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Mechanisms involved in the maintenance of inbreeding depression in gynodioecious *Silene vulgaris* (Caryophyllaceae): an experimental investigation

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par

Mélanie Glaettli

Biologiste diplômée
de l'Université de Lausanne

Jury

Prof. Yves Poirier, Président et Rapporteur
Prof. Jérôme Goudet, Directeur de thèse
Prof. David E. McCauley, expert
Prof. Lukas F. Keller, expert
Dr. Luc Gigord, expert

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Rapporteur	*Monsieur*	*Prof.*	*Yves* **Poirier**
Experts	*Monsieur*	*Prof.*	*David E.* **McCauley**
	Monsieur	*Prof.*	*Lukas F.* **Keller**
	Monsieur	*Dr*	*Luc* **Gigord**

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Madame Mélanie Glaettli

Biologiste diplômée de l'Université de Lausanne

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Contents

Summary

Gynodioecy, the joint occurrence of females and hermaphrodites within natural populations, is a widely studied mating system ever since Darwin (1877). It is an exceptional mating system because continuous selection is necessary to maintain it. Since females only reproduce through ovules whereas hermaphrodites transmit genes through ovules and pollen, larger female fitness, in terms of seed output, is required to allow their maintenance. Two non-exclusive mechanisms can account for the maintenance of females. First, as females do not produce pollen they can reallocate their resources towards a higher ovule production. Second, hermaphrodites can self- and cross-fertilize whereas females are obligate outcrossers. Thus hermaphrodites should partly suffer from inbreeding depression (i.e.: the fitness decline of inbred relative to outbred individuals) and thereby produce less fit progeny than females.

This thesis investigated the effects of self- and cross-fertilization of hermaphrodites over two consecutive generations. Inbreeding depression increased across the successive stages of the life-cycle (i.e.: from "seed traits" to "reproductive traits") displaying large inbreeding depression estimates (up to 0.76). This investigation not only detected large inbreeding depression estimates but also detected mechanisms involved in the maintenance of inbreeding depression. For instance cryptic self-incompatibility which is here a larger *in vivo* pollen performance of distant pollen compared to self-pollen; the expression of inbreeding depression especially in late life-cycle stages, and the appearance of females in the progeny of selfed hermaphrodites.

The female biased sex ratio in the progeny of selfed hermaphrodites was a surprising result and could either come from the sex determining mechanisms (complex nucleo-cytoplasmic interaction(s)) and/or from inbreeding depression. Indeed, we not only got females and hermaphrodites but also partial male-sterile (PMS) individuals (i.e.: individuals with differing number of viable stamens). We detected that inbred pollen bearing plants (excluding females) have less viable stamens per flower than outbred plants. A positive correlation was detected between inbreeding depression for the number of viable stamens *per* flower and the difference in sex ratio between inbred and outbred individuals. A positive relationship was also detected between inbreeding depression for pollen viability and inbreeding depression for number of viable stamens *per* flower. Each correlation can either account for pleiotropic effects (a major gene acting on the two considered traits) or linkage disequilibrium between genes controlling each of the two related traits. If we hypothesize that these correlations are due to a major gene with pleiotropic effects, the positive relationship between inbreeding depression for number of viable stamens *per* flower and inbreeding depression for pollen viability showed that deleterious alleles present on a major gene coding for pollen production and viability depressed male fitness within inbred plants. The positive relationship between sex ratio difference between inbred and outbred individuals and inbreeding depression for number of viable stamens *per* flower indicates that (1) either number of viable stamens *per* flower is, in addition to inbreeding, also affected by the loci coding for sex determinism or, (2) the presence of females within the progeny of selfed hermaphrodites is a consequence of large inbreeding depression inhibiting pollen production, or (3) sex is here determined by a combination of loci coding for sex expression and inbreeding depression for male reproductive traits.

In conclusion, *Silene vulgaris* has been shown to be a good model for understanding the evolution of mating systems that promote outbreeding.

Résumé

La gynodïoécie est définie comme étant la présence simultanée d'hermaphrodites et de femelles au sein de populations naturelles d'une même espèce. Ce système de reproduction a toujours fasciné le monde scientifique depuis Darwin, comme en témoigne ses écrits (1876, 1877) sur les systèmes de reproduction chez les plantes. Les femelles ne transmettent leurs gènes qu'à travers leurs ovules alors que les hermaphrodites transmettent leurs gènes à la fois par la voie mâle (le pollen) et la voie femelle (les ovules). La condition pour que la gynodïoécie se maintienne nécessite donc une fitness de la fonction femelle plus élevée chez les femelles que chez les hermaphrodites. Deux mécanismes mutuellement non exclusifs peuvent expliquer le maintien des femelles au sein de ces populations gynodioïques. D'une part, les femelles peuvent réallouer les ressources non utilisées pour la production de pollen et peuvent par conséquent produire plus d'ovules. D'autre part, la reproduction des femelles ne peut se faire que par allo-fécondation alors que les hermaphrodites, peuvent se reproduire à la fois par auto- et allo-fécondation. L'autofécondation s'accompagne en général d'une diminution de fitness de la descendance relativement à la progéniture issue d'allo-fécondation ; ce phénomène est connu sous le nom de dépression de consanguinité.
Cette thèse avait pour but de mettre en évidence une éventuelle dépression de consanguinité chez *Silene vulgaris*, une espèce gynodioïque. Des hermaphrodites, issus de trois vallées alpines, ont été auto- et allo-fécondés sur deux générations successives. La dépression de consanguinité pouvant s'exprimer à tous les stades de vie d'un individu, plusieurs traits de fitness, allant du nombre de graines par fruit à la production de gamètes ont été mesurés sur différents stades de vie successifs. L'estimation de la dépression de consanguinité totale atteignait des valeurs allant de 0.52 à 0.76 selon la vallée considérée, ce qui indiquerait que les hermaphrodites ont tout intérêt à limiter l'autofécondation et que les femelles ne devraient pas avoir de peine à subsister dans les vallées étudiées. Par la même occasion des mécanismes diminuant la purge potentielle du fardeau génétique, et permettant ainsi le maintien du « niveau » de dépression de consanguinité et par conséquence le maintien de la gynodïoécie ont été mis en évidence. En effet, nos résultats montrent que la dépression de consanguinité s'exprimait tard dans le cycle de vie permettant ainsi à un certain nombre individus consanguins de transmettre leurs allèles délétères à la génération suivante. D'autre part, la croissance *in vivo* des tubes polliniques d'auto-pollen était plus lente que celle de l'allo-pollen et donc en situation de compétition directe, les ovules devraient plutôt être issus d'allo-fécondation, diminuant ainsi les chances de purges d'allèles délétères. Enfin, l'apparition de femelles dans la progéniture d'hermaphrodites autofécondés diminue aussi les chances de purge d'allèles délétères. Il nous a été impossible de déterminer si l'apparition de femelles dans la descendance d'hermaphrodites autofécondés était due au déterminisme génétique du sexe ou si la différence de sexe ratio entre la descendance auto- et allo-fécondée était due à une éventuelle dépression de consanguinité inhibant la production de pollen. Nous avons observé que *S. vulgaris* ne présentaient pas uniquement des hermaphrodites et des femelles mais aussi toute sorte d'individus intermédiaires avec un nombre variable d'étamines viables. Nous avons pu mettre en évidence des corrélations positives entre (1) la différence de sexe ratio (la proportion d'individus produisant du pollen) entre individus consanguins et non consanguins et une estimation de la dépression de consanguinité pour le nombre d'étamines viables d'individus produisant du pollen, ainsi qu'entre (2) la dépression de consanguinité pour le nombre d'étamines viables et celle estimée pour la viabilité du pollen. Chaque corrélation indique soit l'effet d'un (ou plusieurs) gène(s) pléiotropique(s), soit un déséquilibre de liaison entre les gènes. En considérant que ces corrélations sont le résultat d'effet pléiotropiques, la relation entre le nombre d'étamines viables par fleur et la viabilité du pollen, indiquerait un effet négatif de la consanguinité sur la production et la viabilité du pollen due partiellement à un gène majeur. La seconde corrélation indiquerait soit que les gènes responsables de la détermination du sexe agissent aussi sur l'expression de la fonction mâle soit que l'expression du sexe est sujette à la dépression de consanguinité, ou encore un mélange des deux.
Aux regards de ces résultats, *Silene vulgaris* s'est avéré être un bon modèle de compréhension de l'évolution des systèmes de reproduction vers la séparation des sexes.

