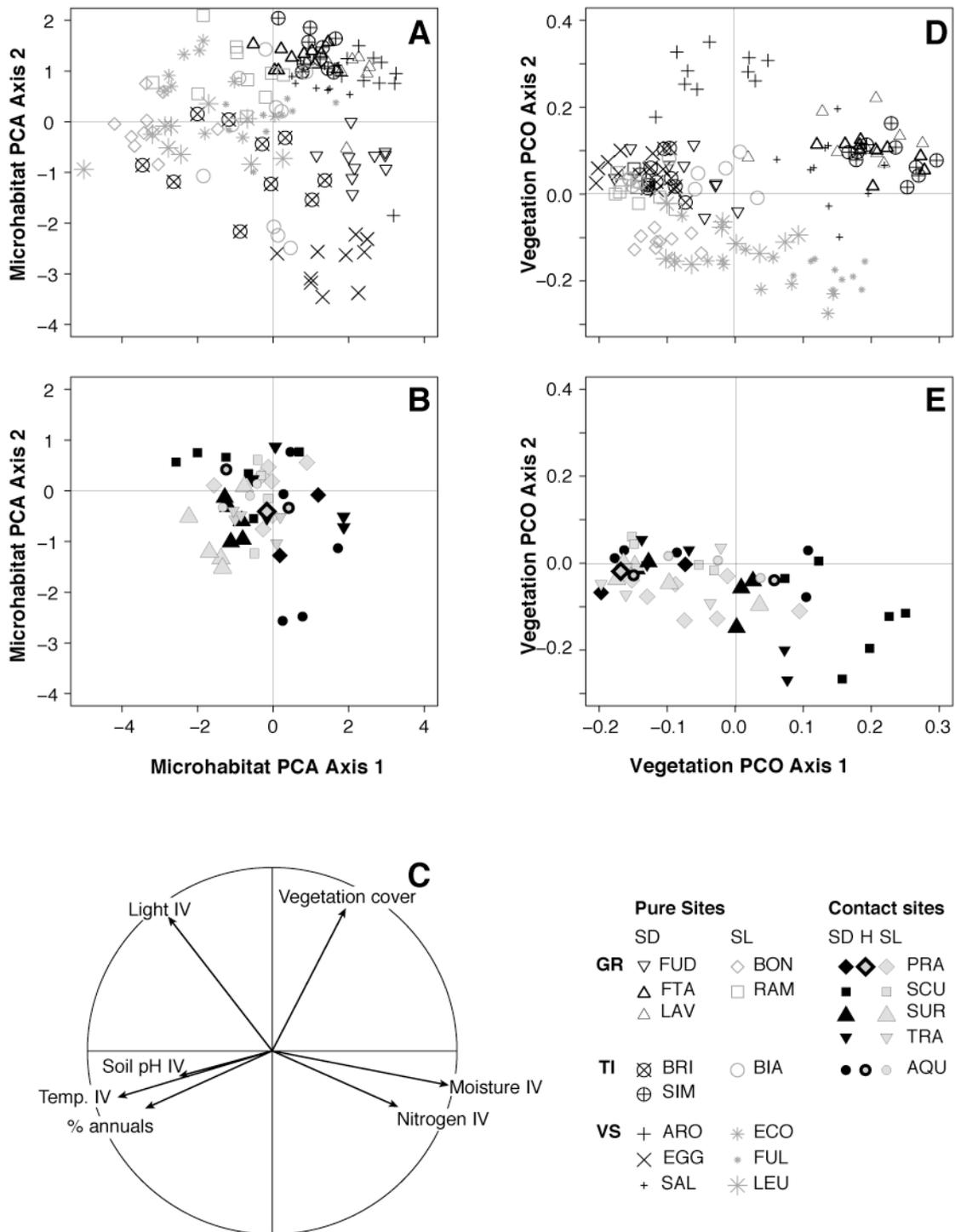


Figure 3 Principal components analysis (PCA, **A–C**, Axis 1: $\lambda = 2.86$, 41% of variation explained, Axis 2: $\lambda = 1.41$, 20% of variation explained) of seven habitat variables (Ellenberg indicator values [IV], % annuals and % vegetation cover,) and principal coordinates analysis (PCO, **D–E**, Axis 1: $\lambda = 0.019$, 4% of variation explained, Axis 2: $\lambda = 0.014$, 3% of variation explained) of vegetation composition using the Jaccard index in 1 m² vegetation relevés around individuals of *Silene dioica* (SD) and *S. latifolia* (SL) in pure sites (**A, D**) and within contact sites (**B, E**) where both taxa and sometimes hybrids (H) were found. Panels A-C and E-F correspond the same PCA and PCO, respectively, but site types were separated for clarity.

The distinction between *S. latifolia* and *S. dioica* habitats is also evident in ordinations of habitat variables (Fig. 3A) and floristic distance (Fig. 3D). However, both ordinations, especially floristic distances, also show overlap between sites of the two taxa and exhibit strong differentiation between sites of the same taxon, particularly within *S. dioica*. The position of contact sites is intermediate with regard to habitat variables (Fig. 3B) as may be expected from the univariate tests (Table 2, see above). In the floristic ordination (Fig. 3D), however, contact sites clearly group with *S. latifolia* sites and the floristic overlap zone, whereas *S. dioica* sites from subalpine meadows/pastures from all three regions (SAL, FTA, SIM, LAV) and the highest-elevation *S. dioica* site ARO (top and right, Fig. 2D) are clearly distinct from all other sites. The *S. latifolia* sites are more homogeneous in floristic composition and less separated from the overlap zone.

Table 4 Within-sites analysis of habitat variables (five Ellenberg indicator values [IV], % annuals, % vegetation cover) in five contact sites between *S. dioica* and *S. latifolia* in the Swiss Alps. All explanatory variables were tested over the residual error with 41 df. Three genetically intermediate individuals were excluded from the analysis.

Habitat variable	F-value	P-value
Moisture IV	7.989	0.0072*
Light IV	0.575	0.453
Nitrogen IV	0.732	0.397
Soil pH IV	0.253	0.618
Temperature IV	0.004	0.953
% Vegetation cover	0.737	0.396
% annuals	2.509	0.121

* marginally significant with false discovery rate controlled $\alpha=0.05/7=0.0071$ for the lowest P-value.

Habitat differentiation within contact sites

Evidence for ecological differentiation of *S. dioica* and *S. latifolia* within contact sites is weak. After correction for multiple testing, one habitat variable, the indicator

value for moisture requirement, was marginally significantly different between the two taxa within contact sites (Table 4). From the ordination results (Fig. 3B, E), it is also evident that contact sites exhibit diverse habitat conditions without clear separation of the two taxa.

Discussion

The two related and fully cross-fertile taxa *S. dioica* and *S. latifolia* show strong and consistent differences in AFLP banding patterns as shown by PCO and cluster analyses and AMOVA. This differentiation is clearly not only due to the 18 out of 179 discriminating AFLP bands detected, but is also evident in multivariate associations of AFLP bands when discriminating bands are removed from the dataset. Geographically distant (>300km) populations of each taxon are genetically similar to each other and differentiated from populations of the other taxon growing nearby or even in the same site. Current massive introgression would lead to a grouping by regions rather than by species. Thus, current or recent introgression has not led to genome-wide admixture across the studied range of populations but it cannot be ruled out with the data at hand that historical introgression events contributed to the similarity of the two species (Minder et al. 2007). The low percentage of discriminating AFLP bands (10 percent, frequency difference >70 percent) is similar to the results of Minder et al. (2007) and to studies in other hybridizing taxa (e.g., Choler et al. 2004; Hansen et al. 2003). Apart from historical introgression, such weak differentiation could be due to shared ancestry in these closely related taxa (Desfeux and Lejeune 1996), non-homology of AFLP bands, high variability within taxa or any combination of these factors (Meudt and Clarke 2007). The discriminating bands, on the other hand, may represent candidate loci for adaptive genetic differences between the taxa (compare Gavrillets and Vose 2005), but further genetic and functional analyses are necessary to separate such adaptive differences from selectively neutral differences that can arise from genetic drift. Our findings support the current designation of the two taxa as species. However, species definitions are under fervent debate and little consensus has been reached thus far on how the hybridizing species should be dealt with (Hey 2001; Rundle et al. 2001).

Within contact sites, all but three individuals clearly grouped with either *S. dioica* or *S. latifolia* indicating that hybrid individuals are either rarely produced or do not survive to reproductive age that was sampled in this study. This bimodal pattern is typical for many hybrid zones, such as those found in *Iris* and *Gryllus* (Jiggins and Mallet 2000). The genetically intermediate individuals probably correspond to early-generation hybrids that were similarly rare in a study of Minder et al. (2007). Early-generation backcross individuals are expected to lie between intermediate hybrid and pure taxa in the PCO, and indeed some individuals from contact sites are at the margin of the *S. latifolia* cluster in the PCO and may be later-generation backcrosses. The genotypic status of these individuals is difficult to judge because the number of differentiating AFLP bands for *S. dioica* and *S. latifolia* is low. Simulation studies indicate that 50–70 species-specific markers are desirable for unequivocal identification of advanced generation backcrossed genotypes (Boecklen and Howard 1997). For the habitat analysis, this distinction between pure individuals and advanced-generation backcrosses is not as important because, in bimodal hybrid zones such as this one, individuals that closely resemble one species are likely have ecological preferences very similar to that species (Barton and Hewitt 1985; Cruzan and Arnold 1993; Johnston et al. 2001).

This study shows a clear habitat differentiation of the two taxa between pure sites, but not within contact sites. The between-site habitat differences are consistent with the morphological differences between the taxa: The lower indicator values for moisture for relevés around *S. latifolia* are likely related to this taxon's deep, thickened taproot that may allow the plant to survive dry spells while *S. dioica* grows thinner, more shallow roots (Baker 1947a; Baker 1947b; Friedrich 1979). The higher temperature requirement of plants growing near *S. latifolia* as compared to those growing near *S. dioica* could be related to differences in growth at low temperatures. Alternatively, this relationship could be a corollary of spatial differentiation because temperature requirement was correlated with altitude, unlike the other environmental variables. Such spatial differentiation by altitude could result from reduced availability of suitable open habitats for *S. latifolia* at higher altitudes or be due to an incomplete colonization of the valleys after the Ice Ages (compare Baker 1948). The higher incidence of annuals in *S. latifolia* sites speaks for higher disturbance at those

sites that is currently due to agriculture or other human practices. *Silene dioica* might have a higher competitive ability than *S. latifolia* because it produces a wintergreen rosette (Baker 1947a) that allows it to persist in dense vegetation. From these results, we predict that differentiating ecological traits of the two taxa include higher drought tolerance in *S. latifolia*, higher competitive ability and possibly higher growth rates at low temperatures in *S. dioica*.

Despite this habitat differentiation it is also evident that the two taxa share habitat conditions. All five contact sites were found in intermediate ecological conditions, but resembled *S. latifolia* sites in terms of indicator values for moisture, disturbance regime (percent annuals) and floristic composition. This asymmetry might contribute to the higher incidence of introgression from *S. dioica* to *S. latifolia* than vice versa (Minder et al. 2007). All five contact sites were associated with massive disturbance either natural or artificial (Table 1) and thus, human activities probably increase the incidence of contact sites between this pair of *Silene* taxa (Baker 1948) as has also been reported for *Banksia* (Lamont et al. 2003) and *Rorippa* (Bleeker and Hurka 2001). Hybridization in the two *Silene* taxa is by no means a new phenomenon though, and has been observed for several centuries (Baker 1948; Friedrich 1979). Several sites with one of the taxa were also found on intermediate conditions similar to those of contact sites (e.g., BRI, BIA) and these are candidates for the development of new contact zones should human or natural disturbance disperse the other taxon to these sites. Other sites of both taxa were strongly differentiated from the contact sites and the intermediate sites and may serve as habitat refuges for example the higher altitude meadow sites of *S. dioica* (ARO, LAV, FTA, SIM) and dry, disturbed sites of *S. latifolia* (BON, FUL). Habitat-mediated selection against immigrants or hybrids likely occurs on these differentiated sites.

In contrast to between-sites habitat differentiation, evidence for habitat differences within contact sites is weak, paralleling the situation in hybrid zones of *Chorthippus* (Bridle et al. 2002) and some *Bombina* hybrid zones (Vines et al. 2003). In *Silene*, indicator values for moisture requirement that differentiated between the pure sites of the two taxa had marginally significant differences between taxa within sites. While it is arguable how and whether P-values given should be adjusted

(Moran 2003; Verhoeven et al. 2005) and our analysis is conservative, this might indicate that the habitats of the two taxa are differentiated by minute differences in moisture availability within certain contact sites. However, because this difference is very small and data from pure populations indicates that both taxa can grow under such conditions, we have little evidence for within-site habitat differentiation. Therefore, hybrid inferiority in both habitats (Anderson 1948; Nosil et al. 2005) as a possible reproductive barrier between *S. dioica* and *S. latifolia* is not supported for the contact sites investigated.

The remaining possible explanations for the low incidence of hybrids at contact sites despite full cross-fertility and low pollinator fidelity (Goulson and Jerrim 1997; van Putten et al. 2007) include low hybrid fitness under field conditions, pollen competition, spatial aggregation of taxa within sites (Barton and Hewitt 1985), and dispersal limitation. Under greenhouse conditions, F_1 and early generation backcrosses are vigorous and fertile (personal observation) but neither their relative success under natural circumstances nor a possible role of pollen competition or spatial aggregation in hybridization has been tested to date. The presence of both pure and mixed populations at intermediate conditions speaks for dispersal controlling the presence of each taxon at these sites (compare Metcalf and Pavard 2007). Neither of the two taxa has specialized means of dispersal. In *S. dioica*, allozyme analysis revealed small breeding units in the range of several meters and strong differences between populations (Giles et al. 1998). In *S. latifolia* in its invasive range in North America, cpDNA patterns in a 5m grid suggested limited seed dispersal, while allozyme analysis did not reveal patterns at that scale, probably due to pollen dispersal over longer distances (McCauley et al. 1996). At larger scales of 1–25 km, however, population differentiation was significant also for allozymes (McCauley 1994). Similar results were obtained in the native range of *S. latifolia* using allozymes (Delmotte et al. 1999) and microsatellites (Jolivet and Bernasconi 2007). Limited exchange of genotypes between populations could substantially slow down the spread of introgressed genotypes and thus contribute to the maintenance of the taxa. The rarity of early generation hybrids within contact sites, on the other hand, could also be due to the ephemeral nature of several of these sites. Because both taxa are short-lived (Baker 1947a; Baker 1947b; Friedrich 1979) it is difficult to

assess the number of generations that has passed since the establishment of the populations. In all contact sites investigated, individuals of both taxa as old as three to four years were found, as indicated by root growth rings (unpublished results).

This study shows how detailed investigations of habitat parameters together with genetic analyses at the between-sites and the within-sites scale can help to refine the understanding habitat-mediated selection in hybridizing taxa. We conclude that the genetically differentiated taxa *S. dioica* and *S. latifolia* are also ecologically differentiated but considerable, asymmetric habitat overlap occurs. Both taxa currently meet and mate at highly disturbed intermediate sites that resemble *S. latifolia* sites that may have led to the asymmetrical introgression from *S. dioica* into *S. latifolia* (Minder et al. 2007). Transplant experiments that are currently in progress will reveal whether this habitat differentiation is due to different ecological tolerances of the two taxa as their morphological differences suggest or, alternatively, the result of historical range shifts. Within contact sites, there is little habitat-genotype association making habitat-mediated selection against intermediate phenotypes of hybrids at unlikely at contact sites suggesting that other forms of reproductive barriers could be active. At the more differentiated sites of the two taxa, however, habitat-mediated selection against the non-resident taxon and hybrids could occur. Furthermore, dispersal limitation likely decelerates introgression between the two taxa. These processes could act to preserve the genetic integrity of both taxa in the absence of habitat differentiation at contact sites.