GENERAL INTRODUCTION

In the plant kingdom, a wide diversity of breeding systems exists and has been of great interest since Darwin's books: "*The effects of cross and self-fertilization in the vegetable kingdom*" (1876) and,"*The different forms of flowers on plants of the same species*" (1877). These books not only provided data about variation in the plant mating systems but also considered their importance for the production of genetically diverse and vigorous offspring. Indeed, inbreeding depression (i.e.: the relative larger fitness of cross-fertilized progeny compared to self-fertilized progeny) is generally recognized as the main selective force shaping the evolution of mating strategies (Charlesworth and Charlesworth, 1987; Holsinger, 2000; Barrett, 2002; Keller and Waller, 2002). Darwin (1876, 1877) also indicated the possible evolutionary pathways between mating systems and suggested that gynodioecy, the joint occurrence of females (male-sterile) and hermaphrodites within natural populations, is an intermediate step between hermaphroditism and dioecy. Indeed, the evolution of two separate sexes requires a minimum of two mutations: one inducing male-sterility (creating females) and, a second inducing female-sterility (producing males) (Charlesworth, 1999). This thesis deals with inbreeding depression, the way different mechanisms can maintain large levels of inbreeding depression and its importance in the maintenance of gynodioecy.

The advantages and disadvantages of self-fertilization

In contrast to the majority of animals, and especially vertebrates, in which individuals are unisexual and can only reproduce through cross-fertilization, a large majority of flowering plants are hermaphroditic (i.e.: bearing both sexual functions, anthers (male) and stigmas (female)) and can potentially reproduce through self-fertilization, the fusion of gametes from the same parent. There are several advantages to self-fertilization. First, self-compatible

hermaphroditic individuals are not subject to the "Allee effect", the difficulty of finding a sexual partner in low density populations, because they can self-fertilize (Begon, Harper, and Townsend, 1996). This is consistent with Baker's law (Baker, 1953) which states that self-compatible hermaphrodites are more likely to establish new populations after long distance dispersal than self-incompatible or dioecious species (Heilbuth, 2000). The reproductive assurance through selfing of self-compatible hermaphrodites has also been shown to allow the maintenance of species in metapopulation dynamics with frequent extinction and recolonization (Pannell and Barrett, 1998; Pannell, 2000). Secondly, self-fertilization has a two-fold transmission advantage over cross-fertilization since it allows the transmission of both ovules and pollen to their progeny whereas cross-fertilization only transmits one set of genes, either ovules or pollen. However, there also are disadvantages to self-fertilization which explains why many mechanisms have evolved to prevent self-fertilization. For instance self-incompatibility (Hiscock and McInnis, 2003) is a mechanism preventing self-fertilization or reproduction with a related individual through genetically determined pollen recognition. Also, separation of sexes such as dioecy (i.e.: plants are either producing male or female) or dichogamy in which the female and male reproductive phases do not overlap, are mechanisms preventing self-fertilization (Barrett, 2002, 2003). The major selective disadvantage of self-fertilization, which leaded to the evolution of mating systems promoting cross-fertilization, is inbreeding depression.

The genetic basis of inbreeding depression and its dynamics

Inbred mating, and its extreme form: self-fertilization, increases the amount of homozygous loci within the offspring and, thereby lowers their fitness. This phenomena is known as inbreeding depression and is expressed as the relative fitness decline of inbred compared to oubred individuals (Charlesworth and Charlesworth, 1987; Keller and Waller, 2002). Two

hypotheses can explain the genetic basis of inbreeding depression: overdominance or partial dominance. Under the overdominance hypothesis, outbred individuals which are more likely to be heterozygous, have increased vigor relative to both homozygotes (Charlesworth and Charlesworth, 1987). According to the partial dominance hypothesis, inbreeding depression is the expression of recessive deleterious mutations (i.e.: genetic load) in homozygous individuals (Charlesworth and Charlesworth, 1987; Lynch and Walsh, 1998). The main difference between the two mechanisms is that under partial dominance, inbreeding exposes deleterious recessive alleles to natural selection and, through purging, can eventually lower the genetic load (Lande and Schemske, 1985). The study by Barrett & Charlesworth (1991) on *Eichhornia paniculata* (Pontedriaceae) was the first to clearly showed purging. After one generation of self-fertilization, flower production decreased but no further decline was detected in subsequent generation of selfing. Moreover, after five generations of self-fertilization, the number of flowers per plant of progeny derived from intercrosses between inbred lines was larger than in the original outbred population. This clearly shows that the genetic load has decreased through purging. Thus, under the partial dominance hypothesis, a negative correlation between the levels of inbreeding depression and the degree of inbreeding is expected: highly selfing species should have low levels of inbreeding depression because deleterious mutations have already been (or are continuously) purged compared to predominantly outcrossing species (Lande and Schemske, 1985).

Lande and Schemske (1985) estimated inbreeding depression (δ) as $(w_o-w_s)/w_o$ where w_o and w_s are, respectively, the fitness of outcrossed and selfed individuals. Thus, $\delta>0$ indicates that fitness of oucrossed individuals is larger than fitness of selfed individuals and, if $\delta<0$ the fitness of outcrossed individuals is lower than the one of selfed individuals. The theoretical model by Lande and Schemske (1985) showed that only two stable equilibria exist: selfing species with low inbreeding depression and outcrossing species with large levels of

inbreeding. A third non-stable equilibrium exists when δ=0.5, here the two-fold transmission advantage of self-fertilization is just balanced by outcrossed individuals that are twice as fit as selfed ones. Therefore a small increase or decrease in inbreeding depression should either favor cross-fertilization or self-fertilization, respectively. Thus predominantly selfing species should have an inbreeding depression coefficient (δ) lower than 0.5 and outcrossing species should display inbreeding levels larger than 0.5. These results were confirmed by experimental investigations (*reviewed in* Husband and Schemske, 1996).

Gynodioecy, its maintenance and the way sex is determined

Gynodioecy, the co-occurrence of females (i.e.: male-sterile plants) and hermaphrodites within populations, is an exceptional and widely studied mating system (Darwin, 1877; Couvet, Ronce, and Gliddon, 1998; Bailey, Delph, and Lively, 2003; Delph and Mutikainen, 2003; Shykoff et al., 2003). Since females only reproduce through ovules whereas hermaphrodites reproduce through ovules and pollen, the maintenance of gynodioecy requires a higher fitness of females relative to hermaphrodites, in terms of seed output (Pettersson, 1992b; Sakai et al., 1997). The larger fitness of female can come from two non-exclusive mechanisms: reallocation of resources and avoidance of inbreeding depression. Because females lack the male function, they can allocate more resources to other traits, for instance higher ovule production or increased longevity. The offspring of females should also be fitter than hermaphrodites' progeny because females cannot self-fertilize whereas hermaphrodites can, and do in some species (Jolls and Chenier, 1989; Pettersson, 1992b; Mutikainen and Delph, 1998; Rankin, Weller, and Sakai, 2002; Shykoff et al., 2003).

Although, there are mechanisms that allow females to compensate for the loss of the male function, the way sex is inherited is also important in determining the threshold fitness advantage females must have to be maintained. The mode of sex inheritance in gynodioecious

species has often been shown to be nucleo-cytoplasmic (Saumitoulaprade, Cuguen, and Vernet, 1994; Charlesworth and Laporte, 1998): a cytoplasmic male-sterility (CMS) allele (inherited maternally) suppresses the male function and, specific corresponding nuclear genes (either inherited through the male or the female function) can restore pollen production. In this context nuclear genes are called restorers. Models investigating the maintenance of polymorphism at both cytoplasmic and nuclear genes had to assume inbreeding depression, a difference in seed production between sex morphs and a fitness effect of restorer genes. Indeed, restorers were favored when interacting with the cytoplasmic allele they specifically restore but had a "cost of restoration" in presence of alien cytoplasmic alleles they do not restore (Frank, 1989; Gouyon, Vichot, and Van Damme, 1991; Couvet, Ronce, and Gliddon, 1998). Now, if polymorphism is lost either at cytoplasmic or nuclear genes, sex determinism becomes respectively nuclear or cytoplasmic. If sex is inherited through nuclear genes, the fitness of females must be twice that of hermaphrodites providing no pollen limitation (in terms of female function; i.e.: seed production) whereas if sex is inherited partly or totally through cytoplasmic genes only a slight advantage for females, not necessary two-fold, is needed (Charlesworth and Ganders, 1979).

When sex is only inherited through cytoplasmic genes, the slight advantage of females will allow them to rapidly increase in frequency within populations. However, they cannot outcompete hermaphrodites because when females become dominant they also become pollen limited and the fitness of hermaphrodites can thus exceed that of females. Therefore fitness of both sexes depends on the sex ratio (i.e.: the proportion of hermaphrodites) of a population or more precisely on the amount of hermaphrodites in the neighborhoods (McCauley and Brock, 1998; Graff, 1999). Fitness of both females and hermaphrodites have been shown to be frequency-dependent in *Silene vulgaris* (McCauley and Brock, 1998; Taylor, Trimble, and McCauley, 1999). When hermaphrodites are rare both sexes are affected: females are pollen

limited and produce less seeds, and hermaphrodites are more likely to self-fertilize since outcrossing opportunities are small and, thus produce more inbred progeny that is likely to suffer from inbreeding depression. Thus fitness of both sexes increase with the proportion of hermaphrodites.

Study species

Silene vulgaris is a gynodioecious herbaceous perennial plant distributed in the Holarctic. Its sex determinism has been shown to be nucleo-cytoplasmic (Charlesworth and Laporte, 1998). The pollinators of *S. vulgaris* are moths (Noctuidae and Geometridae) which generally forage within the same flowering plant before flying to a nearby plant (Pettersson, 1991, 1992b). Indeed pollen movements have been shown to be limited (Pettersson, 1992b; Taylor, Trimble, and McCauley, 1999). Also, seeds have limited dispersal since they disperse through gravity (McCauley, 1998; Olson and McCauley, 2002). The limited dispersal of both pollen and seeds provides opportunities for population structure and non-negligible selfing rates. Indeed, selfing rates have been shown to vary between 0.15 and 0.61 (Emery and McCauley, 2002). And, McCauley (1998) showed that population structure is large since F_{ST} values for chloroplastic DNA and allozymes were respectively 0.62 and 0.22, showing that pollen movements are larger than seed movements. His sampling design consisted of patches where all individuals were not more than 20m away within an area of 20km.

This study was conducted on three valleys of the western Swiss Alps in which different populations occur. In each population we defined several patches and, with the help of microsatellites we detected a $F_{indpatch}$ (corresponding to F_{IS}) of 0.3 in all patches corresponding to a selfing rate of about 50% per patch (Box. 1).

Box 1. Hierarchical genetic structure of our *Silene vulgaris* populations

Sampling

Samples were collected in summer 2000 from populations located in three distinct valleys (Mont d'Or, Dent de Jaman, Val Ferret) in the western Swiss Alps. Valleys are separated by 10-55km. In each valley, sampling was performed in 4 (Mont d'Or), 5 (Val Ferret) and 6 (Dent de Jaman) populations, respectively. Populations within each valley were separated by less than 4km and at altitudes varying between 1550-2150m. Population sex ratios (i.e.: proportion of hermaphrodites) were varying between 0.6 and 1. Within populations, patches were defined as groups of individuals located within a range of 5-10m and separated from each other by 20-100m. From each patch, 6-13 individuals were randomly collected for genetic analysis.

Hierarchical design and F-statistics

Genetic diversity was partitioned for microsatellites (nDNA) and chlorotypes (chlDNA) using a hierarchical design. Individual (ind, only relevant for microsatellites) are nested within patches (patch), themselves in populations (pop) which are nested in valley (val). Finally we have the overall layer, named total. In order to estimate the variance components, we implemented the algorithm described by Yang (Evolution, 1998) in the R statistical package. The overall diversity σ_t is then the sum of the σ_w, σ_ind σ_patch, σ_pop and σ_val. The way diversity is apportioned can be investigated with the help of hierarchical F-statistics. We used the following statistics:

$F_{indpatch}$=(σind)/(σw+σind) (only for microsatellites, for chlorotypes, σw=0), which measures departure from random mating at the level of the patch

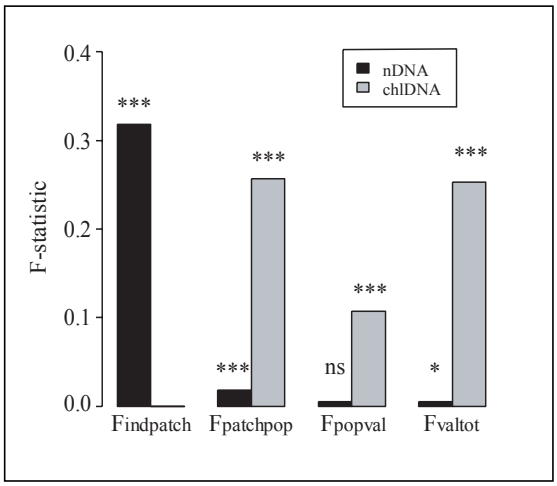
$F_{patchpop}$=(σpatch)/(σw+σind+σpatch) quantify differences in allele frequencies among patches relative to the population

F_{popval}=(σpop)/(σw+σind+σpatch+σpop) quantify differences in allele frequencies among population relative to the valley

$F_{valtotal}$=(σval)/(σw+σind+σpatch+σpop+σval) quantify differences in allele frequencies among valleys relative to the total

Statistical significance of F-statistics

The significance of each component was tested using permutations. With this method, one generates the distribution of the appropriate component under the null hypothesis, and compares the observed value to the distribution under the null hypothesis. The probability associated with the test is the proportion of values under the null distribution that are larger than the observed. One essential step in this procedure is to identify the adequate unit to permute: for σind, alleles were permuted among individuals within patch. For σpatch, individuals were permuted among patches within populations. For σpop, whole patches were permuted among populations, within valleys. Last, for valleys, whole populations were permuted among valleys.



ns: p>0.05, *: p<0.05, ***: p<0.001

Main results

1) The high genetic correlation of alleles within an individual shows that selfing is important, selfing rate estimated as: S= 2*$F_{indpatch}$ / (1+$F_{indpatch}$) (Hartl and Clark, 1997) is around 0.5 for all patches.

2) Maternally inherited markers (chlDNA) are highly structured already at the lowest scale.

3) Microsatellites and chloroplastic markers show tremendous differences at all levels of structure. These differences are unlikely due to pollen transfer which has been shown to be limited in *S. vulgaris* (Pettersson, 1992). It is more likely to come from our highly polymorphic microsatellite markers (Juillet et al., 2003).