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Supplementary Material

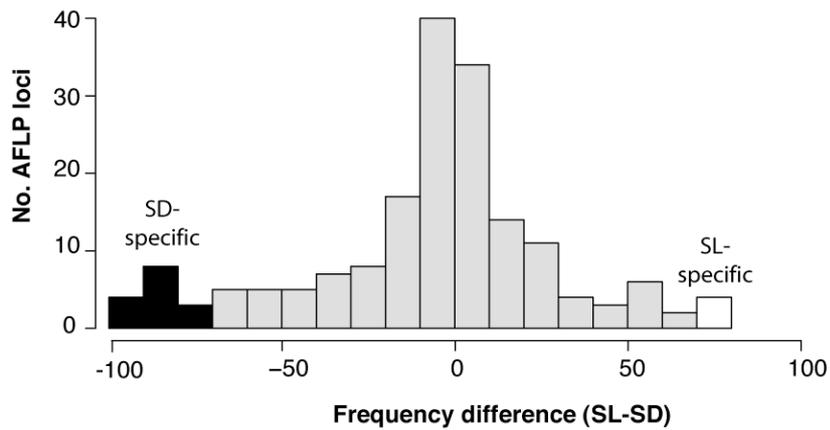


Figure S1 Distribution of frequency differences of 179 AFLP bands between populations of *Silene dioica* (SD, 80 individuals from eight populations) and *S. latifolia* (SL, 53 individuals from six populations) in Switzerland.

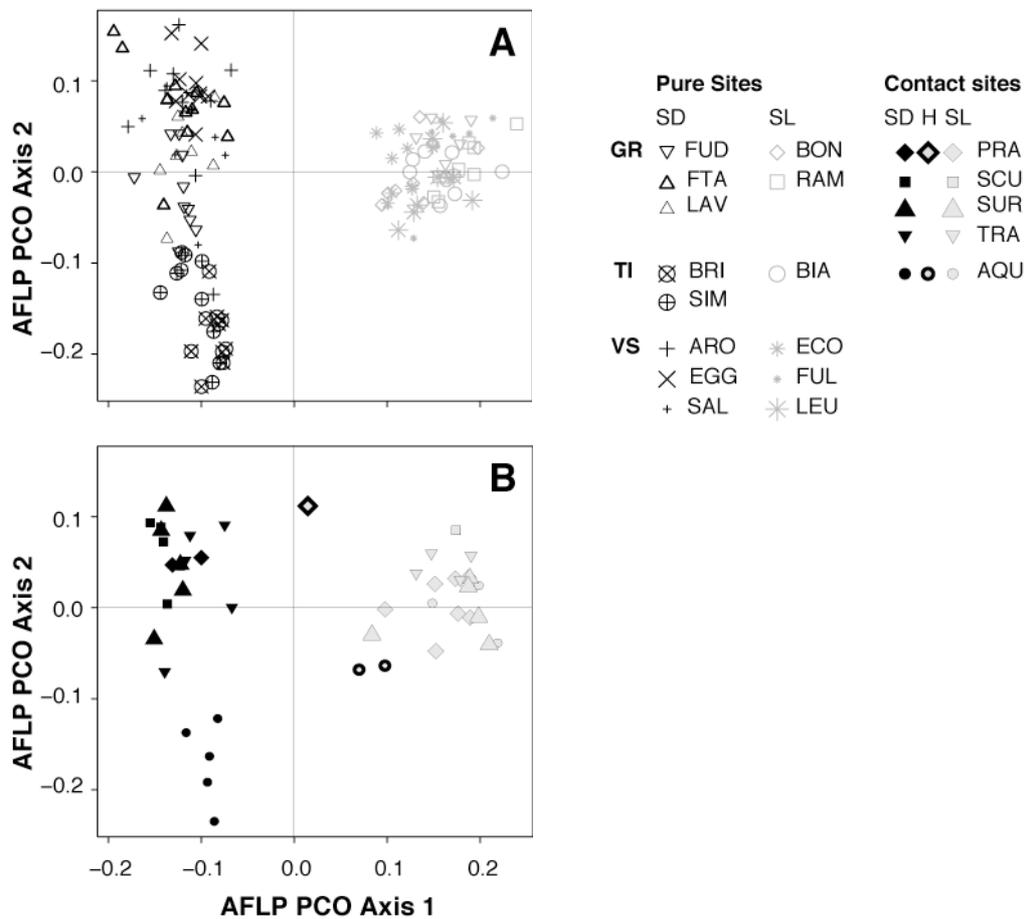




Figure S3 Hierarchical cluster analysis (complete linkage) using simple mismatch coefficients of 179 polymorphic AFLP bands obtained from in pure populations of *Silene dioica* and *S. latifolia*, as well as from contact sites with both taxa within 30m. Bootstrap support values are reported to the right of selected nodes, and were <60% where not given.

Chapter II

Stress tolerance in *Silene dioica*, *S. latifolia* (Caryophyllacea) and first-generation hybrids between them

Manuscript:

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Abstract

Hybridization is common in natural plant populations and its effect on gene flow depends on the ecological performance of hybrids. We studied responses to shade and drought stress in crosses between and within the naturally hybridizing species *Silene dioica* and *S. latifolia*. Responses to drought stress did not differ between cross types. Shade stress, in contrast, led to a reduced flowering incidence in *Silene dioica* and the hybrids but not in *S. latifolia*. All cross types had an increased specific leaf area (SLA) under shade stress, however, *Silene latifolia* had the strongest plastic response, while both hybrid crosses produced extremely large leaves under shade conditions. A high plasticity and rapid flowering under stress conditions in *S. latifolia* could be an adaptation to higher disturbance in its habitat, whereas a delay of reproduction might be adaptive in the more predictable environment of *S. dioica*.

Key words: plasticity, *Silene dioica*, *Silene latifolia*, stress tolerance

Introduction

Hybridization is common in plants and animals and may lead to extensive gene flow between taxa or even to genetic assimilation (Burgess and Husband, 2006; Levin et al., 1996; Rhymer and Simberloff, 1996). Especially in taxa that are adapted to different habitats, the effect of hybridization in natural populations depends to a large extent on the performance of hybrids in the different habitats. Indeed, hybrids often are confined to disturbed habitats (Anderson, 1948; Baker, 1948; Bleeker and Hurka, 2001; Lamont et al., 2003; Levin et al., 1996) or may occur only in the habitat of one of the species involved, which may lead to asymmetric gene flow (Daehler and Strong, 1997; Karrenberg and Favre, 2008; Mahelka et al., 2007). Asymmetric gene flow can also be caused by maternal effects that result in differences in the performance of hybrids depending on which species is the maternal parent (Burgess and Husband, 2004; Burke et al., 1998; Campbell and Waser, 2001; Sultan et al., 1998; Wu and Campbell, 2005). Ecological differences between species or hybrids can be expressed under all environmental conditions and can also include differences in plastic reactions to environmental stress (Nicotra et al., 1997; Ryser and Eek, 2000; Valladares et al., 2000). Yet, the performance of hybrids is only rarely studied under different conditions (Stift *et al.*, 2008) and most inferences on the consequences of hybridization are made in studies in benign conditions (Brock and Galen, 2005; Burgess and Husband, 2004; Etterson et al., 2007; Kirk et al., 2005; Rhode and Cruzan, 2005) or in few natural habitats (Arnold, 1997; Campbell et al., 2008; Johansen-Morris and Latta, 2006; Miglia et al., 2007). In this study, we use a greenhouse experiment to directly investigate responses to stress in the naturally hybridizing species *S. dioica* and *S. latifolia* and in their reciprocal first-generation hybrids.

Silene dioica and *S. latifolia* are both short-lived perennials and dioecious. These species occupy different environments, with *S. dioica* growing in rich meadows, pastures or forests and *S. latifolia* preferring drier and more disturbed habitats (Baker, 1947a, 1947b; Friedrich, 1979; Karrenberg and Favre, 2008). *Silene latifolia* and *S. dioica* come into contact at sites similar to *S. latifolia* habitat (Karrenberg and Favre, 2008; Minder et al., 2007). Despite their complete cross-fertility, early generation hybrids are hardly ever found in nature (Goulson and

Jerrim, 1997; Karrenberg and Favre, 2008; Minder et al., 2007). Partial barriers to hybridization have been described (Rahme et al., 2009; Waelti et al., 2008) but are not sufficient to explain the rarity of hybrids.

In this study we investigate stress tolerance in *S. dioica* and *S. latifolia* and their first-generation hybrids. Stress factors were chosen from the conditions that differ between the species habitats, i.e. drought and shade (Karrenberg and Favre, 2008). As *S. latifolia* grows in drier habitats, we hypothesize that this species will tolerate drought conditions better than *S. dioica*. On the other hand, forest populations of *S. dioica* are common and shade adaptation has been suggested for this species (McKiernan and Baker, 1991). Therefore, we expect *S. dioica* to better withstand shade stress than *S. latifolia*. If the stress tolerance of the hybrids is lower than that of the two species this may help to explain the rarity of hybrids in natural populations. Alternatively, the performance of hybrids could be intermediate between the species or depend strongly on the stress applied and it is also possible that maternal effects lead to strong differences between the reciprocal hybrids. These latter results would have strong implications for the direction of gene flow between *S. dioica* and *S. latifolia*.

Specifically, we use fitness proxies (survival, flowering incidence, biomass production) and measurements of leaf morphology and root diameter to ask: (1) How do *S. dioica*, *S. latifolia* and their reciprocal hybrids differ in their fitness reduction under drought and shade stress? (2) Does plasticity for leaf morphology and root diameter differ between the two species and their hybrids?

Material and methods

Plant material, crossing design and cultivation

A full-sib-half-sib crossing design was used to obtain nine families of each of four cross types (Fig. 1): crosses within *Silene latifolia* Poiret (SL), crosses within *S. dioica* (L.) Clairv. (SD) and reciprocal crosses between the two species (*S. dioica* female x *S. latifolia* male [HD] and *S. latifolia* female x *S. dioica* male [HL]). Parental

Table 1 Populations of *Silene dioica* and *S. latifolia* in the Swiss Alps from which seed were collected.

	Location	Canton	Coord. (N)	Coord. (E)	Altitude	Habitat
<i>S. dioica</i>						
GOT	Gotthard	UR	46° 35'28.72	8°33'32.81	1800 m	Megaphorbia
DAV	Davos	GR	46°48'10.46	9°53'47.68	1800 m	Open river side
SCH	Susch	GR	46°44'39.74	10°04'45.37	1430 m	Meadow /pasture
<i>S. latifolia</i>						
LEU	Leuk	VS	46°19'12.13	7°38'57.32	950 m	Terrassed fields/fallow
GOR	Gordemo	TI	46°11'12.63	8°50'59.25	400 m	Vinyards
SUR	Sur En	GR	46°48'52.73	10°21'34.70	1120 m	Gravel pit

Experimental design and treatments

The experiment was set up in a greenhouse at Zurich, Switzerland for two growing seasons (2006-2007) under ambient temperatures. The experimental array was a strip-split-plot, composed of nine whole-plots (Cochran and Cox, 1968). Each whole-plot contained four sub-plots to which the following treatments were assigned: benign, drought stress, shade stress and combined stress. Each sub-plot contained one male and one female plant per cross-type, from families related through half-sibs. A whole-plot thus contained 4 treatments x 4 cross types x 2 sexes (32 plants), the entire experiment was comprised of 288 plants. The shading treatment was applied using wooden cages (100 x 100 x 80 cm) covered with shading cloth that retained about 65 % of the ambient light (ST 52, Hortima, AG, Switzerland). Plants under the benign and the shade stress treatments received a trivet that was regularly refilled, whereas for plants under the drought stress and under the combined stress treatments, we withheld watering until six or more of the eight plants in one of the drought stressed sub-plots exhibited severe wilting. At this time, top watering until flow-through was applied to all plants of the whole-plot (all treatments) to avoid differences in nutrient availability. For the benign and shade stress treatments trivets were removed during top watering to allow flow-through. Females were pollinated by hand throughout their flowering time using randomly chosen pollen donors of additional plants of the same families used for the experiment. This treatment is expected to lead to full seed set because seed set and seed weight do not differ between intra and interspecific pollinations (Goulson and Jerrim, 1997; Rahme et al.,

Table 2 Logistic regression (survival, flowering incidence) and mixed model (other traits) results from a stress tolerance experiment in *Silene dioica*, *S. latifolia* and reciprocal first-generation hybrids between them. Terms removed during stepwise backward model simplification are indicated with a hyphen.

	Survival			Flowering incidence			Total biomass		
	Df	F-value	p-value	Df	F-value	p-value	denDf	F-value	p-value
Treatment	3	18.0967	<0.001 ***	3	35.131	<0.001 ***	24	97.7966	<0.001 ***
Cross type	3	0.2484	0.86246 ns	3	14.072	<0.001 ***	24	29.4072	<0.001 ***
Sex	1	3.2502	0.07249 .	1	6.8299	0.009546 **	200	1.2544	0.2639 ns
Treat * Cross	-	-	-	9	2.7383	0.004667 **	-	-	-
Treat* Sex	-	-	-	-	-	-	-	-	-
Cross * Sex	-	-	-	-	-	-	-	-	-
	Seed biomass			Seed number			Seed size		
	denDf	F-value	p-value	denDf	F-value	p-value	denDf	F-value	p-value
Treatment	24	26.8652	<0.001 ***	24	25.569	<0.001 ***	24	2.857	0.0582 ns
Cross type	24	1.85787	0.1637 ns	24	0.972	0.4222 ns	24	2.008	0.1399 ns
Sex	-	-	-	-	-	-	-	-	-
Treat * Cross	-	-	-	-	-	-	-	-	-
Treat* Sex	-	-	-	-	-	-	-	-	-
Cross * Sex	-	-	-	-	-	-	-	-	-
	Flower number			Flower size			Total flower biomass		
	denDf	F-value	p-value	denDf	F-value	p-value	denDf	F-value	p-value
Treatment	24	23.733	<0.001 ***	24	0.49	0.6955 ns	24	23.61557	<0.001 ***
Cross type	24	1.866	0.1624 ns	24	27.39	<0.001 ***	24	3.009124	0.05 *
Sex	-	-	-	-	-	-	-	-	-
Treat * Cross	-	-	-	-	-	-	-	-	-
Treat* Sex	-	-	-	-	-	-	-	-	-
Cross * Sex	-	-	-	-	-	-	-	-	-
	SLA			Leaf area			Leaf dry weight		
	denDf	F-value	p-value	denDf	F-value	p-value	denDf	F-value	p-value
Treatment	24	160.33	<0.001 ***	24	27.842	<0.001 ***	24	7.168	0.00134 **
Cross type	24	13.11	<0.001 ***	24	9.146	<0.001 ***	24	18.85	<0.001 ***
Sex	122	2.07	0.1532 ns	122	2.348	0.1269 ns	122	3.814	0.0531 ns
Treat * Cross	72	6.52	<0.001 ***	72	3.692	<0.001 ***	72	3.699	<0.001 ***
Treat* Sex	-	-	-	-	-	-	-	-	-
Cross * Sex	-	-	-	-	-	-	122	3.539	0.0168 *
	Root cross sectional area			Root mass ratio					
	denDf	F-value	p-value	denDf	F-value	p-value			
Treatment	24	48.277	<0.001 ***	24	4.3742	0.0136 *			
Cross type	24	32.193	<0.001 ***	24	1.6296	0.2068 ns			
Sex	185	0.963	0.3276 ns	189	0.0489	0.8253 ns			
Treat * Cross	-	-	-	-	-	-			
Treat* Sex	185	2.685	0.0476 *	189	2.8641	0.0376 *			
Cross * Sex	-	-	-	-	-	-			

2009). Moreover first generation hybrids are fully fertile (A. Favre and S. Karrenberg, personal observation).