Population structure inferred from microsatellites was low ($F_{patchpop}$=0.02) but significant between patches within populations and even lower for higher hierarchical levels (Box. 1). However, the use of chloroplastic DNA displayed more structure already at the lowest geographical scale ($F_{patchpop}$=0.26).

The large difference of structure between chloroplastic DNA and nuclear DNA (microsatellites) comes either from long-distance pollen dispersal and/or the high levels of polymorphism among the microsatellite markers. Since pollen movement have been described to be locally restricted in *Silene vulgaris* (Pettersson, 1992b; McCauley and Brock, 1998), the discrepancy between chloroplastic and nuclear DNA is rather due to the large levels of polymorphism of the microsatellite markers (Juillet et al., 2003). To compare our chloroplastic DNA structure with the study by McCauley (1998) we assessed F_{ST} as $F_{patchtot}$. We estimated $F_{patchtot}$ to be 0.5 ($p<0.001$) which falls within the confidence interval of McCauley (1998).

Thus, the high selfing rates and the population structure detected in our *S. vulgaris* populations showed that inbred matings are substantial and show that experimental investigations on inbreeding depression are relevant. Inbreeding depression was already detected in *S. vulgaris*, but was not assessed on the whole life-cycle of the plant (Jolls and Chenier, 1989; Pettersson, 1992b; Emery and McCauley, 2002).

Outline of the thesis

This thesis assessed the importance of inbreeding depression across the successive life-cycle stages, from seed production to pollen viability. For this purpose, I experimentally investigated the effects of self- and cross-fertilized on different hermaphrodites that originated from seeds collected in the three alpine valleys for which we assessed population structure (Box. 1). In chapter I, investigations on the effects of one generation of self- and cross-

fertilization of hermaphrodites were conducted. Since both pollination treatments were applied on each individual (called family) we further compared estimates of inbreeding depression for each family and at the valley level. In chapter II, I describe the results of a second generation of self- and cross-fertilizing hermaphrodites from the previous generation. Since three levels of inbreeding (F=0, 0.5 and 0.75) were obtained, we investigated whether synergistic epistasis could be detected. In chapter III, I present results for the effect of inbreeding on visual cues for the potential attractiveness of plants and flowers to pollinators. Finally, chapter IV investigates the effect of crossing distance on differential pollen performance within the stigma and style. The results presented here show different mechanisms that prevent successive generations of self-fertilization. This hinders the purging process to take place and thus gives insight into mechanisms that can maintain high levels of inbreeding depression.

Population and family levels of inbreeding depression increase along the life-cycle in hermaphrodites of *Silene vulgaris* (Caryophyllaceae)

Abstract – We investigated the effect of one generation of selfing on the fitness of Silene vulgaris' hermaphrodites originating from three different valleys within the Western Swiss Alps. In each valley, inbreeding significantly depressed fitness and this inbreeding depression was increasing along the successive life-cycle stages: from number of seeds per fruit to flowering rates, through the proportion of aborted seeds per fruit and germination rates. The combination of these different life-cycle stages resulted in estimations of cumulative inbreeding depression from 0.46 to 0.56 for the different valleys. Progeny issued from self-fertilization also resulted in late germination and flowering as well as biased progeny sex ratio towards females. Our family-structured design allowed us to compute family and valley inbreeding depression estimates and, both estimates were found to be statistically similar for each fitness trait within each valley. Moreover family-level estimates allowed comparisons among the different life-cycle stages. Across life-cycle stages correlations were not significant for most traits, indicating that the genetic basis of inbreeding depression varies across the life-cycle.

INTRODUCTION

Inbreeding depression is the reduction in fitness of inbred relative to outbred individuals and is considered as the primary force opposing the transmission advantage associated with selfing (Holsinger, 2000; Keller and Waller, 2002). Theoretical models (Lande and Schemske, 1985) have shown that a weak fitness difference between inbred and outbred individuals, with an inbreeding depression coefficient (δ) lower than 0.5, should favor the evolution of self-fertilization since the gain of transmitting two gametes to a single offspring is larger than the cost of inbreeding. However, if an outbred individual which only transmits one gamete *per* offspring, is more than twice as fit as inbred ones (δ>0.5); the loss of inbred progeny is too costly to balance the gain of transmitting more genes and, cross-fertilization should be favored. Selfing rate and inbreeding depression are negatively correlated and the threshold value between the evolution of outcrossing and the one of selfing is 0.5. Thus, the intensity of overall inbreeding depression is thought to play a major role in the evolution of mating systems, and for instance separation of sexes such as dioecy or gynodioecy (Charlesworth and Charlesworth, 1987; Charlesworth, 1999).

The study species, *Silene vulgaris*, is gynodioecious; hermaphrodites and females (i.e.: male-sterile individuals) co-occur within populations. Since females can only transmit their genes through the female function whereas hermaphrodites can contribute genes via ovules and pollen, a condition needed to introduce or to maintain females in populations is an improved fitness through seeds compared to hermaphrodites (Charlesworth and Ganders, 1979; Frank, 1989; Couvet, Ronce, and Gliddon, 1998). Reproductive compensation of females for the loss of male fitness can appear through two non exclusive mechanisms. First, females could produce a larger quantity of ovules and/or seeds, via reallocation of resources not used by the

male function. Secondly, females may also produce seeds of better quality than hermaphrodites, since they are obligatory outcrossers. Indeed, hermaphrodites have been shown to be more inbred than females and could therefore suffer from inbreeding depression (Jolls and Chenier, 1989; Pettersson, 1992b; Mutikainen and Delph, 1998; Baker, Thompson, and Barrett, 2000; Emery and McCauley, 2002).

In selfing as well as in predominantly outcrossing species, inbreeding depression can be expressed at early and/or at late life-cycle stages (Husband and Schemske, 1996), and not considering later stages could substantially underestimate effective inbreeding depression (Willis, 1999b; Goodwillie, 2000). It is therefore crucial to estimate the fitness of inbred and outbred individuals at various stages along the life-cycle to obtain accurate estimates of the total inbreeding depression. Furthermore, Husband and Schemske (1996) showed that inbreeding depression is generally of important magnitude in late life-cycle stages such as reproductive stages, whatever the reproductive system is. In predominantly selfing species, intensity of inbreeding depression was increasing throughout the life-cycle: late life-cycle traits such as size at maturity and/or reproductive stages contribute more to cumulative inbreeding depression estimates than early traits like number of seeds and/or germination rate. For outcrossing species, inbreeding depression was more homogeneously distributed along the life-cycle: inbreeding depression was large at seed production and reproductive stages. Their review also confirmed Lande and Schemske's (1985) theoretical expectations since cumulative inbreeding depression for selfing species (δ=0.23) was lower than the threshold value and also significantly smaller than inbreeding depression estimates for outcrossing species (δ=0.53). Thus, the combination of cumulative inbreeding depression and its intensity throughout the life-cycle, if congruent, could help to infer the predominant mating system (self- vs. cross-fertilization) with more confidence.