Data collection

Fitness proxies: Survival and flowering incidence were recorded in the second season. At the end of the experiment, total biomass was determined to the nearest 0.1 mg (Mettler AE 240, Mettler-Toledo AG, Greifensee, Switzerland) after drying shoots and carefully washed roots (see below) were dried separately for three days at 40°C. Female reproductive output was determined by weighing and counting seeds of previously bagged capsules (total seed weight, total seed number, weight per seed). Male flowers were collected after wilting and dried for 48 hours at 40°C to determine male reproductive output (total flower weight, flower number and weight per flower).

Leaf traits: We determined leaf area, leaf dry weight and specific leaf area (SLA, leaf area/dry weight) from one basal leaf of each surviving plant the second year. As SLA reaction to treatments can vary between leaves of a plant according to their developmental stage (Gunn *et al.*, 1999), we chose comparable leaves that were fully expanded but not yet senescent. Fresh weight and dry weight (after 48 hours at 40°C) were determined on a 1 mg precision scale (Mettler AE 240, Mettler-Toledo AG, Greifensee, Switzerland). Pressed leaves were scanned (Epson V750 Pro, Epson Deutschland GmbH, Dietlikon, Switzerland) and pictures were treated with the open source ImageJ software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA) to estimate leaf area to the nearest 0.01 cm².

Root traits: root mass ratio (root biomass/total biomass) and main root cross sectional area were determined. Root dry biomass was determined as described above. Root cross sectional area was estimated using two perpendicular diameters measured just below the root collar to the nearest 0.01 mm.

Statistical analysis

Survival and flowering incidence (of the surviving plants) were analyzed as binary data using a generalized linear model with quasibinomial error distribution (Crawley, 2007). Leaf and root traits were analyzed with linear mixed models (Pinheiro and Bates, 2000) using the *nlme* package (Pinheiro *et al.*, 2007) in R (R Development Core Team, 2006). Cross type, treatment, sex and their two-way interactions were used as fixed factors; blocks, lines and cages within blocks were random effects. Note that these random effects are not fully nested, instead, lines and treatments within blocks are crossed (Pinheiro and Bates, 2000). Model simplification proceeded using stepwise backward selection and likelihood ratio tests (Crawley, 2007). When significant interactions between treatment and cross type (or sex) occurred, the data were split by cross type (or sex) for further analysis. Significant main effects were further analyzed using TukeyHSD tests. Residuals and model fit were analyzed graphically and response variables were log transformed to yield normally distributed residuals (Venables and Ripley, 2002). Least square means \pm 1 standard error were obtained using JMP 7.0.2 (SAS Institute Inc., Cary, USA) and back-transformed to the original scale.

Results

Fitness proxies: Survival did not differ among cross types or sexes but in terms of flowering incidence, cross types reacted differently to stress treatments (significant cross type-by-treatment interaction, Table 2, Fig. 2b). All plants except one survived under the benign and drought stress treatments whereas under the shade and combined stress treatments survival was generally reduced to 75% on average (Table 2, Fig. 2a). In separate analyses per cross type, flowering incidence was significantly affected by stress treatments in all cross types (Table 3). In *S. latifolia*, all plants flowered except three (in the combined stress treatment), whereas *S. dioica* and both hybrid cross types had a strongly reduced flowering incidence under both shade stress and combined stress treatments (Fig. 2b). Overall, significantly fewer females (65%) than males (80%) flowered, however, sex-by-treatment and sex-by-cross types interactions were not significant (Table 2).

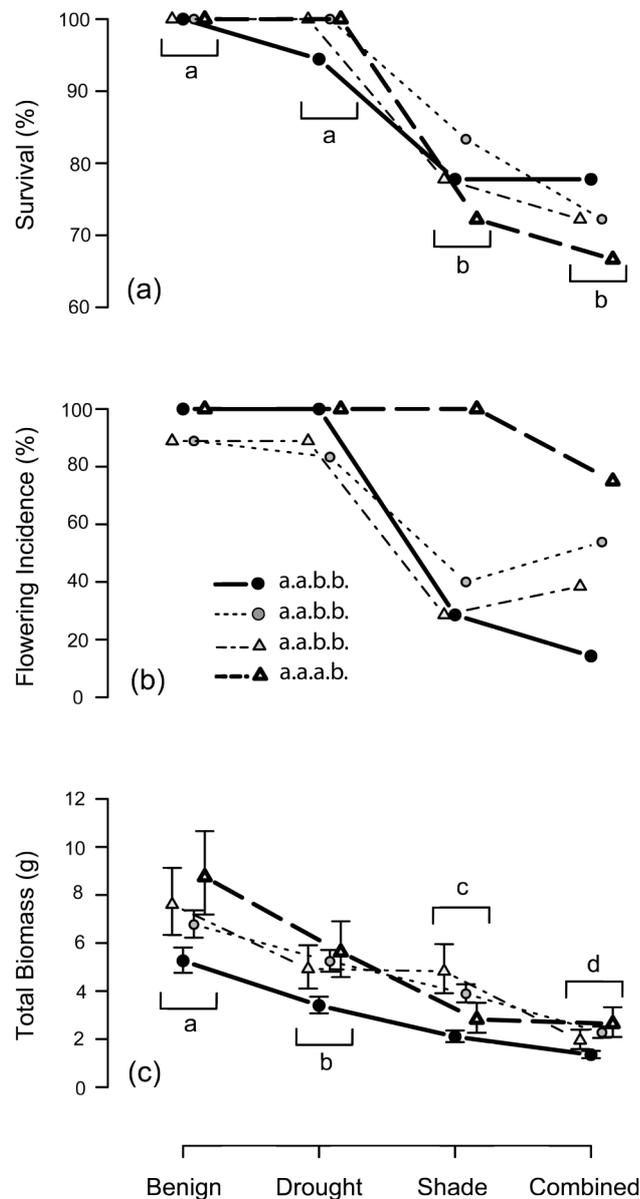


Fig 2 Percentage of survival (a), percentage of flowering incidence (b), and back-transformed least square means \pm SE of total biomass (c) of *Silene dioica* (black circles, bold line), *Silene latifolia* (open triangle, dashed bold line), F₁ hybrid with *S. dioica* as maternal parent (grey circle, dotted line), F₁ hybrids with *S. latifolia* as maternal parent (grey triangle, dashed dotted line) under four treatments (benign, drought, shade, combined stresses) in a greenhouse experiment. Significant differences between means are indicated with different letters.

Biomass production was greatest in *S. latifolia* under all treatments, as compared to *S. dioica* and both hybrid cross types (Fig. 2c). All cross types suffered similar reductions in biomass under stress treatments (treatment effect significant, cross type-by-treatment interaction not significant, Table 2); they generally produced progressively less biomass under drought stress, shade stress and combined stress treatments (Fig. 2c). Male reproductive output did not differ between the cross types in terms of flower numbers. *Silene latifolia* generally produced larger flowers than *S. dioica*, while the HD hybrid cross type produced flowers of intermediate size (Table 2 and 4). Flower size of the HL hybrid was similar to flower size in its maternal parent *S. latifolia* (Table 2 and 4). Stress treatments led to a general reduction in flower number and total flower biomass while the mass of individual flowers remained unaffected (Table 4). Female reproductive output did not differ between cross types either and was also generally affected by stress treatments in the same way as males (Table 2 and 4).

Leaf traits: Leaf traits (SLA, dry weight, leaf area) were affected differently by stress treatments in the four cross types (significant treatment-by-cross type interactions, Table 2, for within cross type models see Table 3). In all cross types, drought stress had little effect on SLA, while shade stress and the combined stress treatment resulted in increased SLA as compared to the benign treatment. This reaction was much stronger in *S. latifolia* than in *S. dioica* and the hybrids exhibited an SLA increase similar to their maternal parent (Fig. 3a, SLA increase of 44.5 % [SD], 54.5 % [HD], 114 % [HL] and 147 % [SL]). Variation in SLA resulted from changes in one or both of its components (leaf area and leaf dry weight). While *S. latifolia* significantly increased leaf area in shade stress and combined stress treatments in comparison to benign and drought stress treatments, the other three cross types had an increased leaf area only under the shade stress treatment (Fig. 3b).

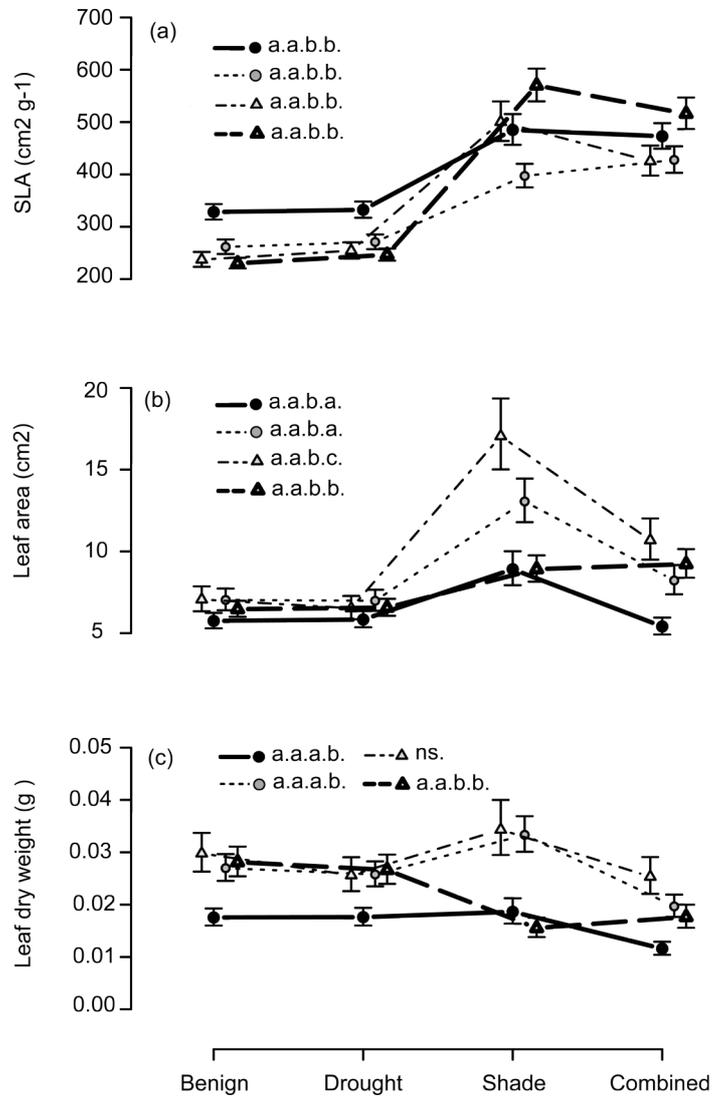


Fig 3 Back-transformed least square means \pm SE of specific leaf area (SLA, a), leaf area (b), and leaf dry weight (c) of *Silene dioica* (black circles, bold line), *Silene latifolia* (open triangle, dashed bold line), F₁ hybrids with *S. dioica* as maternal parent (grey circle, dotted line), F₁ hybrid with *S. latifolia* as maternal parent (grey triangle, dashed dotted line) under benign and stress treatments in a in a greenhouse experiment. Significant differences between means are indicated with different letters.

The increase of leaf area was strikingly larger in the two hybrid cross types than in *S. latifolia* and *S. dioica* (Fig. 3b, increased leaf surface between benign and shade treatments: 55 % [SD], 85% [HD], 141 % [HL] and 38 % [SL]). A decrease in leaf dry weight under shade and combined stress treatments together with a increase in SLA was observed only in *S. latifolia* (Fig. 3c). Plants of *S. dioica* and of the HD hybrid cross type reduced leaf dry weight only under the combined stress treatment, while the in the HL cross type leaf dry weight was not affected by the treatments (Table 3, Figure 3c).

Root traits: All cross types allocated about a third of their biomass to roots, regardless of the treatments (Table 2). *Silene latifolia* generally had a significantly larger root cross sectional area than *S. dioica* while root cross sectional area was intermediate in the HD hybrid cross type and indistinguishable from *S. latifolia* in the HL hybrid cross type.

Table 3 Results of mixed model analyses on reproductive vegetative traits in a stress tolerance experiment with *Silene dioica*, *S. latifolia* and reciprocal hybrids between them (HD, *S. dioica* mothers; HL, *S. latifolia* mothers). Significant differences between means are indicated with different letters.

	Flowering incidence			SLA			AREA			Leaf Dry Weight		
	denDf	F value	Pr(>F)	denDf	F value	P value	denDf	F value	P value	denDf	F value	P value
SD	label= aabb			label= aabb			label= aaba			label= aaab		
treat	59	34.8866	<0.001***	43	24.33	<0.001***	43	4.7686	0.0059**	43	3.933	0.0145*
sex	59	0.5838	0.4481	43	7.83	0.0077**	43	4.3447	0.0431*	43	8.653	0.0052**
treat x sex	59	0.3345	0.8005	43	2.78	0.0524	43	0.2348	0.8716	43	0.854	0.472
HD	label= aabb			label= aabb			label= aaba			label= aaab		
treat	60	3.6706	0.01741*	46	28.738	<0.001***	46	9.1034	<0.001***	46	4.456	0.0079**
sex	59	3.2996	0.0747	46	0.509	0.479	46	0.2617	0.6114	46	0.719	0.4010
treat x sex	56	0.1578	0.9242	46	4.457	0.0078*	46	2.0409	0.1213	46	2.361	0.0836
HL	label= aabb			label= aabb			label= aabc			label= ns		
treat	59	7.4418	<0.001***	45	38.53	<0.001***	45	20.7242	<0.001***	45	1.3885	0.2585 ns
sex	58	1.0432	0.3115	45	0.992	0.3246	45	0.9467	0.3358	45	1.4794	0.2302
treat x sex	55	0.6672	0.5758	45	0.591	0.6239	45	2.4172	0.0787	45	1.6659	0.1878
SL	label= aaab			label= aabb			label= aabb			label= aabb		
treat	57	26.317	<0.001***	44	9.30E+01	<0.001***	44	6.4391	0.0010**	44	9.141	<0.001***
sex	56	29.796	<0.001***	44	6.15E-03	0.9378	44	0.1899	0.6651	44	0.2	0.6567
treat x sex	53	0	1	44	7.45E-02	0.9734	44	0.4916	0.6899	44	0.438	0.7272

On the next page:

Table 4: Least square means of reproductive and vegetative traits with one standard error intervals in stress tolerance experiment with *Silene dioica* (SD), *S. latifolia* (SL), hybrids with *S. dioica* mothers (HD) and hybrids with *S. latifolia* mothers (HL). Significant differences between means are indicated with different letters.

		Treatments				Shade				Combined				Tukeys			
		Benign				Drought				Shade				cross type treatment			
		100	%		100	%		83.33	%	72.22	%	72.22	%	72.22	%	72.22	%
Survival	HD	6.767	(6.232 - 7.349)	5.240	(4.815 - 5.702)	3.889	(3.538 - 4.276)	3.889	(3.538 - 4.276)	2.280	(2.059 - 2.525)	2.280	(2.059 - 2.525)	2.280	(2.059 - 2.525)	2.280	(2.059 - 2.525)
	HL	7.606	(6.351 - 9.109)	4.924	(4.115 - 5.893)	4.795	(3.902 - 5.892)	4.795	(3.902 - 5.892)	1.952	(1.596 - 2.388)	1.952	(1.596 - 2.388)	1.952	(1.596 - 2.388)	1.952	(1.596 - 2.388)
	SD	5.261	(4.801 - 5.765)	3.395	(3.093 - 3.728)	2.104	(1.897 - 2.334)	2.104	(1.897 - 2.334)	1.330	(1.200 - 1.475)	1.330	(1.200 - 1.475)	1.330	(1.200 - 1.475)	1.330	(1.200 - 1.475)
	SL	8.746	(7.117 - 10.747)	5.622	(4.541 - 6.960)	2.805	(2.233 - 3.522)	2.805	(2.233 - 3.522)	2.613	(2.049 - 3.332)	2.613	(2.049 - 3.332)	2.613	(2.049 - 3.332)	2.613	(2.049 - 3.332)
Total Biomass (g)	HD	1.30180	(1.09219 - 1.53513)	0.62338	(0.47040 - 0.79537)	0.40373	(0.19769 - 0.65409)	0.40373	(0.19769 - 0.65409)	0.096	(0.00000 - 0.42804)	0.096	(0.00000 - 0.42804)	0.096	(0.00000 - 0.42804)	0.096	(0.00000 - 0.42804)
	HL	1.01328	(0.83525 - 1.21116)	0.64205	(0.49854 - 0.80192)	0.50452	(0.28851 - 0.76501)	0.50452	(0.28851 - 0.76501)	0.225	(0.08023 - 0.39396)	0.225	(0.08023 - 0.39396)	0.225	(0.08023 - 0.39396)	0.225	(0.08023 - 0.39396)
	SD	0.90402	(0.76817 - 1.05194)	0.44762	(0.35217 - 0.55126)	0.34454	(0.22611 - 0.47719)	0.34454	(0.22611 - 0.47719)	NA		0.304	(0.20357 - 0.41562)	0.304	(0.20357 - 0.41562)	0.304	(0.20357 - 0.41562)
	SL	1.06754	(0.91620 - 1.23255)	0.86819	(0.72597 - 1.02402)	0.86819	(0.72597 - 1.02402)	0.86819	(0.72597 - 1.02402)	0.54927	(0.43040 - 0.68001)	0.54927	(0.43040 - 0.68001)	0.54927	(0.43040 - 0.68001)	0.54927	(0.43040 - 0.68001)
Seed Number	HD	2203.80	(1821.62 - 2634.12)	819.97	(572.69 - 1101.48)	677.61	(319.92 - 1118.74)	677.61	(319.92 - 1118.74)	329.29	(0.00 - 934.51)	329.29	(0.00 - 934.51)	329.29	(0.00 - 934.51)	329.29	(0.00 - 934.51)
	HL	1626.16	(1341.31 - 1942.78)	908.21	(692.45 - 1148.39)	752.11	(449.23 - 1110.18)	752.11	(449.23 - 1110.18)	479.81	(250.92 - 744.48)	479.81	(250.92 - 744.48)	479.81	(250.92 - 744.48)	479.81	(250.92 - 744.48)
	SD	1575.19	(1333.63 - 1839.67)	747.78	(585.65 - 924.52)	588.20	(363.13 - 845.39)	588.20	(363.13 - 845.39)	NA		494.45	(317.91 - 691.33)	494.45	(317.91 - 691.33)	494.45	(317.91 - 691.33)
	SL	1617.97	(1375.64 - 1882.99)	1224.59	(999.18 - 1472.97)	792.99	(597.41 - 1009.71)	792.99	(597.41 - 1009.71)	NA		0.00049	(0.00040 - 0.00060)	0.00049	(0.00040 - 0.00060)	0.00049	(0.00040 - 0.00060)
Seed Weight (g)	HD	0.00060	(0.00055 - 0.00066)	0.00077	(0.00070 - 0.00084)	0.00071	(0.00062 - 0.00081)	0.00071	(0.00062 - 0.00081)	0.00069	(0.00060 - 0.00078)	0.00069	(0.00060 - 0.00078)	0.00069	(0.00060 - 0.00078)	0.00069	(0.00060 - 0.00078)
	HL	0.00062	(0.00056 - 0.00068)	0.00070	(0.00063 - 0.00077)	0.00069	(0.00060 - 0.00081)	0.00069	(0.00060 - 0.00081)	0.00056	(0.00051 - 0.00061)	0.00056	(0.00051 - 0.00061)	0.00056	(0.00051 - 0.00061)	0.00056	(0.00051 - 0.00061)
	SD	0.00057	(0.00054 - 0.00061)	0.00059	(0.00055 - 0.00062)	0.00056	(0.00051 - 0.00061)	0.00056	(0.00051 - 0.00061)	0.00073	(0.00065 - 0.00081)	0.00073	(0.00065 - 0.00081)	0.00073	(0.00065 - 0.00081)	0.00073	(0.00065 - 0.00081)
	SL	0.00065	(0.00059 - 0.00072)	0.00069	(0.00062 - 0.00077)	0.00073	(0.00065 - 0.00081)	0.00073	(0.00065 - 0.00081)	0.00073	(0.00065 - 0.00081)	0.00073	(0.00065 - 0.00081)	0.00073	(0.00065 - 0.00081)	0.00073	(0.00065 - 0.00081)
Flower Biomass (g)	HD	1.41278	(1.14510 - 1.74303)	0.66710	(0.54215 - 0.82086)	0.56592	(0.41281 - 0.77581)	0.56592	(0.41281 - 0.77581)	0.22873	(0.17218 - 0.30386)	0.22873	(0.17218 - 0.30386)	0.22873	(0.17218 - 0.30386)	0.22873	(0.17218 - 0.30386)
	HL	1.30165	(1.11322 - 1.52198)	0.74481	(0.63699 - 0.87088)	0.99308	(0.73524 - 1.34133)	0.99308	(0.73524 - 1.34133)	0.45216	(0.33477 - 0.61073)	0.45216	(0.33477 - 0.61073)	0.45216	(0.33477 - 0.61073)	0.45216	(0.33477 - 0.61073)
	SD	0.84956	(0.71490 - 1.00959)	0.47408	(0.40055 - 0.56110)	0.39637	(0.25061 - 0.62691)	0.39637	(0.25061 - 0.62691)	0.22052	(0.13942 - 0.34878)	0.22052	(0.13942 - 0.34878)	0.22052	(0.13942 - 0.34878)	0.22052	(0.13942 - 0.34878)
	SL	1.36335	(1.07804 - 1.72418)	0.90223	(0.72177 - 1.12780)	0.33409	(0.26103 - 0.42759)	0.33409	(0.26103 - 0.42759)	0.28857	(0.21479 - 0.38770)	0.28857	(0.21479 - 0.38770)	0.28857	(0.21479 - 0.38770)	0.28857	(0.21479 - 0.38770)
Flower Number	HD	243.974	(207.032 - 284.783)	141.561	(114.135 - 171.880)	96.518	(65.930 - 131.524)	96.518	(65.930 - 131.524)	65.054	(39.549 - 94.068)	65.054	(39.549 - 94.068)	65.054	(39.549 - 94.068)	65.054	(39.549 - 94.068)
	HL	187.855	(162.478 - 215.320)	115.249	(95.395 - 136.737)	174.888	(129.096 - 228.308)	174.888	(129.096 - 228.308)	74.257	(42.835 - 110.913)	74.257	(42.835 - 110.913)	74.257	(42.835 - 110.913)	74.257	(42.835 - 110.913)
	SD	206.277	(170.829 - 245.693)	110.970	(88.959 - 135.045)	84.842	(34.097 - 149.902)	84.842	(34.097 - 149.902)	34.827	(0.000 - 85.779)	34.827	(0.000 - 85.779)	34.827	(0.000 - 85.779)	34.827	(0.000 - 85.779)
	SL	177.519	(149.437 - 208.272)	131.050	(107.793 - 156.440)	71.234	(51.707 - 92.691)	71.234	(51.707 - 92.691)	36.645	(17.931 - 57.500)	36.645	(17.931 - 57.500)	36.645	(17.931 - 57.500)	36.645	(17.931 - 57.500)
Flower Weight (g)	HD	0.00611	(0.00567 - 0.00657)	0.00559	(0.00516 - 0.00603)	0.00578	(0.00520 - 0.00640)	0.00578	(0.00520 - 0.00640)	0.00540	(0.00485 - 0.00596)	0.00540	(0.00485 - 0.00596)	0.00540	(0.00485 - 0.00596)	0.00540	(0.00485 - 0.00596)
	HL	0.00733	(0.00685 - 0.00783)	0.00687	(0.00641 - 0.00736)	0.00593	(0.00524 - 0.00665)	0.00593	(0.00524 - 0.00665)	0.00705	(0.00630 - 0.00784)	0.00705	(0.00630 - 0.00784)	0.00705	(0.00630 - 0.00784)	0.00705	(0.00630 - 0.00784)
	SD	0.00437	(0.00415 - 0.00459)	0.00468	(0.00445 - 0.00491)	0.00453	(0.00380 - 0.00531)	0.00453	(0.00380 - 0.00531)	0.00413	(0.00342 - 0.00488)	0.00413	(0.00342 - 0.00488)	0.00413	(0.00342 - 0.00488)	0.00413	(0.00342 - 0.00488)
	SL	0.00836	(0.00787 - 0.00885)	0.00748	(0.00704 - 0.00793)	0.00726	(0.00679 - 0.00774)	0.00726	(0.00679 - 0.00774)	0.00775	(0.00719 - 0.00833)	0.00775	(0.00719 - 0.00833)	0.00775	(0.00719 - 0.00833)	0.00775	(0.00719 - 0.00833)
Root Mass Ratio	HD	0.32557	(0.29031 - 0.36279)	0.37554	(0.33880 - 0.41424)	0.33379	(0.29700 - 0.37268)	0.33379	(0.29700 - 0.37268)	0.32142	(0.28378 - 0.36130)	0.32142	(0.28378 - 0.36130)	0.32142	(0.28378 - 0.36130)	0.32142	(0.28378 - 0.36130)
	HL	0.38724	(0.35585 - 0.42003)	0.38619	(0.35497 - 0.41880)	0.34736	(0.31573 - 0.38050)	0.34736	(0.31573 - 0.38050)	0.34922	(0.31694 - 0.38307)	0.34922	(0.31694 - 0.38307)	0.34922	(0.31694 - 0.38307)	0.34922	(0.31694 - 0.38307)
	SD	0.34639	(0.31940 - 0.37446)	0.33361	(0.30787 - 0.36037)	0.28790	(0.26152 - 0.31543)	0.28790	(0.26152 - 0.31543)	0.25902	(0.23300 - 0.28621)	0.25902	(0.23300 - 0.28621)	0.25902	(0.23300 - 0.28621)	0.25902	(0.23300 - 0.28621)
	SL	0.37312	(0.34886 - 0.39822)	0.34389	(0.31812 - 0.37066)	0.25919	(0.23549 - 0.28385)	0.25919	(0.23549 - 0.28385)	0.31764	(0.29099 - 0.34540)	0.31764	(0.29099 - 0.34540)	0.31764	(0.29099 - 0.34540)	0.31764	(0.29099 - 0.34540)
Root section (mm ²)	HD	242.253	(217.320 - 268.782)	139.946	(121.833 - 159.172)	150.338	(130.239 - 171.771)	150.338	(130.239 - 171.771)	100.582	(82.757 - 119.652)	100.582	(82.757 - 119.652)	100.582	(82.757 - 119.652)	100.582	(82.757 - 119.652)
	HL	292.561	(266.025 - 320.702)	184.785	(165.889 - 204.736)	223.900	(200.666 - 248.581)	223.900	(200.666 - 248.581)	93.480	(77.494 - 110.489)	93.480	(77.494 - 110.489)	93.480	(77.494 - 110.489)	93.480	(77.494 - 110.489)
	SD	132.404	(117.496 - 148.078)	62.877	(51.467 - 74.869)	59.454	(47.024 - 72.588)	59.454	(47.024 - 72.588)	38.579	(26.929 - 50.910)	38.579	(26.929 - 50.910)	38.579	(26.929 - 50.910)	38.579	(26.929 - 50.910)
	SL	328.657	(287.959 - 372.951)	202.177	(168.632 - 239.020)	208.192	(174.444 - 245.223)	208.192	(174.444 - 245.223)	128.896	(101.752 - 158.725)	128.896	(101.752 - 158.725)	128.896	(101.752 - 158.725)	128.896	(101.752 - 158.725)

Discussion

In this study, we compared stress responses to shade and drought between the closely related species *Silene dioica* and *S. latifolia* and reciprocal first-generation hybrids between them. The most striking difference in the stress response of the two species and hybrids was a failure to induce flowering under shade stress in *Silene dioica* and in the hybrids but not in *S. latifolia*. This is unexpected, because *S. dioica* is found in forest conditions while *S. latifolia* is more common under open, drier conditions (Baker, 1947a, 1947b; Friedrich, 1979; Goulson and Jerrim, 1997; Karrenberg and Favre, 2008). Likewise, we did not observe that *S. latifolia* was more tolerant to drought conditions as expected, rather, drought generally reduced biomass and reproductive output.

The failure to induce flowering under shade stress in *S. dioica* but not in *S. latifolia* could either be due to differences in flower induction pathways or in threshold flowering size. The direct effect of the green shading cloth on light quality could also have influenced flower induction. As found for *Arabidopsis thaliana*, a lower r/fr ratio can be a signal for growing neighboring vegetation and might induce flowering (Wollenberg *et al.*, 2008). This mechanism could be more effective in *S. latifolia* than in *S. dioica*. Alternatively, *Silene dioica* might have a higher or less plastic threshold flowering size than *S. latifolia*, and may delay reproduction until enough resources have been gathered as has been observed in other species (Bender *et al.*, 2002; Metcalf *et al.*, 2003).

In both species, specific leaf area (SLA) increased under shade stress as expected (Mommer *et al.*, 2005; Steinger *et al.*, 2003), however, leaf morphology in *Silene latifolia* was considerably more plastic than in *S. dioica* with an SLA increase of 147 % from benign to shade stress conditions as compared to 44 % in *S. dioica*. Phenotypic plasticity in plant morphology is often associated with spatially or temporally heterogeneous habitats (Bradshaw, 1965; Donohue *et al.*, 2001; Valladares *et al.*, 2000; van Kleunen and Fischer, 2001). In *Psychotria* species, for example, species growing in clearings showed a higher mean phenotypic plasticity and had a faster leaf turnover than constantly shaded understory species (Valladares *et al.*, 2000). Similarly, a high plasticity and flowering under stress conditions in *S.*

latifolia could be an adaptation to the higher disturbance in its habitat, whereas delaying reproduction might be adaptive in the more predictable environment of *S. dioica*.

Hybrids were similar to *S. latifolia* in terms of biomass production but had a strongly reduced flowering incidence under shade stress conditions as did *S. dioica*. This may be because genes for flower induction from *S. dioica* are dominant under shade stress in the hybrids, whereas heterosis effects or dominance of *S. latifolia* genes shape their morphology. Hybrids also had an altered plastic response to shade resulting in extremely large leaves under shade stress. This may be due to disturbed epistatic interactions in stress-activated genes (Dobzhansky, 1936; Kawecki and Ebert, 2004; Orr, 1995; Orr and Turelli, 2001; Turelli and Orr, 2000). In nature, very large leaves could be selected against (herbivory, pathogens) and represent a non-beneficial use of resources. On the other hand, such transgressive traits could provide variation might be advantageous in new or ephemeral habitats (Ellstrand and Schierenbeck, 2000).

Maternal effects were found in two size related traits independent of the growing conditions: root cross sectional area and for male flower size the HD hybrid (*S. dioica* mother) was intermediate between the parental species and the HL hybrid (*S. latifolia* mother) had larger values for both traits and was indistinguishable from its maternal parent. These findings could suggest a dominant effect of the cytoplasm or maternally inherited nuclear genes on flower size and root cross sectional area. Such an asymmetry in F_1 hybrids has been observed in first generation hybrids both in greenhouse experiments or common garden (Burgess and Husband, 2004; Iida et al., 2007; Kimball et al., 2008; Kirk et al., 2010) and in natural conditions (Campbell and Waser, 2001; Kimball et al., 2008). In our study we did not find any fitness differences between the reciprocal hybrids. However, under natural conditions, larger roots and male flowers could be associated with higher fitness in the *S. latifolia* habitat leading to an advantage of HL hybrids over HD hybrids that would result in asymmetric gene flow as suggested by Minder *et al.* (2007). We are currently testing this hypothesis in a reciprocal transplant experiment (see chapters III and IV).