Since *S. vulgaris* is self-compatible, hermaphrodites can self and outcross while females have to outcross. Selfing rate estimations for different gynodioecious populations of *S. vulgaris* have been shown to vary widely between 0.15 and 0.6 (Emery and McCauley, 2002; Charlesworth, unpublished manuscript) and points out to a mixed mating system. Despite the theoretical work of Lande and Schemske (1985) claiming that intermediated selfing rates are not stable, different mechanisms have been shown to maintain stable partially self-fertilizing populations. For instance, population structure with density dependence (Ronfort and Couvet, 1995), spatial heterogeneity and temporal fluctuations (Cheptou and Mathias, 2001) or the occurrence of a genetic association between viability and mating system loci (Holsinger, 1991; Uyenoyama and Waller, 1991a, b, c; Uyenoyama, Holsinger, and Waller, 1993). An association between fitness and the mating system loci can occur as follows: selfing results in high proportions of homozygous loci through segregation and produces progeny at both fitness extremes. Some individuals will have high fitness with many homozygous loci for the wild-type allele and others will have low fitness with many homozygous deleterious mutations. Hence, high and low fitness genotypes can become associated with highly selfing genotypes. Low fitness individuals will then be removed by selection and only high fitness individuals will remain in the population. Thus, lineages with a history of selfing could exhibit higher fitness and lower inbreeding depression than lineages that have been outcrossed. These models emphasize that population inbreeding depression estimates are alone insufficient to predict whether a selfing variant can invade in a particular population and therefore highlight the importance of determining the variation in inbreeding depression among individuals. Variation of inbreeding depression at the family level has been explored in a few studies (Holstford and Ellstrand, 1990; Pray and Goodnight, 1995; Carr and Dudash, 1997; Koelewijn, 1998; Mutikainen and Delph, 1998; Fowler and Whitlock, 1999; Takebayashi and Delph, 2000). These studies all found among family variation for

inbreeding depression. But only Takebayashi and Delph (2000) found an association between inbreeding depression and mating system loci: herkogamy (i.e.: anther-stigma distance) was positively correlated with the intensity of inbreeding depression in cumulative fitness. On the other hand, Schultz & Willis (1995) showed through simulations that the random occurrence of deleterious additive or recessive mutations produce large variation in inbreeding depression among individuals (or families) and claimed that variation among individuals in inbreeding depression was not a sufficient indicator of among individuals differences in their history of inbreeding. Moreover, these mutations can also be more or less deleterious, and it follows that the genetic load and its expression (i.e.: inbreeding depression) are different in each family. Large among family variation in inbreeding depression was only related to history of inbreeding under restricted conditions (dominant selfing modifier, high selfing rate, recessive lethal as genetic mechanisms responsible for inbreeding depression) (Schultz and Willis, 1995). Despite family level estimates of inbreeding depression being controversial and population estimates being thought to be more relevant, whether these two estimates are comparable has not been assessed. Family level estimates of inbreeding depression can be relevant to see whether selfing variants could invade a population, to test whether populations are different in their intensity of inbreeding depression, but also to estimate correlations between the different life-cycle stages. A positive correlation between two life-cycle stages would either suggest positive pleiotropy, in which genes coding for both traits are partially the same, or that genes are different but under strong linkage disequilibrium. A negative correlation would point to a pleiotropic gene, that increases the value of the first trait but decrease the value of the second trait. Finally, no correlation would show that different genes are involved in the different life-cycle stages (Rao, Widen, and Andersson, 2002).

To quantify inbreeding depression, we a) sampled a large number of families (Lynch, 1991) and b) got estimates of inbreeding depression at different stages throughout the life-cycle.

Since only hermaphrodites can self, we manipulated experimentally the levels of inbreeding in hermaphrodites of *Silene vulgaris* coming from three different valleys within the Western Swiss Alps. To obtain family-structured data, we performed self- and cross-pollinations within dams. Our questions are: 1) Does inbreeding have negative effects on the fitness of progenies? 2) If there is inbreeding depression, at which life-cycle stage(s) is it expressed and can we infer a mating system? 3) Is the genetic basis for inbreeding depression similar across the life-cycle stages? 4) Are estimations of inbreeding depression at the valley level comparable to inbreeding depression estimates at the family level?

MATERIAL & METHODS

Study species

Silene vulgaris (Caryophyllaceae) is a gynodioecious short-lived perennial, weedy plant. Sex determinism is most likely under cytonuclear control (Charlesworth and Laporte, 1998; McCauley, Olson, and Taylor, 2000), where a cytoplasmic mutation induces male-sterility (CMS) and specific dominant nuclear restorers can restore the male function. Hence, a hermaphrodite can either come from a hermaphroditic mother that does not carry a CMS or it can carry nuclear restorers that correspond to the CMS inherited from its mother (hermaphrodite or female). Sex ratio, defined as the proportion of hermaphrodites, can vary among populations. Flowering occurs from May to September. Hermaphrodites are protandrous: stamens dehisce within the first 2 days and then stigmas develop and remain receptive 3 days long. Since individuals bear numerous flowers (up to 100 flowers/individual) that develop asynchronously over the flowering period, hermaphrodites can self through geitonogamy because pollinators tend to forage within a plant (Pettersson, 1992a). Fruits can contain up to 100 seeds.

Material collection

In autumn 2000, fruits containing seeds from natural populations were collected in 3 different valleys in the Western Swiss Alps: Mont D'Or (MO: 570389N, 137416E, altitude: 1880m), Dent de Jaman (DJ: 546398N, 144389E, altitude: 1543m) and Val Ferret (VF: 574761N, 084844E, altitude: 1895m). In MO valley, we sampled seeds from one large mountain population of approximately 500 individuals with overall sex ratio of 0.8 (calculated over 393 individuals), though females were locally clustered. Seeds coming from the two other valleys were sampled within different populations of smaller size (10-50 individuals for DJ and 50-100 individuals for VF). Distances between populations did not exceed 2km and their sex ratios were varying between 0.8 and 1 for both valleys.

In March 2001, viable seeds from the collected fruits were sawn in Jiffy pots (Ø 5cm) in a greenhouse with controlled conditions (temperature: 18-20°C, relative humidity: 50-60%). Seedlings were watered daily and grown under natural light conditions. About a month after germination, seedlings were transplanted in larger pots (Ø 15cm). In June 2001, when plants were flowering, we sexed them and randomly choose 20 hermaphrodites within each valley, each of those being the progeny of a different hermaphroditic mother on the field (Fig. 1).

Pollination treatments, growth conditions & fitness components

To control for genetic and dam effects, we performed self- and cross-pollinations within each selected plant (Lynch, 1988). Each of the 60 hermaphrodites, which will subsequently be referred as a family (20 families/valley), was handled in the same following way: 12 flowers were emasculated and each flower was then isolated in a small transparent philatelist envelope to prevent uncontrolled pollinations. These 12 flowers were then submitted to three pollination treatments: 2 were not pollinated and served as controls to assess envelop efficiency in preventing any free pollination; 5 were self-pollinated and 5 were cross-

pollinated (each with a different sire coming from the same valley) and then re-isolated within envelops. Pollinated flowers were saturated with pollen to avoid uncontrolled effects of different pollen load and/or pollen viability and, envelops were considered as efficient in preventing uncontrolled pollinations, since none of the unpollinated flowers produced seeds. Envelops also enabled us to wait until the fruits opened without loosing any seed. In July 2001, seeds were mature and, for each pollination treatment, we measured the following fitness components for "seed traits": fruit set, number of viable seeds *per* fruit, number of aborted seeds *per* fruit (seeds that looked dry and that did not germinate) and mean weight of viable seeds (Fig. 1). The latter was estimated from the total weight of viable seeds within a fruit divided by the number of seeds.