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Chapter III

Reproductive barriers between the naturally hybridizing champions *Silene dioica* and *S. latifolia* (Caryophyllaceae): habitat adaptation and flowering phenology

Manuscript:

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Abstract

Reproductive isolation allows for lineage divergence and speciation and is therefore a process of great importance in evolution. Reproductive barriers often are complex, asymmetric and may depend on the environment. We studied reproductive barriers in the naturally hybridizing champions *S. dioica* and *S. latifolia*. We established a transplant experiment at six sites, three within each species' habitat, and assessed cumulative fitness and flowering phenology of *S. latifolia*, *S. dioica* and their first- and second-generation hybrids. In each habitat, the resident species had the highest fitness suggesting that habitat adaptation limits possibilities for contact between the two species. Furthermore, flowering times of the two species overlapped only partially further reducing possibilities for mating. While first-generation hybrids performed intermediate between the two species, second-generation hybrids had a generally low performance that may be due to a disruption of epistatic interactions. A stronger fitness reduction of *S. latifolia* in *S. dioica* habitats than vice versa and a longer flowering overlap of hybrids with *S. latifolia* than with *S. dioica* further suggest that reproductive barriers between *S. dioica* and *S. latifolia* lead to preferential gene flow from *S. dioica* into *S. latifolia*.

Key words: Reproductive barriers, *Silene dioica*, *Silene latifolia*, transplant experiment

Introduction

In the process of speciation, reproductive isolation evolves between formerly interbreeding groups of individuals (Schluter, 2000, 2001). Such barriers to gene flow can occur before or after zygote formation and are referred to as pre- or post-zygotic reproductive barriers (Rieseberg and Willis, 2007; Templeton, 1989; Widmer et al., 2009). Pre-zygotic barriers include geographical, ecological or temporal differentiation that prevents mating (Lowry et al., 2008), as well as an advantage of con-specific pollen or sperm, i.e. pollen or sperm competition (Carney et al., 1996; Howard, 1999; Rahme et al., 2009; Rieseberg et al., 1995). Post-zygotic barriers include low hybrid fitness or hybrid sterility (Arnold, 1997; Coyne and Orr, 2004; Rieseberg and Carney, 1998). In general, pre-zygotic barriers are assumed to have a relatively greater impact on the reduction of gene flow as they occur first (Martin and Willis, 2007; Ramsey et al., 2003; Schemske, 2000). Different barriers to gene flow often act in succession and can be asymmetric, resulting in a higher level of gene flow from one species into the other than vice versa (Bacilieri et al., 1996; Emms et al., 1996; Rahme, 2009; Rieseberg et al., 1995).

The strength and direction of both pre- and post-zygotic barriers can strongly depend on the environment. In plants, divergent flowering phenologies can act as a strong pre-zygotic barrier between populations or species (Hall and Willis, 2006; Husband and Schemske, 2000; Lowry et al., 2008; Ramsey et al., 2003). However, when such phenologically diverged species come into contact in disturbed or new habitats, their flowering times might shift causing flowering overlap and intermating as has been observed in two species of *Banksia* (Lamont et al., 2003). Post-zygotic barriers such as inferior performance of hybrids can be due to intrinsic genetic mechanisms, but can also depend to a large extent on the interaction of hybrid genotypes with their environment (Nosil et al., 2005; Rundle and Whitlock, 2001; Schluter, 2000). If hybrids between species adapted to different habitats have intermediate phenotypes they are expected to have low fitness in both habitats (Dudley, 1996; Lexer et al., 2003; Nagy, 1997) and higher fitness in intermediate habitats (Campbell and Waser, 2001; Emms and Arnold, 1997; Fritsche and Kaltz, 2000; Johnston et al., 2001; Wang et al., 1997). On the other hand, if habitat

differentiation between hybridizing taxa is absent or weak, a subset of hybrids might be broadly fit (Kawecki et al., 1997). Thus, it is vital to assess reproductive barriers over a range of realistic conditions as we do in this study for a naturally hybridizing species pair of *Silene*.

We studied *Silene dioica* and *S. latifolia*, both short-lived perennials and dioecious. The two species have clear habitat preferences and contact sites are found in habitats more similar to the *S. latifolia* habitat than to the *S. dioica* habitat, however, intermediate forms and early-generation hybrids are very rare (Baker, 1947b, 1948; Friedrich, 1979; Goulson and Jerrim, 1997; Karrenberg and Favre, 2008; Minder et al., 2007). Nonetheless, gene flow between the two species has been documented (Minder et al., 2008). Both a study on natural hybrids zones (Minder et al., 2007) and direct observations of the realized hybrid production in artificial populations (Rahme, 2009) suggested that this gene flow is asymmetric, favoring gene flow from *S. dioica* into *S. latifolia*. Here, we hypothesize that in this system, habitat adaptation and flowering time act as reproductive barriers and reduce opportunities for the formation of hybrids and may lead to preferential gene flow from *S. dioica* into *S. latifolia*.

Potential habitat-related reproductive barriers between *S. dioica* and *S. latifolia* are both pre-zygotic and post-zygotic. *Silene dioica* occurs in moist forests, pastures or meadows while *S. latifolia* grows in dryer, more disturbed and open habitats such as arable fields, roadsides, and hedge banks (Baker, 1947a, 1947b; Friedrich, 1979). Thus, habitat adaptation may not allow the two species to survive and reproduce in each other's habitat, alternatively (or in addition), dispersal limitation could reduce mating opportunities between the two species. Moreover, phenological barriers could prevent or reduce gene flow. *Silene dioica* flowers earlier and for a shorter period of time than *S. latifolia*, but the flowering periods of the two species have been reported to overlap to varying degrees in different studies (Biere and Honders, 1996; Bopp and Gottsberger, 2004; Rahme, 2009; van Putten et al., 2007). *Silene dioica* and *S. latifolia* are fully cross-fertile, can be artificially crossed and both first- and second-generation hybrids exhibited no obvious growth reduction or sterility (Baker, 1947a,

1947b; Clapham et al., 1987; Rothenbuehler, 2008). In natural environments, however, performance of hybrids likely is altered and reduced hybrid performance could thus constitute a post-zygotic reproductive barrier.

To investigate these reproductive barriers we assessed cumulative fitness and flowering phenology of *S. latifolia*, *S. dioica* and first- and second-generation hybrids between them in a transplant experiment using six different sites within three natural populations of each species. Specifically, we asked: **(1)** Are *Silene latifolia* and *S. dioica* more fit in their respective habitat? **(2)** Do hybrids have reduced fitness at any of the sites? **(3)** How does flowering time of the species and their hybrids overlap?

Material and Methods

Study Sites and Plant Material

We established six study sites within typical natural populations of *Silene latifolia* Poiret and *S. dioica* (L.) Clairv. in the Swiss Alps, three sites for each species habitat (Table 1, compare to Karrenberg and Favre (2008)).

Table 1 Sites and origin of seed used in a reciprocal transplant experiment with *Silene dioica* and *S. latifolia* and hybrids between them.

	Name	Elevation	Coord N	Coord E	Resident species	Habitat and use
<i>Transplant sites</i>						
	Fully	455m	46°06'59.90	7°04'39.28	<i>S. latifolia</i>	Dry meadows, mowed
	Gampel	626m	49°18'47.55	7°43'15.20	<i>S. latifolia</i>	Dry meadows, mowed, pastured
	Leuk	962m	49°19'15.89	7°38'45.56	<i>S. latifolia</i>	Agricultural fields, mowed
	La Fouly	1248m	45°58'32.94	7°07'09.03	<i>S. dioica</i>	Rich meadows, mowed, pastured
	Bodmen	1402m	49°21'41.44	7°36'37.14	<i>S. dioica</i>	Rich meadows, mowed and pastured
	Arolla	1816m	46°02'43.71	7°29'13.64	<i>S. dioica</i>	Rich meadows, mowed, pastured
<i>Seed origin</i>						
	Leuk	950	46°19'12.13	7°38'57.32	<i>S. latifolia</i>	Terraced fields, fallow
	Gordemo	400	46°11'12.63	8°50'59.25	<i>S. latifolia</i>	Vinyards
	Sur En	1120	46°48'52.73	10°21'34.70	<i>S. latifolia</i>	Gravel pit
	Gotthard	1800	46° 35'28.72	8°33'32.81	<i>S. dioica</i>	Megaphorbia
	Davos	1800	46°48'10.46	9°53'47.68	<i>S. dioica</i>	Open river side
	Susich	1430	46°44'39.74	10°04'45.37	<i>S. dioica</i>	Rich meadow, mowed, pastured

We used five different cross types: within-species crosses (*Silene dioica* and *S. latifolia*, 18 families each), reciprocal hybrid crosses (*S. dioica* female x *S. latifolia* male and *S. latifolia* female x *S. dioica* male, 18 families each) and second-

generation hybrids (F_2 , 36 families). The crosses were generated from 36 plants cultivated from seeds collected in three populations of each species in the Swiss Alps (Table 1). The source populations showed no evidence of introgression and were free of intermediate phenotypes (Minder et al., 2007). We used a full-sib-half-sib crossing design for a first generation and generated experimental plants by further intercrossing of these plants in a second generation in a way that each family descends from three or four populations. In spring 2007, experimental plants were pre-cultivated in multi-pot trays in a commercial greenhouse (Mottiez Fleur, 1903 Collonges, Valais, Switzerland) and transplanted with their soil plugs (diameter and height of 5cm) directly into the existing vegetation. Transplant sites were mown shortly prior to transplantation (corresponding to the regular mowing regime) and resident *Silene* plants within the transplant area were removed. Only during the two first weeks, when necessary, the experiment was watered. Pre-cultivation for each site was timed such that experimental plants were of approximately similar size as juveniles of the resident *Silene* population at transplantation. In 2008 and 2009, we mowed experimental sites once after the local and experimental plants had completed reproduction and once at the very end of the season (end of October).

Table 2 Cumulative fitness calculation for each plant of the transplant experiment. To each category was attributed a number of points to which was added the reproductive output in 2008 (R08) divided by the mean reproductive output 2008 (R) and the size by autumn 2009 (S09) divided by the mean size in 2009 (S).

Categories	Fitness calculation
0. No reproduction	$0 + S09/S$
1. Dead after one reproduction	$1 + 1 \cdot R08/R$
2. Dead after two reproductions	$2 + 2 \cdot R08/R$
3. Alive after one reproduction	$3 + 1 \cdot R08/R + S09/S$
4. Alive after 2 reproductions	$4 + 2 \cdot R08/R + S09/S$
5. Three reproductions	$5 + 3 \cdot R08/R$

Experimental setup

A complete randomized blocks design was used with five blocks per site and 216 plants per block (1080 plants per site). Each block received one plant of each of the 18 families of each of the two within species crosses and each of the two reciprocal first-generation hybrids and four plants of each of the 36 F₂ families. Plants within blocks were labeled with numbered aluminum tags and organized in a 9 plant x 12 rows array with 20cm distance between plants within rows, and alternating 20 and 40 cm distances between rows to allow access for measuring.

Data collection

Plants that died until two weeks after transplantation were removed from further analyses (transplant shock). Survival was scored twice a year (spring and fall) between 2007 and 2009, and we assessed flowering in autumn (presence of flowering shoots). Fertility was investigated in detail in the second season, 2008. We visited sites every seven to ten days and recorded flowering status. Reproductive output was estimated as the total number of open flowers for males and the number of capsules for females.

Cumulative Fitness

For each plant, we estimated a cumulative fitness value as: *cumulative fitness* = *rank* + *reproductive output/mean reproductive output in 2008* + *size prediction/mean size in 2009* (see Table 2). Each individual was first assigned to a rank from 0 to 5 according to its survival and number of reproduction events. To this rank we added the reproductive output estimated as the number of capsules or flowers (for females and males respectively) produced in 2008 multiplied by the number of flowering events (one to three). We used the mean per site of 2008 as substitute for plants that flowered in 2009 only. We had to take into account that plants that were still alive by the end of the experiment might reproduce again. For this reason, we added the size in 2009 to predict the probability to reproduce in 2010, because larger plants were more likely to reproduce in the following year (data not shown). We standardized reproductive output and size by dividing them by their mean in 2008 and 2009 respectively, taking sex into account for reproductive output. This calculation resulted in a continuous cumulative fitness measure.

Analysis

After removal of plants that died because of transplant shock, 6196 plants were included in the analysis. Cumulative fitness was log transformed and analyzed

Table 3 Mixed Model results of cumulative fitness in a reciprocal transplant experiment affected by cross type (*S. dioica*, *S. latifolia*, reciprocal F₁ hybrids and F₂ hybrids), habitat type (*S. dioica* or *S. latifolia* habitat) and sites within habitat types. Significant cross type-by-habitat or the cross type-by-site interactions lead to further analysis within habitats and within sites.

		Df	DenDf	F-ratio	p-value
<i>All data</i>					
	cross	4	4.03	5.19	0.0846 ns
	hab	1	171.21	23.62	<0.001 ***
	hab*cross	4	1416.77	78.73	<0.001 ***
<i>S. dioica sites</i>					
	cross	2	13.71	18.83	<0.001 ***
	site	4	184.44	50.07	<0.001 ***
	cross*site	8	582.67	1.93	0.0526 .
<i>S. latifolia sites</i>					
	cross	2	14.20	20.22	<0.001 ***
	site	4	230.39	45.10	<0.001 ***
	cross*site	8	590.41	2.30	0.0199 *
<i>Cross effect per site</i>					
	Arolla	4	99.13	30.31	<0.001 ***
	Fully	4	176.73	36.54	<0.001 ***
	La Fouly	4	284.70	9.46	<0.001 ***
	Leuk	4	261.35	10.25	<0.001 ***
	Gampel	4	257.87	23.09	<0.001 ***
	Fully	4	188.22	24.22	<0.001 ***

in an ANOVA performed in JMP version 7 (SAS Institute Inc., Cary, NC, 1989-2007). We used a mixed effect model to test whether cumulative fitness depended on the cross type, habitat and their interaction (fixed factors). Random factors were blocks, sites nested within habitat, lines nested within cross types and lines nested within sites. When the interaction was significant, the data were split by habitat and site (Table 3). Cross-type means were compared with a Tukey's HSD test and reported

as back-transformed least square means. Flowering overlap between the two species and hybrids was analyzed graphically.

Results

Cross type performance: The relative performance of each cross type depended on the habitat: *Silene dioica* and *S. latifolia* had the highest cumulative fitness in their own habitat and the lowest in the other species' habitat (Fig. 1). The cumulative fitness largely differed between sites of *S. latifolia* habitat, whereas sites only marginally differed for this fitness measure within *S. dioica* habitat (Table 3). Reciprocal hybrids performed equally but their relative position to the parental species varied between sites. F₁ hybrids displayed intermediate values between the parental species F₁ in Arolla, Bodmen, Gampel and Fully, were similar to *S. dioica* in La Fouly and similar to *S. latifolia* in Leuk (Fig.1). The cumulative fitness of the F₂ hybrids was always low and similar to the foreign species at all sites (Fig. 1).

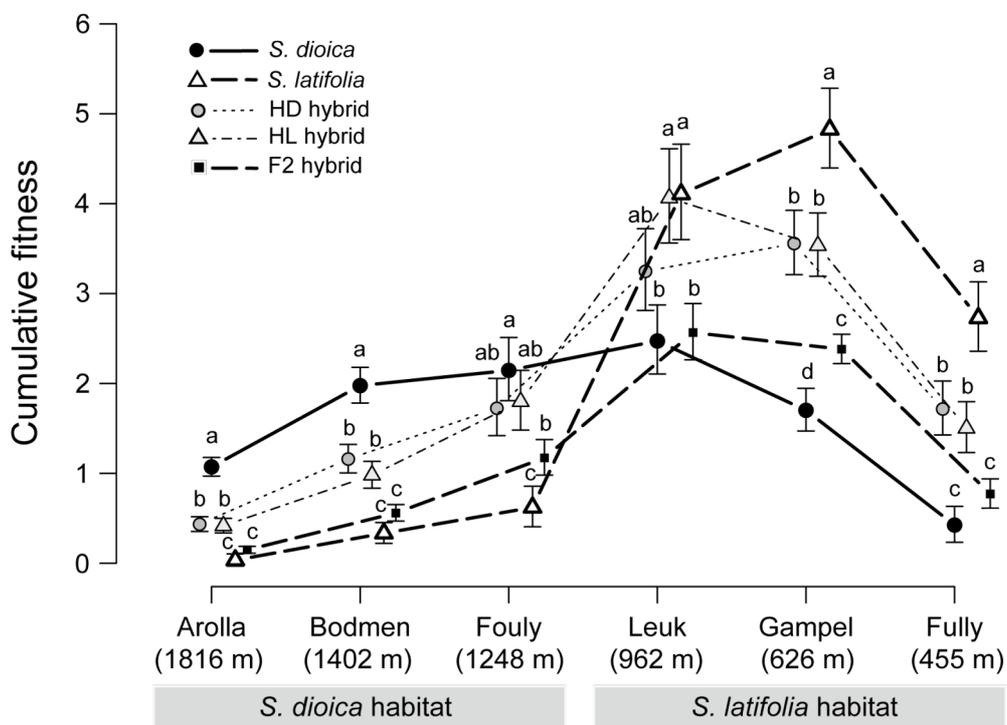
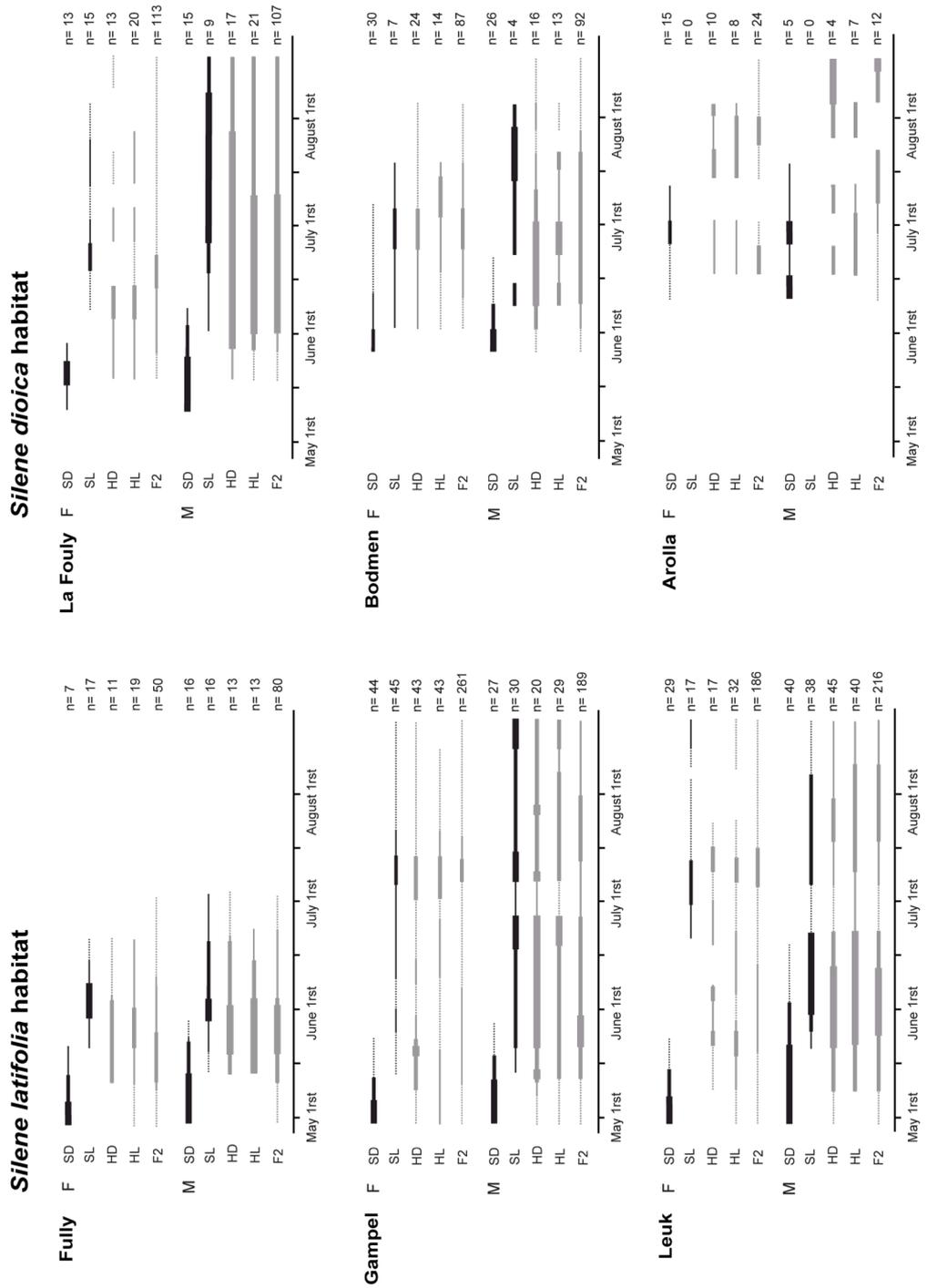


Fig 1 Cumulative fitness (back-transformed least square means with one standard error) in a reciprocal transplant experiment with *Silene dioica*, *S. latifolia* and their first- and second-generation hybrids (F₁ and F₂). Significant differences between cross types within sites are indicated by different letters.

Fig 2 Flowering phenology of *S. dioica* (SD), *S. latifolia*(SL), F₁ hybrids with *S. dioica* as maternal parent (HD), F₁ hybrids with *S. latifolia* as maternal parent (HL) and F₂ hybrids in six sites of a transplant experiment. The thickness of the lines represents the percentage of flowering individuals (n) within cross types with dashed lines (<5%), thin continuous line (<20%), bold line (<40%) and thick line (<40%). Sexes are displayed separately, F (females) and M (males).



Flowering time: Plants starting to bloom the first week of May 2008 in all *S. latifolia* sites, mid-May in La Fouly, end of May in Bodmen and beginning of June in Arolla (Fig. 2). At all sites, the first cross type to flower was *S. dioica* and the last to induce flowering was *S. latifolia* (in Arolla, *S. latifolia* did not flower). *Silene dioica* finished flowering three to five weeks after flower initiation whereas *S. latifolia* flowered throughout the later season. Flowering time of both species overlapped for male plants in all sites but not for females. In Leuk and La Fouly, there was a gap between the flowering of *S. dioica* females and *S. latifolia* females (one month in Leuk, 2 weeks in La Fouly). At the remaining sites, females of both species co-flowered at least for a short time including peak flowering of males of both species. In Arolla and Bodmen very few or even no *S. latifolia* achieved reproduction. Plants of hybrid cross types started to flower throughout the season at each site, clearly overlapping with all other cross types. Reciprocal F₁ hybrids were indiscernible from each other in terms of flowering time.

Discussion

We found evidence for ecologically dependent reproductive barriers in the naturally hybridizing species pair *S. dioica* and *S. latifolia*. In each habitat, the resident species had the highest fitness. This is a clear indication of habitat adaptation that likely reduces possibilities for contact site formation, as has also been reported in other systems (Campbell and Waser, 2007; Fritsche and Kaltz, 2000; Leimu and Fischer, 2008; Wang et al., 1997). Secondly, flowering phenologies differed between the two species at all sites with *S. dioica* flowering earlier and for a shorter time than *S. latifolia* suggesting that mating opportunities between the species are further reduced by phenological divergence. First-generation hybrids (F₁) generally had at least intermediate fitness, but second-generation hybrids (F₂) exhibited reduced fitness at all sites, and this may act as a third reproductive barrier. The generally low performance of F₂ hybrids may be due to a disruption of epistatic interactions (Dobzhansky, 1936; Orr, 1995; Orr and Turelli, 2001; Turelli and Orr, 2000) and has also been detected in similar studies using F₂ or later-generation hybrids in other plant species (Campbell et al., 2008; Fritz et al., 2006; Johansen-Morris and Latta, 2006; Rhode and Cruzan, 2005; Wright and Stanton, 2007).

Several lines of evidence further suggest that these barriers to gene flow may be asymmetric. Although the relative fitness of the native species was always superior to that of the foreign species, *S. latifolia* suffered a stronger fitness reduction in *S. dioica* habitats than vice versa. In fact, *Silene latifolia* may be excluded from sites at higher altitudes, as shown by the near-complete failure to reproduce at the two highest-elevation sites Arolla and Bodmen in our study. This result is consistent with previous reports of contact sites in the habitat of *S. latifolia* (Karrenberg and Favre, 2008) and supports the hypothesis that introgression preferentially occurs from *S. dioica* into *S. latifolia*. (Minder et al., 2007). This hypothesis is further corroborated by the relatively high fitness of first-generation hybrids in one of the *S. latifolia* sites, Leuk, situated at an intermediate altitude. In *Ipomopsis*, *Penstemon* and *Artemisia* hybrid fitness is also known to vary across environments, and was highest at intermediate altitudes (Campbell and Waser, 2007; Kimball et al., 2008; Miglia et al., 2007; Wang et al., 1997). Our results suggest that sites of the *S. latifolia* habitat at intermediate altitudes favour contact site formation and allow for sustained gene flow between *S. dioica* and *S. latifolia*.

Flowering times of males of both species partially overlapped with that of females of the other species. In a study using artificial populations, Rahmé (2009) detected that *S. latifolia* produces more hybrid offspring than *S. dioica* when males of both species occur in equal numbers, even though pollen competition reduces the number of hybrids produced in *S. latifolia* but not in *S. dioica* (Montgomery et al., 2010; Rahme et al., 2009). This may be due to insect pollinators with a higher constancy towards the first-flowering species, *S. dioica*, a process that could also be active in natural contact sites. In addition, asymmetrical gene flow from *S. latifolia* into *S. dioica* likely is favored by a longer flowering time overlap of hybrids with *S. latifolia* than with *S. dioica* as we report in this study.

In conclusion, we show clear evidence that habitat adaptation, phenological divergence and F₂ hybrid breakdown may act as reproductive barriers between *S. dioica* and *S. latifolia*. A stronger fitness reduction of *S. latifolia* in *S. dioica* habitats than vice versa, high first-generation fitness in an intermediate *S. latifolia* site and longer flowering overlap of hybrids with *S. latifolia* than with *S. dioica* further suggest that the remaining gene flow preferentially occurs from *S. dioica* into *S. latifolia*.

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Chapter IV

Mechanisms of habitat adaptation in *Silene dioica* and *S. latifolia* (Caryophyllaceae)

Manuscript:

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Abstract

Habitat adaptation allows species to survive and reproduce in certain habitats but not in others. We investigated the relative importance of survival, flowering and the production of an overwintering rosette in *S. latifolia*, *S. dioica* and hybrids between them in their respective habitats. We transplanted *Silene dioica*, *Silene latifolia* as well as two classes of hybrids (F₁ and F₂) in six natural populations of both species. We scored survival and flowering over three years as well as the occurrence of the rosette in the first year. Differences in survival were the main restriction to the establishment of one species in the other species habitat. The overwintering rosette was under positive selection and might be a key trait limiting the invasion of *S. dioica* habitat by *S. latifolia* and the hybrids. F₁s usually survived comparably to the resident species, whereas F₂s were intermediate of the number of months survived. Flowering was intermediate for the F₁s and generally low for the F₂ hybrids at each site. Differences in survival and flowering varied across sites and cross types suggesting a high heterogeneity of selection regimes in natural populations.

Key words: flowering, hybrids, overwintering rosette, selection, *Silene dioica*, *Silene latifolia*, survival, transplant experiment

Introduction

Environmental differences between habitats, whether they are abiotic (climate, nutrient availability, soil characteristics) or biotic (presence of competitors or predators) can impose divergent selection (Schluter, 2000). Species that are adapted to contrasting habitats likely display different traits resulting from this selection. Identifying these traits is necessary to understand how differences in habitat adaptation contribute to the reproductive isolation of cross-fertile species and to ecological speciation (Rundle and Nosil, 2005; Schluter, 2001, 2009). Genetically-based trait correlations within species are a major difficulty in detecting such traits under selection (Jordan, 1991; Lande and Arnold, 1983; Lexer et al., 2003; Nagy, 1997). A promising method to investigate selection on individual traits is to generate recombinant hybrids between ecologically differentiated species (for example F₂ hybrids) and expose these to natural selection in a transplant experiment (Lexer et al., 2003). This method generates phenotypic variation in traits that are additively controlled by several or many genes as is assumed for most ecologically relevant traits (Lander and Botstein, 1989; Wu et al., 2007). Our present transplant experiment uses F₂ hybrids and aims at investigating habitat adaptation in naturally hybridizing champions (*Silene*).

We studied *Silene dioica* and *S. latifolia*, two closely related campion species that have clear habitat preferences (Goulson and Jerrim, 1997; Karrenberg and Favre, 2008). *Silene dioica* occurs in moist habitats such as forests, pastures or meadows. *S. latifolia* grows in arable fields, roadsides, and hedge banks – typically dryer, more disturbed and open habitats (Baker, 1947a, 1947b; Friedrich, 1979). They are fully cross-fertile and early generation hybrids are rarely found at contact sites that are similar to the *S. latifolia* habitat (Goulson and Jerrim, 1997; Karrenberg and Favre, 2008; Minder et al., 2007). *Silene dioica* and *S. latifolia* morphologically differ in many ways. For example, *S. latifolia* has white scented flowers that open at night and *S. dioica* has red or pink flowers that open mainly during the day. *Silene dioica* has a shallow root system, while the *S. latifolia* grows a thickened taproot (Baker, 1947a, 1947b; Friedrich, 1979; Goulson and Jerrim, 1997). *Silene dioica* produces a compact rosette with ground leaves that are not pedunculate (Baker,

1947b) whereas *S. latifolia* is a “partial rosette plant” with more upright winter leaves (Baker, 1947a). In previous work, we reported that both species had a higher cumulative fitness in their respective habitats and that hybrids were relatively less fit than the resident species (chapter 3). Our cumulative fitness estimate was based on survival, flowering incidence, reproductive output, and plant size, fitness components which could all variably contribute to the cumulative fitness differences between cross types and habitats.

Here, we are interested in the mechanisms underlying the observed advantage of *S. dioica* and *S. latifolia* in their respective habitats (see Chapter III). We compare two fitness components (survival and flowering), using both species as well as their reciprocal (F_1) and F_2 hybrids in transplant sites replicated within the habitat of each species. We specifically ask: 1) Do *S. latifolia*, *S. dioica* and their hybrids differ in their survival and/or flowering between the contrasting habitats? 2) Is the production of an overwintering rosette a trait under selection in either of these habitats?

Material and Methods

Plant Material, study sites and experimental setup

We used five cross types resulting from a two generations of crosses between and within *Silene dioica* and *Silene latifolia*: within-species crosses, F_1 and F_2 hybrids. A total of 6480 plants were pre-cultivated under greenhouse conditions (Mottiez Fleur, 1903 Collonges, Valais, Switzerland) and then transplanted in six naturally occurring typical *Silene* populations in the Swiss Alps (see Table 2 in Chapter III, and Fig. 2 in general introduction of this thesis). Three study sites were situated within *S. dioica* habitats, and three were within *S. latifolia* habitats. Within sites, a complete randomized block design was used with five blocks, each receiving 18 plants of each within-species and F_1 hybrid crosses, and 144 F_2 plants. Further details on seed sources for crosses, crossing design and characteristics of the transplant sites are available in chapter 2 of this thesis.

Table 1 The effect of the cross type (*S. dioica*, *S. latifolia*, reciprocal F₁ hybrids and F₂ hybrids), the habitat and their interaction on (1) the number of months survived analyzed with a linear mixed model with blocks, sites nested within habitat, families nested within cross type and families by site interaction as random factors and (2) on the flowering incidence analyzed as binary data using a generalized linear model. When the cross type by habitat interaction was significant, the data were split by habitat and re-analyzed as above with site as fixed effect. When the cross type by site interaction was significant, the data were split by site and the effect of cross type alone as fixed effect was analyzed within each site. Stars are with significant results, ns with non-significant result.