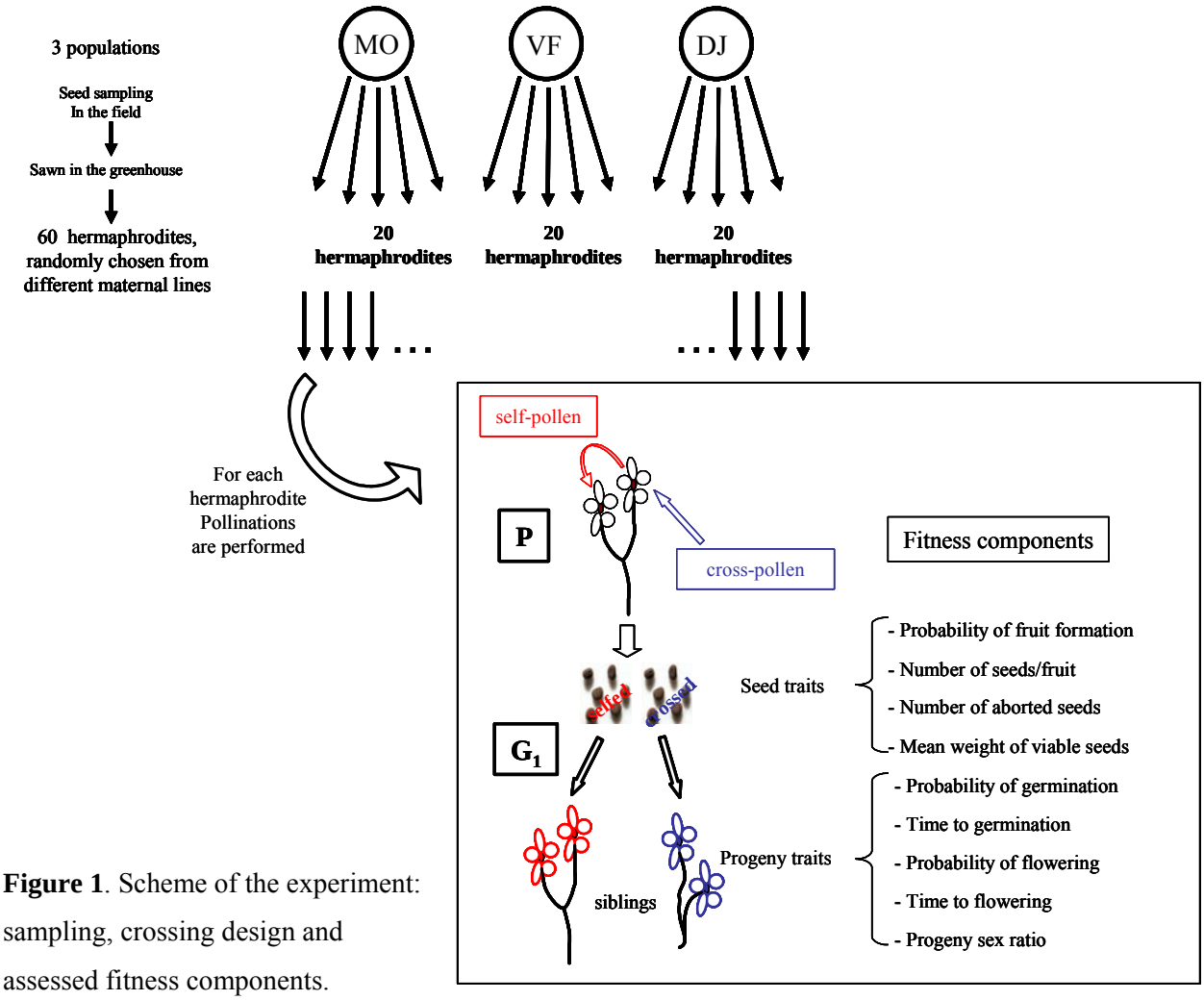


Figure 1. Scheme of the experiment: sampling, crossing design and assessed fitness components.

In September 2001, for practical space availability reasons, we planted seeds from half of the families (10 families/valley) for. For each family, we planted 32 seeds: 16 seeds from each pollination treatment (self- vs. cross-) in Jiffy pots (Ø 5cm). Once sown in the greenhouse (temperature: 18-20°C, relative humidity: 50-60%, natural light conditions) seeds were watered and checked daily for germination, flowering and sex expression. December 2001, seeds from the 30 remaining families (10 families/valley) were planted (16 from self- and 16 from cross-pollination treatment) and censused in the same way. Greenhouse conditions were similar (temperature: 18-20°C, relative humidity: 50-60%) apart from the light conditions. Since natural light (or photoperiod) in late autumn is generally insufficient and could alter germination rates or further flowering, artificial light was installed (a light:dark photoperiod of 15:9 hours). Differing light conditions between the two cultures was accounted by adding a block effect. We assessed fitness components on "progeny traits" as probability of germination, time to germination, probability of flowering, time to flowering and progeny sex ratio (Fig. 1).

Statistical Analysis

Inbreeding depression was tested as the difference between pollination treatments within each family separately. Either two-tailed paired t-tests or wilcoxon signed rank tests were used to test for a within family pollination treatment effect on the different fitness components.

We used mixed-model ANOVA with nested effects in order to see how variance was distributed between our predictive variables: pollination treatment, valley, block and family. Two models of ANOVA were used. The first was used for "seed traits": pollination treatment was considered as a fixed effect while valley and, family nested within valley, were considered as random. Boxcox transformations were performed when necessary (Sokal and

Rohlf, 1995). To accommodate for block effect, a second model was used to analyze "progeny traits": block effect was random and family was nested within the block*valley interaction (valleys occurred in each block but families were different). None of these characters fulfilled the ANOVA conditions. Apart from time to flowering when boxcox transformed, all other variables were transformed into rank. The statistical package R (version 1.8.1) was used for all statistical analyses (R Development Core Team, 2003).

Inbreeding depression coefficients

Inbreeding depression δ was estimated as $(w_o - w_s)/ w_{max}$, where w_o and w_s are the fitness of outcrossed and selfed progeny, respectively and w_{max} is the maximum of both (Agren and Schemske, 1993). This estimate of inbreeding depression varies between -1 and 1; where -1 implies zero fitness for outcrossed plants and, 1 zero fitness for selfed plants. Positive values of inbreeding depression are not different from the more classical inbreeding depression expression: $\delta=(w_o-w_s)/w_o$ which ranges between $]-\infty; 1]$ (Lande and Schemske, 1985). The advantage of Ågren & Schemske's (1993) estimates for inbreeding depression is the symmetrical distribution of values; equal weight is given to "inbreeding- and outbreeding-depression".

We computed an inbreeding depression coefficient δ_F for each family and its mean $\overline{\delta}_F$ (family mean inbreeding depression), for each valley. This allowed comparisons with the "valley inbreeding depression estimate" (δ_V), using the following formula (Johnston and Schoen, 1994) (*n* is the number of families/valley):

$$\overline{\delta}_F = \frac{1}{n}\sum \frac{w_{oi}-w_{si}}{w_{\max i}} \qquad \text{and} \qquad \delta_V = \frac{\sum w_{oi}-\sum w_{si}}{\sum w_{\max i}}$$

Inbreeding depression estimates *per* family (δ_F) were also used to test for correlations (Spearman) between the different life-cycle stages fitness components. Total fitness was

computed as the product of fitness components for size of progeny and its "survival" probability: (number of seeds/fruit)*(probability of germination)*(probability of flowering) and allowed us to estimate cumulative mean inbreeding depression coefficients for family ($\overline{\delta}_F$) and for valley (δ_V). Kruskal-Wallis tests were used to detect among valley differences in inbreeding coefficients.