	(1) Number of months survived				(2) Flowering incidence		
	Df	DenDf	F-ratio	p-value	Df	F-ratio	p-value
All data							
cross type	4	171.50	12.77	<0.001 ***	4	132.00	<0.001 ***
hab	1	4.03	1.92	0.2380 ns	1	455.05	<0.001 ***
cross type * habitat	4	1188.35	66.03	<0.001 ***	4	82.01	<0.001 ***
<i>S. dioica</i> habitat							
cross type	4	263.82	61.13	<0.001 ***	4	136.05	<0.001 ***
site	2	16.11	15.38	<0.001 ***	2	187.57	<0.001 ***
cross type * site	8	596.19	7.43	<0.001 ***	8	23.47	0.0028 **
<i>S. latifolia</i> habitat							
cross type	4	202.50	20.49	<0.001 ***	4	119.71	<0.001 ***
site	2	15.94	45.76	<0.001 ***	2	221.50	<0.001 ***
cross type * site	8	600.57	5.84	<0.001 ***	8	13.23	0.1040 ns
Cross effect per site							
Arolla	4	316.73	50.13	<0.001 ***	4	58.95	<0.001 ***
Bodmen	4	197.53	15.98	<0.001 ***	4	76.09	<0.001 ***
Fouly	4	299.18	11.69	<0.001 ***	4	18.56	<0.001 ***
Leuk	4	294.99	5.63	<0.001 ***	-	-	-
Gampel	4	219.55	11.32	<0.001 ***	-	-	-
Fully	4	203.20	14.92	<0.001 ***	-	-	-

Data collection

Two weeks after each site was set up (June, July 2007), we assessed transplant shock. Dead plants were removed from further analyses (284 plants). From fall 2007 to fall 2009, survival was scored twice a year (spring and fall). We here express survival as the number of months survived, standardized by the mean of each site. In summer 2008, flowering incidence was monitored every 10 days,

whereas dry stems were used to assess flowering in 2009. Shortly before winter 2007, the shape of the overwintering rosette was assessed in the F₂ hybrids.

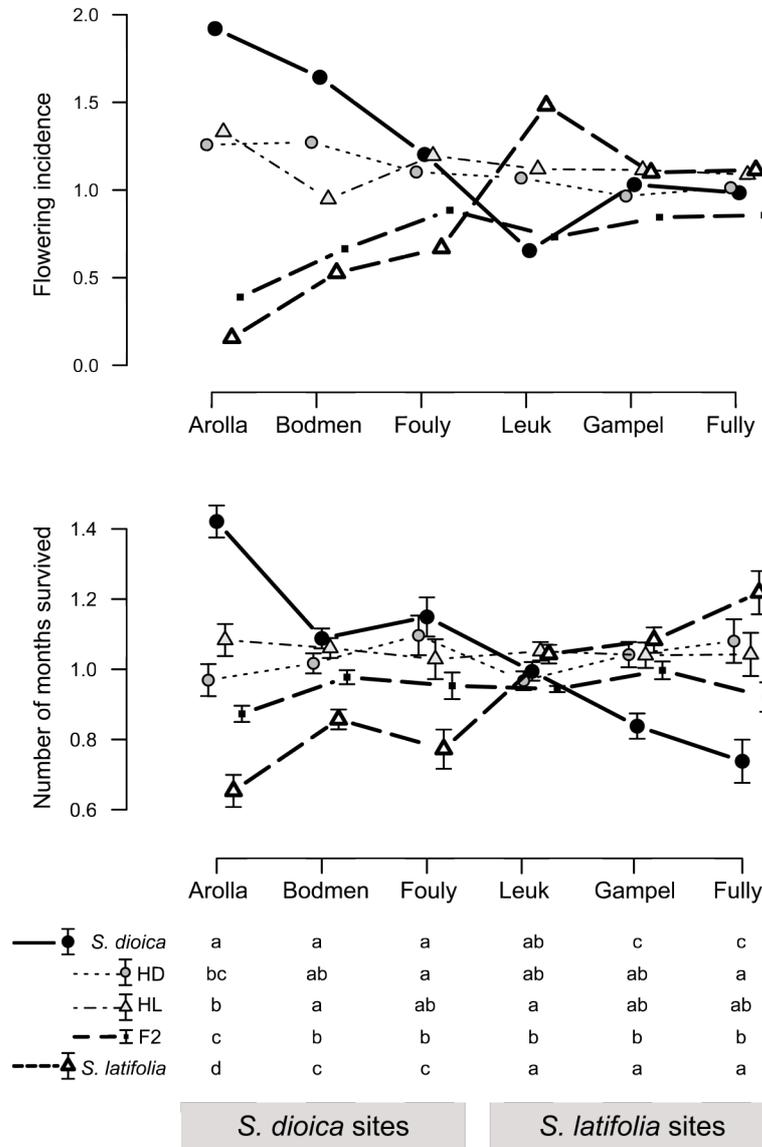


Fig. 1: Relative flowering incidence and number of months survived \pm SE of five cross types in the 6 sites of the transplant experiment: *S. dioica* (black circles, bold line), *S. latifolia* (open triangle, dashed bold line), F₁ hybrids with *S. dioica* as maternal parent (grey circle, dotted line), F₁ hybrid with *S. latifolia* as maternal parent (grey triangle, dashed dotted line) F₂ hybrids (small black square, dashed bold line). Both flowering incidence and number of months survived are divided by their mean of each site. For the number of months survived, cross type means that differed within sites in Tukey's HSD test are indicated by different letters.

Both species can display green structures in winter, but their shape differs. We defined the overwintering rosette as a compact group of leaves that are not pedunculate (*S. dioica*-like).

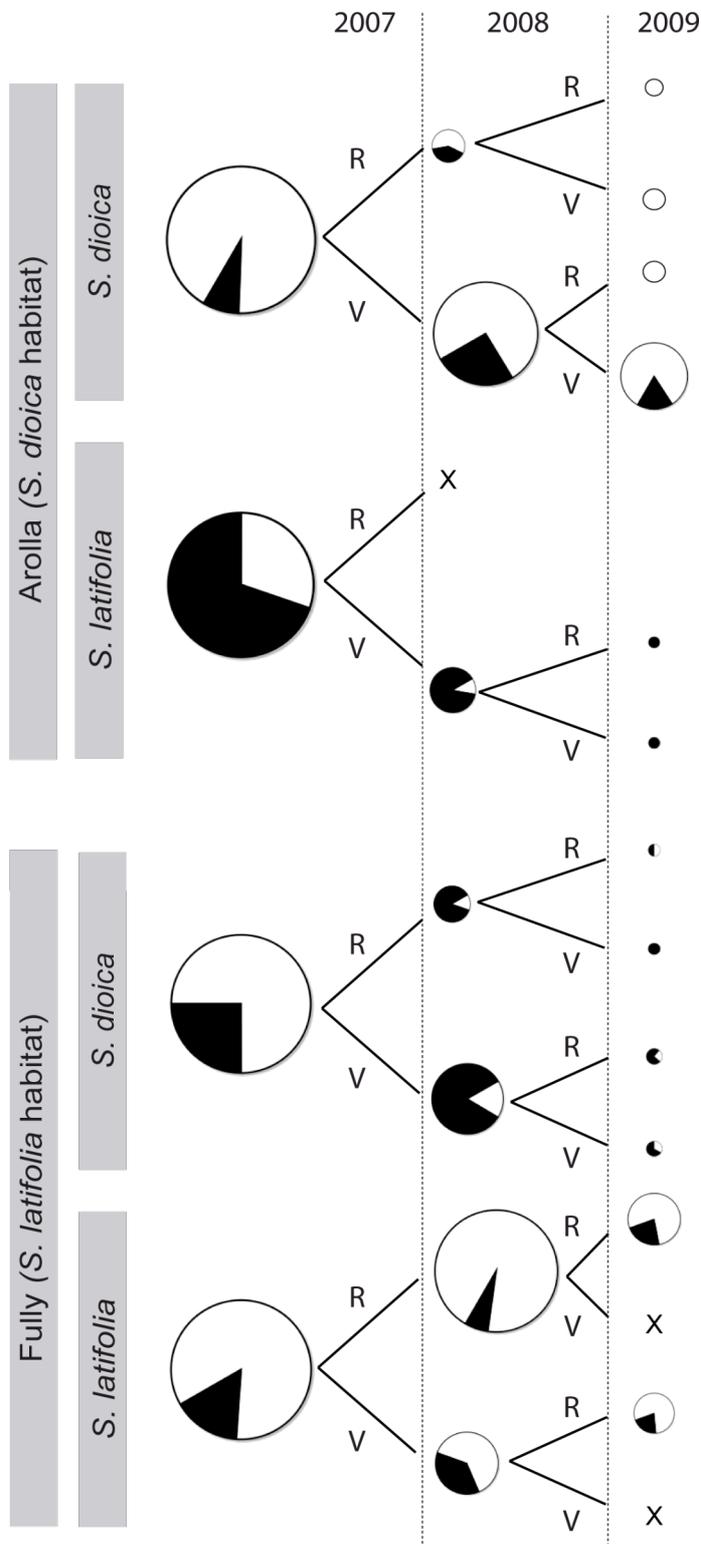


Fig. 2 Survival and flowering of *Silene dioica* and *S. latifolia* in one transplanting site of *S. dioica* habitat (Arolla) and one transplanting site of *S. latifolia* habitat (Fully). Circle size indicates the number of plants in spring, with sectors corresponding to plants that survived (white) or died (black) throughout the season. A further distinction is made between the plants that reproduced (R) or remained vegetative (V).

In contrast, *S. latifolia*-like winter leaves were neither compact nor pedunculate. We attributed the overwintering rosette shape to three categories: *S. dioica*-like, intermediate, and absent. Cumulative fitness was defined as in chapter 3 of this thesis. For flowering phenology, we recorded the date at first flowering, the date when flowering stopped and the date when plants re-flowered the same season if it occurred.

Analysis

The number of months survived was analyzed with a linear mixed model (Pinheiro and Bates, 2000). We tested whether the number of months survived depended on the cross type, the habitat and their interaction (fixed factors). Blocks, sites nested within habitat, families nested within cross types and families by site interaction were set as random factors. Cross type means of the number of months survived were compared with Tukey multiple comparisons test and reported as back-transformed standardized least square means. Flowering incidence was analyzed as binary data using a generalized linear model (Crawley, 2007). To test if the shape of the overwintering rosette had an influence on cumulative fitness, we analyzed cumulative fitness within the F_2 with the rosette shape, the habitat and their interaction as fixed factor. Random factors were kept as above, excluding the effect of the family nested within cross types. The cumulative fitness was log-transformed after addition of the overall mean. Mean cumulative fitness of the three categories of rosette index (absent, intermediate, *S. dioica*-like) were compared with a Tukey multiple comparisons test. Whenever the interaction was significant, the data were split by habitat or site and the effect of the cross type was re-analyzed within habitat or site. All analyses were performed in JMP version 7 (SAS Institute Inc., Cary, NC, 1989-2007). We graphically assessed the differences between the cross type in the production of *S. dioica*-like overwintering rosette at each site.

Results

Number of months survived and flowering: *Silene latifolia* and *S. dioica* survived longer in their own habitat, whereas both species flowered better in sites within the *S. latifolia* habitat (Fig. 1-2). We chose to display only Arolla and Fully

populations in figure 2 because they contrast the best the differences between the species in each habitat. Differences in the number of days survived between the resident and foreign species were progressively larger towards both extreme sites (Arolla and Fully). However, the two species attained very similar number of months survived in Leuk, a site of intermediate altitude (Fig. 1). As shown in figure 2, *S. latifolia* died mostly in the first winter after transplanting in Arolla (*S. dioica* habitat). In contrast in Fully (*S. latifolia* habitat), three quarters of *S. dioica* plants survived throughout the first winter but many died the following summer. Over two years, the mean flowering incidence of *S. latifolia* was as high as 84 % in its own habitat, and 20 % in *S. dioica* habitat, and *Silene dioica* flowered slightly better in *S. latifolia* habitat (67 %) than in its own habitat (54 %). For flowering incidence, cross types differed between sites within *S. dioica* habitat but not between those within *S. latifolia* habitat (Table 1).

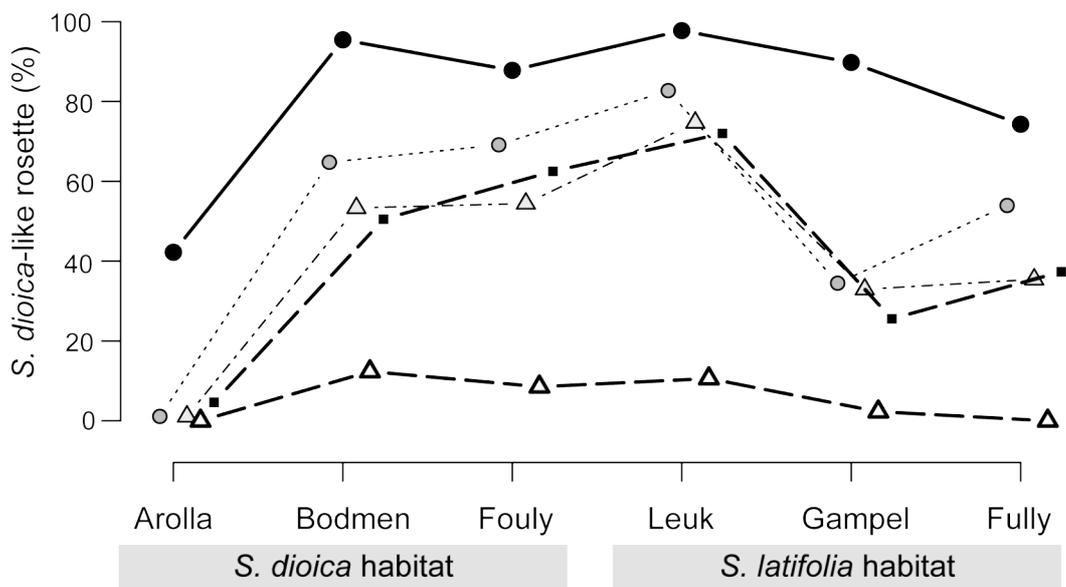


Fig.3 Percentage of *S. dioica*-like rosette production of five cross types in the six sites of a reciprocal transplant experiment: *S. dioica* (black circles, bold line), *S. latifolia* (open triangle, dashed bold line), F₁ hybrids with *S. dioica* as maternal parent (grey circle, dotted line), F₁ hybrid with *S. latifolia* as maternal parent (grey triangle, dashed dotted line) F₂ hybrids (small black square, dashed bold line).

The differences between the cross types in *S. dioica* habitat increased with the altitude of the sites. Reciprocal hybrids never differed from each other for the number of months survived or for flowering: they were comparable to the resident species for the number of months survived at each site except in Arolla where they were intermediate. The F₁ hybrids flowered comparably to the resident species in Fully, Gampel and Fouly and, the percentage of flowering F₁ hybrids was intermediate in the other sites. A similar pattern was found for flowering. F₂ hybrids always had an intermediate number of months survived between the parental species, but flowered at a similar rate than the foreign species at each site (Fig. 1).

Table 2 The effect of the rosette category (absent, intermediate, *S. dioica*-like), the habitat and their interaction on the cumulative fitness analyzed with a linear mixed model with blocks, sites nested within habitat, families nested within cross types and families by site interaction as random factors. Because the cross type by habitat interaction was significant, the data were split by habitat and re-analyzed as above with site as fixed effect. Stars are for significant results, “ns” for non-significant result.