RESULTS

Evidence for inbreeding depression

Almost all fitness related traits measured showed that progenies issued from cross-pollination treatments were significantly fitter than progenies issued from self-pollination treatments. Fitness components that were significantly depressed by inbreeding were: number of seeds/fruit, number of aborted seeds/fruit, probability and time to germination and, probability and time to flowering (Tab. 1, Fig. 3). Number of seeds *per* fruit was larger when coming from cross-pollination treatments (31.9) than when coming from self-pollination treatments (27.3). Number of aborted seeds was lower in fruits issued from cross-pollinations than from self-pollinations (2.7 versus 5.0, respectively). Probability of germination and probability of flowering were larger in progeny issued from outcrossing (0.73 and 0.9, respectively) than in their siblings issued from selfing (0.55 and 0.64, respectively). Mean time to germination and to flowering were shorter for seeds issued from outcrossing (10.7 and 89.1 days, respectively) than for seeds issued from selfing (13.2 and 96.7 days, respectively). Mean weight of seed was slightly lighter for seeds coming from self-pollinations than for those coming from cross-pollination treatments although this difference was only marginally significant (Tab. 1). Finally, fruit set was not significantly different between pollination treatments (0.68 versus 0.66, Tab. 1), confirming the absence of a self-incompatibility system in *Silene vulgaris* (Dafni, 1992).

Table 1. Mean (standard deviations) of fitness measures of different stages through the life-cycle for self- and cross-pollinations performed within maternal plants (N= sample size). Comparisons of pollination types within the maternal parents are computed with paired test.

Traits		Pollination Treatment					
	N	OUTCROSSED		SELFED		p-value	Paired test
Percentage of capsule formation	61	0.66	(0.28)	0.68	(0.3)	0.44	wilcoxon
Number of seeds/capsules	61	31.92	(21.45)	27.27	(18.33)	0.028	t-test
Weight of seeds	61	8.5e-04	(2.8e-04)	8.1e-04	(4.3e-04)	0.084	wilcoxon
Non-developped seeds	61	2.65	(3.27)	5.04	(4.74)	2.0e-03	wilcoxon
Probability to germination	57	0.74	(0.23)	0.57	(0.28)	1.3e-06	wilcoxon
Time to germination (days)	55	10.76	(3.5)	13.1	(5.84)	9.3e-06	wilcoxon
Probability to flowering	55	0.9	(0.15)	0.64	(0.29)	3.7e-07	wilcoxon
Time to flowering (days)	52	89.08	(13.36)	96.65	(17.18)	8.6e-06	wilcoxon
Progeny sex ratio (%H)	54	0.94	(0.11)	0.75	(0.31)	2.3e-03	wilcoxon

Progeny sex ratio

The proportion of hermaphrodites (i.e.: sex ratio) in the progeny was lower when coming from self- than from cross-pollination treatments (Tab. 1; Fig. 2). Sex ratio varies from 0 to 1 for selfed progeny and from 0.57 to 1 for outcrossed progeny. Sex ratio differences between cross- and self-pollination treatments vary greatly between families. About half of the families in each valley (40% of families for MO and DJ, and 50% for VF) have similar sex ratio for both pollination treatments (difference <5%). Among the other families, most have a sex ratio difference larger than 25%. When computing a mean throughout all families, we obtained an overall valley sex ratio difference of 20% between both pollination treatments which breaks into 31% for MO and 13% for VF and DJ (Fig. 2).

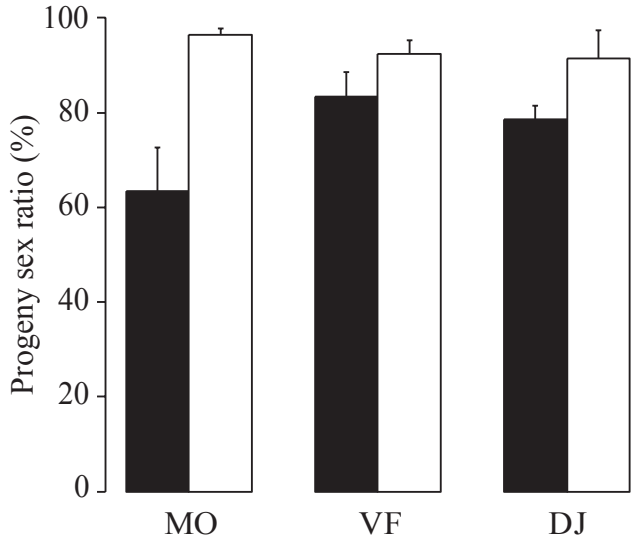


Figure 2. Progeny sex ratio (% of hermaphrodites) mean and standard error, for self- (black bars) and cross-pollination treatments (white bars) in three different valleys (MO, VF & DJ).

Family, Valley and Block variation for fitness components

Analysis of variance shows large and significant family effect for all fitness components (Tab. 2). This indicates that some families can carry fewer deleterious mutations than others. Family*pollination treatment interaction was also significant for most fitness components (Tab. 2). Among valley variation is large for most fitness components, since only number of aborted seeds and time to germination are not significantly different among valleys (Tab. 2). Figures 3 shows how valleys differ for the different seed and progeny traits: plants from VF do better than the two other valleys for fruit set (Fig. 3a) and number of viable seeds (Fig. 3b). However weight of seed has an opposite trend, it is larger for MO and DJ valleys than for VF valley (Fig. 3c). MO valley performs worse than the other valleys for probability of germination (Fig. 3e) and its progeny sex ratio is lower than for VF and DJ valleys (Fig. 2). Valley*pollination treatment interaction is never significant (Tab. 2), indicating that overall valleys respond similarly to pollination treatment. Differences between blocks (both cultures) are significant for probability of germination, time to germination and to flowering and can either account for different lighting conditions or different amount of time of seed storage.

Table 2. Analysis of variance for all considered traits. Number of aborted seeds and time to germination are boxcox transformed. Weight of seed, probability of germination, probability of flowering and time to flowering are transformed into rank.

Levels of significance: + p<0.1; * p<0.05; ** p<0.01; *** p<0.005

A) Seed Traits

	Capsule formation			Number of seeds / fruit			Number of aborted seeds / fruit			Mean weight of seeds		
Source of variation	df	MS	F-value	df	MS	F-value	df	MS	F-value	df	MS	F-value
Pollination treatment	1	0.108	12.00	1	1162	61.16 *	1	0.267	16.22 +	1	563843	11.91 *
Valley	2	2.475	5.23 ***	2	6578	3.49 ***	2	0.028	1.58	2	882735	61.64 *
Pollination treatment*Valley	2	0.009	0.04	2	19	0.05	2	0.016	2.38	2	14321	0.43
Family (Valley)	59	0.473	3.01 ***	58	1884	4.78 ***	58	0.018	3.68 ***	57	47361	2.09 ***
Pollination treatment*Family (Valley)	57	0.256	1.63 **	54	411	1.04	54	0.007	1.43 *	54	33138	1.46 *
Error	470	0.157		310	394		310	0.005		476	22646	

B) Progeny traits

	Probability of germination			Time to germination			Probability of flowering			Time to flowering			Progeny sex ratio		
Source of variation	df	MS	F-value	df	MS	F-value	df	MS	F-value	df	MS	F-value	df	MS	F-value
Pollination treatment	1	867343	23.94	1	1.9E-02	14.88	1	1065320	156.72 +	1	3601	0.33	1	341287	3.64
Block	1	262405	7.47 **	1	5.9E-02	31.65 ***	1	28187	0.69	1	199792	4.60 **	1	132403	3.32 +
Valley	2	302830	4.31 *	2	1.9E-03	0.98	2	264302	3.23 *	2	618317	7.12 ***	2	122533	3.07 **
Pollination treatment*Block	1	765	0.02	1	1.2E-03	0.97	1	517	0.01	1	524	0.09	1	765	0.17
Pollination treatment*Valley	2	39447	0.41	2	9.7E-05	0.08	2	6809	0.08	2	44821	3.88	2	47775	10.46 +
Block*Valley	2	97036	1.38	2	1.2E-03	0.66	2	30826	0.38 +	2	272713	3.14 *	2	97301	2.44 *
Pollination treatment*Block*Valley	2	95266	1.16	2	1.3E-03	2.97 +	2	82836	1.69	2	11562	0.15	2	4568	0.16
Family (Block*Valley)	53	1861738	1.49 *	53	1.9E-03	4.25 ***	53	2168010	1.69 ***	53	2302627	1.75 ***	53	39916	1.63 **
Pollination treatment*Family (Block*Valley)	51	2093291	1.74 **	48	4.3E-04	0.97	51	1246532	1.01	51	1935034	1.53 ***	51	28246	1.15
Error	452	10680875		232	4.4E-04		452	10961121		452	11241772		452	24463	