		Df	DenDf	F-ratio	p-value
All data					
	rosette	1	3756.36	19.19	<0.001 ***
	habitat	1	3.99	4.13	0.1120 ns
	habitat * rosette	1	3287.55	5.06	0.0245 *
<i>S. dioica</i> sites					
	rosette	1	1929.66	20.69	<0.001 ***
	site	2	14.82	21.44	<0.001 ***
	rosette * site	2	1722.10	1.02	0.3611 ns
<i>S. latifolia</i> sites					
	rosette	1	1791.32	2.67	0.1300 ns
	site	2	12.31	23.18	<0.001 ***
	rosette * site	2	1502.68	1.08	0.3402 ns

The overwintering rosette: In average 80% of *S. dioica* plants displayed an over-wintering rosette across all sites whereas only 5% of *S. latifolia* formed such a structure. All hybrid cross types had an intermediate proportion of plants that displayed a *S. dioica*-like rosette except in Arolla where their morphology resembled that of *S. latifolia*. Hybrids never differed from each other (Fig. 3). Within the F₂, the effect of the interaction between the rosette category and the habitat was significant for the cumulative fitness (Table 2). Plants displaying a *S. dioica*-like rosette or an intermediate form had a higher cumulative fitness in *S. dioica* habitats than plants that did not form such a structure. Contrastingly the cumulative fitness of the rosette categories did not differ in the *S. latifolia* habitat (Fig. 4).

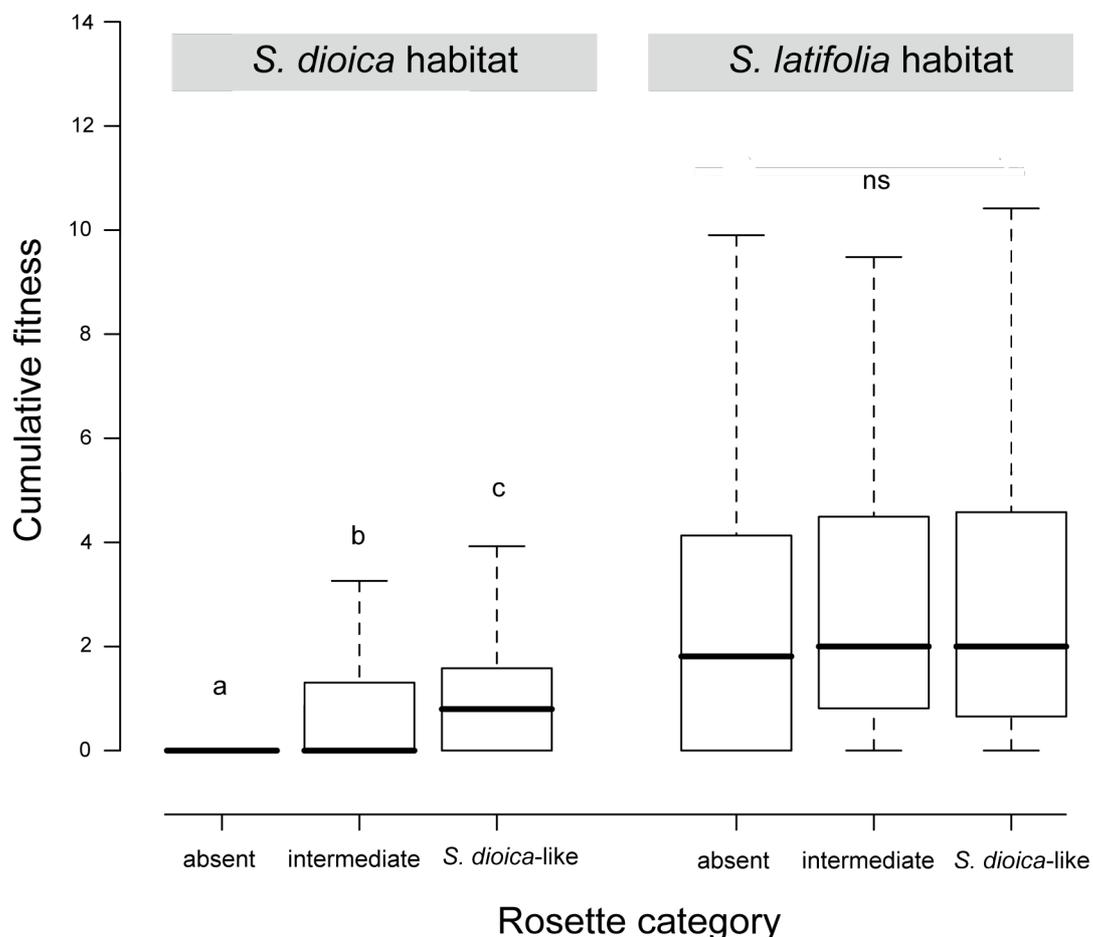


Figure 4 Cumulative fitness of the F₂ hybrids for each rosette category (absent, intermediate or *S. dioica*-like) in the habit of *S. dioica* and *S. latifolia*. Within habitat types, means that differed between sites in the Tukey's HSD test are indicated by different letters.

Discussion

The result of this transplant experiment supports the hypothesis that *Silene latifolia* and *S. dioica* are adapted to their own habitats (chapter 3). Here, we found that the relative contribution of flowering and survival to the fitness advantage of both species in their own habitat differs between the sites of this transplant experiment. The foreign species had a lower survival than the resident at each site except in one *S. latifolia* site, whereas F₁ hybrids survived generally well and F₂ hybrids were intermediate. The flowering incidence is always low for the F₂s and intermediate for the F₁s. The *S. dioica*-like overwintering rosette appears to be under selection in the *S. dioica* habitat.

The number of months survived was higher for *Silene latifolia* and *S. dioica* in their own habitat, whereas both species flowered more in *S. latifolia* habitat. By the end of the experiment, over 90% of *S. latifolia* plants were dead in *S. dioica* habitat and 80% of *S. dioica* individuals did not survive in *S. latifolia* habitat. A failure to survive in the habitat of the other species might compromise successful establishment and be the primary cause to the rarity of contact sites (Karrenberg and Favre, 2008). Differences in survival between ecologically diverged species are not always observed in reciprocal transplant experiments. Indeed, some studies have shown that differences in survival might appear only in one of the transplant habitats (Fritsche and Kaltz, 2000) or may be completely absent (Burgess and Husband, 2006; Byars and Hoffmann, 2009; Emms and Arnold, 1997). In fact, the demonstration of *Iris hexagona* and *I. fulva* being adapted to their own habitat was based on other fitness components than survival (Emms and Arnold, 1997). Similarly in *Ipomopsis*, the low fitness of *I. tenuituba* in *I. aggregata* habitat could not have been predicted on survival alone (Campbell and Waser, 2001, 2007). In our study however, survival strongly shapes the cumulative fitness differences between the species, with the exception of the site Leuk (*S. latifolia* habitat). At sites where survival of *S. latifolia* and *S. dioica* differs, the probability that hybridization occurs is reduced. In Leuk, the number of months survived did not differ significantly between *S. latifolia* and *S. dioica* because differences in survival appeared only two years after transplantation. Because Leuk is at an altitude comparable to that of natural

contact sites between the species, it is possible that differences in flowering incidence play a similar role in such sites.

These strong differences in survival between sites and cross types found in this study contrast with a previous greenhouse experiment (Chapter II), where survival did not differ between the species in different stress treatments. In the greenhouse experiment, the major contrast was the failure to induce flowering for *S. dioica* but not *S. latifolia* under the shade treatment. However, we observed similar indications for contrasting life history between the species in both experiments. First, *Silene dioica* did not flower more in its own habitat but survived well: it might delay flower induction until a threshold flowering size is reached. Moreover, *S. latifolia* flowered well in the greenhouse experiment, and also behaved more like a rapid cycluser in the field (all surviving *S. latifolia* individuals flowered in *S. latifolia* habitat). The life history of the species might differ because of adaptation to different degrees of disturbance, *S. latifolia* habitat being highly disturbed compared to *S. dioica* habitat.

Interestingly, the underlying cause of fitness variation differed between hybrid classes and between transplant sites. While crosses within species and first generation hybrids mainly differed from each other in survival and not in flowering incidence, the F₂ hybrids had intermediate survival times but low flowering incidence. Relative to *S. dioica* and *S. latifolia*, F₁ hybrids survived and flowered variably according to the transplanting site. There were large differences between the sites for survival and flowering, rendering the site replication necessary to distinguish between local selection and habitat-wide selection (Kawecki and Ebert, 2004). For instance, we would not have been able to detect major differences for survival in the *S. latifolia* habitat if only the site Leuk had been considered. Studies using hybrids for transplant experiments in contrasting habitats have found various results on survival and flowering. In *Leavenworthia*, neither survival or flowering were affected by transplantation in both hybrid classes (Koelling and Mauricio, 2010). In *Avena*, F₂ hybrids had an intermediate or slightly lower probability to survive depending on the transplant site considered (Johansen-Morris and Latta, 2006). In *Ipomopsis* and *Iris*, F₁ and F₂ hybrids survived as well as the parental species, but in *Iris* flowering was intermediate or very reduced for these hybrid cross types depending on which the

habitat was considered (Campbell et al., 2008; Emms and Arnold, 1997). Our results are similar to those in *Iris*.

Among the F₂ hybrids, we found that the *S. dioica*-like overwintering rosette was associated with higher cumulative fitness in the *S. dioica* habitat, but not in *S. latifolia* habitat. The lack of an overwintering rosette is a good candidate to explain the low survival of *S. latifolia* in the *S. dioica* habitat because the expression of this trait coincides with drastic differences in survival between *S. dioica* and *S. latifolia* (30% and 92% respectively). The compact structure of the *S. dioica*-like rosette might be efficient to prevent frost damage and serve as an important storage unit (Baker, 1947a, 1947b; Hadley and Bliss, 1964; Reader, 1978). Additionally, it might be an advantage to face the competition for space in the *S. dioica* habitat, where the vegetation cover is higher than in the *S. latifolia* habitat (Karrenberg and Favre, 2008). The advantage of such a structure was also detected in several Fabaceae species and was positively correlated with winter survival (Annicchiarico and Iannucci, 2007). Likewise, winter survival was also negatively correlated with more upright plant growth in *Carthamus tinctorius* (Johnson et al., 2006). A caveat to our analysis is that it was only based on the production of an overwintering rosette in the first year whilst several studies reported that the strength of selection and the mean fitness of hybrids might vary between years (Johansen-Morris and Latta, 2008; Jordan, 1991). Moreover, both fitness and rosette production could be due to difference in resource accumulation. However, in the F₂, the rosette production was not related to the size of a plant (data not shown). We are thus confident that the *S. dioica*-like overwintering rosette is under positive selection in *S. dioica* habitat.

In conclusion, contrasts in survival may account for a major limitation to gene flow between *S. dioica* and *S. latifolia* at most sites. In the *S. dioica* habitat, the *S. dioica*-like overwintering rosette is under positive selection and might be a key trait to limit the establishment of *S. latifolia* in the *S. dioica* habitat. Moreover, both species have probably evolved different life history because of the contrasting degree of disturbance of their respective habitat. *Silene latifolia* behaves more like a rapid cycler, whereas *Silene dioica* delays reproduction. In one *S. latifolia* site situated at the same altitude as natural contact sites, survival did not differ between the species.

Here, low flowering incidence of *S. dioica* and both F₁ and F₂ hybrids might contribute relatively more to reduce gene flow between the species.

Acknowledgments

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General discussion

Plant model systems such as *Helianthus* (Rieseberg et al., 2007), *Ipomopsis* (Campbell et al., 2008) or *Iris* (Arnold et al., 2004) have greatly contributed to our knowledge in the field of ecology and evolution. The genus *Silene*, with a tradition of various studies since Mendel and Darwin, is now in line with such plant systems (Bernasconi et al., 2009). In this thesis, we gathered ecological and genetic data from studies in natural populations, greenhouse and transplant experiments using *S. latifolia* and *S. dioica*. First, we studied the ecological and genetic differentiation of these species by investigating natural populations in the Swiss Alps. We found that *S. dioica* and *S. latifolia* are genetically differentiated both in allopatry and at contact sites. Moreover, these species occupy different habitats that contrasted for several ecological features. These habitats nevertheless overlap and contact sites are similar to *S. latifolia* habitat. There, only few early generation hybrids were found, indicating that hybrid individuals are either rarely produced or that they fail to survive until reproduction. Many hybrid zones show this bimodal pattern, for example in *Gryllus* (Jiggins and Mallet, 2000). With this field study, we characterized and compared the habitats of the two species. We show how detailed investigations of habitat parameters together with genetic analyses can contribute to the understanding of the habitat mediated selection in cross fertile species.

To test if differences in stress tolerance could contribute to the reproductive isolation between these *Silene* species and explain the rarity of hybrids in nature (Goulson and Jerrim, 1997; Minder et al., 2007) we designed a two-year greenhouse experiment involving both species and their reciprocal hybrids. We first hypothesized that *S. dioica* would perform better under shade stress because this plant can occur in forest populations. Secondly, we expected that *S. latifolia* would withstand drought stress better than the other cross types. Our results contrasted with our expectations as the stress tolerance did not differ between the cross types under drought treatment. Moreover, *S. dioica* and the hybrids failed to flower under shade, whereas *S. latifolia* reproduced in this treatment and its plastic response was larger. We attributed these results to different life history strategies of the species related to differences in the degree of disturbance between the species' habitat. A high plastic response and rapid flowering in *S. latifolia* could be an adaptation to higher

disturbance in its habitat, whereas a delay of reproduction might be adaptive in the more predictable environment of *S. dioica*. Genes for flower induction of *S. dioica* might be dominant in the F₁ hybrids, which might lower their life-time fitness as they occur in habitats with a rapid turnover. This thesis contributes to the idea that studies reporting fitness of hybrids should take stress response into consideration as fitness differences might appear only under challenging conditions.

Since several barriers to hybridization can occur between cross-fertile species (Rieseberg and Willis, 2007; Templeton, 1989), we used a transplant experiment to investigate components of reproductive isolation, such as flowering phenology and differences in fitness. We compared *S. dioica*, *S. latifolia* and two classes of hybrids (F₁ and F₂) transplanted into three natural populations of each species. The replication of sites within habitat types was one of the strengths of this study because it took into account the possible variation of the environmental factors between sites (Kawecki and Ebert, 2004). We showed that habitat adaptation, phenological divergence and F₂ hybrid breakdown may act as reproductive barriers between *S. dioica* and *S. latifolia*. We suggest that the remaining gene flow more likely occurs from *S. dioica* into *S. latifolia* for several reasons: first there was a stronger fitness reduction of *S. latifolia* in *S. dioica* habitats than vice versa, then first-generation hybrids had high fitness in an intermediate *S. latifolia* site and finally flowering of hybrids overlaps longer with *S. latifolia* than with *S. dioica*. This work adds new findings to previous studies on reproductive isolation between *S. dioica* and *S. latifolia* (Rahme, 2009). Moreover, this thesis illustrates the need of habitat replication when investigating species wide habitat adaptation.

In the same experiment, we were interested in identifying the mechanisms of habitat adaptation. We compared the cross types for survival and flowering incidence at each site, and used the F₂ hybrids to test if the overwintering rosette was a trait under selection. Such recombinant F₂ hybrids allow to investigate natural selection on individual phenotypic traits and exhibit variation for these traits (Lexer et al., 2003). This method has been successfully used by several authors (Jordan, 1991; Nagy, 1997; Schemske and Bradshaw, 1999; Wright and Stanton, 2007). Our results suggest that differences in survival mainly limit the ability of *S. dioica* and *S. latifolia* to establish populations in the other species habitat. Moreover, the establishment of

S. latifolia in the *S. dioica* habitat might be limited because it lacks the overwintering rosette, a trait under positive selection in the *S. dioica* habitat but not in the *S. latifolia* habitat.

Generally, this thesis adds to our understanding of habitat mediated reproductive isolation between two cross-fertile species. Moreover, it shows how an integrative approach combining field observations, genetic studies, and manipulative experiments contributes to our understanding of plant ecology and evolution.

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