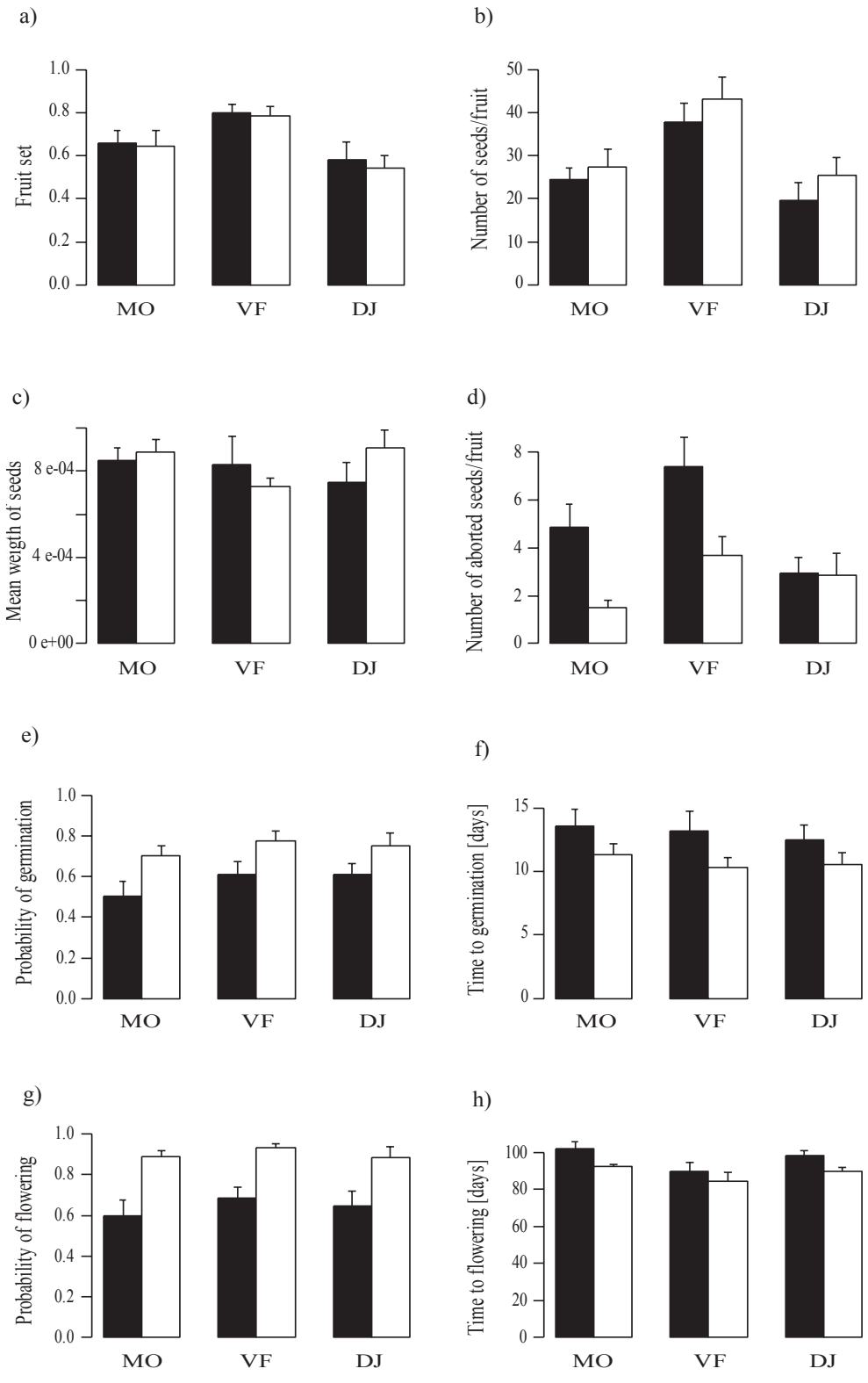


Figure 3. Mean and standard error for self- (black bars) and cross-pollination treatments (white bars) for each valley for: a) Fruit set, b) Number of seeds/fruit, c) Mean weight of seeds, d) Number of aborted seeds/fruit, e) probability of germination, f) time to germination, g) probability of flowering and h) time to flowering.

Inbreeding depression coefficients

We estimated inbreeding depression coefficients for the different components directly related to fitness and, cumulative fitness. For all fitness traits, positive $\overline{\delta}_F$ and δ_V show that, overall, each valley has lower fitness for self- than for cross-pollination treatments (Fig. 4). The magnitude of inbreeding depression increases along the life-cycle. Inbreeding depression is lower for "seed traits" than for "progeny traits": inbreeding depression for number of seeds/fruit was significantly smaller than for probability of germination (Wilcoxon signed rank test, df=55, $p<0.01$) and probability of flowering (Paired T-test, df=54; p=0.01). This increase is more apparent for MO and VF valleys than for DJ valley, where inbreeding depression is more homogeneously distributed across the life-cycle stages.

For number of seeds/fruit, valley means (δ_V) are 0.11, 0.12 and 0.23 for MO, VF and DJ, respectively. δ_F values, computed for each family independently, range from -0.97 to 1 and the means ($\overline{\delta}_F$) are 0.04, 0.10 and 0.15 for MO, VF and DJ, respectively. Inbreeding depression coefficients for proportion of developed seeds are more consistent between families (δ_F varies between -0.25 and 0.53). Thus, valley (δ_V) and family ($\overline{\delta}_F$) means also are more similar than for number of seeds/fruit: δ_V=0.08 and $\overline{\delta}_F$=0.12 for MO, $\delta_V=\overline{\delta}_F$=0.11 for VF and $\delta_V=\overline{\delta}_F$=0.07 for DJ. Inbreeding depression estimates became larger in further life-cycle stages: for probability of germination δ_V is of 0.25, 0.22, and 0.21; and $\overline{\delta}_F$ equals 0.28, 0.24, and 0.27 for MO, VF and DJ, respectively. And for probability of flowering δ_V is of 0.35, 0.30, and 0.27; and $\overline{\delta}_F$ equals 0.33, 0.30, and 0.25 for MO, VF and DJ, respectively. Finally, mean inbreeding depression estimates for cumulative fitness are large: δ_V=0.57, 0.50 and 0.61 and $\overline{\delta}_F$=0.56, 0.53 and 0.46 for MO, VF and DJ respectively (Fig. 4). For MO and VF, mean inbreeding depression coefficients for valley (δ_V) and for family ($\overline{\delta}_F$) are more similar than in DJ, however this difference is not significant (Wilcoxon test, df=14, p=0.68). Kruskal-Wallis tests performed to see whether valleys differ in their family inbreeding depression ($\overline{\delta}_F$) showed no significant differences

among valleys for any fitness component as well as for cumulative fitness. The largest difference in inbreeding depression coefficient for cumulative fitness was 0.10 between MO and DJ. Computation of δ_F allowed between life-cycle stages correlations for inbreeding depression but most life-cycle stages correlations were not significant. Only inbreeding depression for number of seeds correlated positively with inbreeding depression estimates for proportion of aborted seeds (Spearman' correlation, df=54, r=0.274, p=0.04). And a significant negative correlation was detected between inbreeding depression for probability of germination and probability of flowering (Spearman's correlation, df=54, r=-0.37, p=0.005).

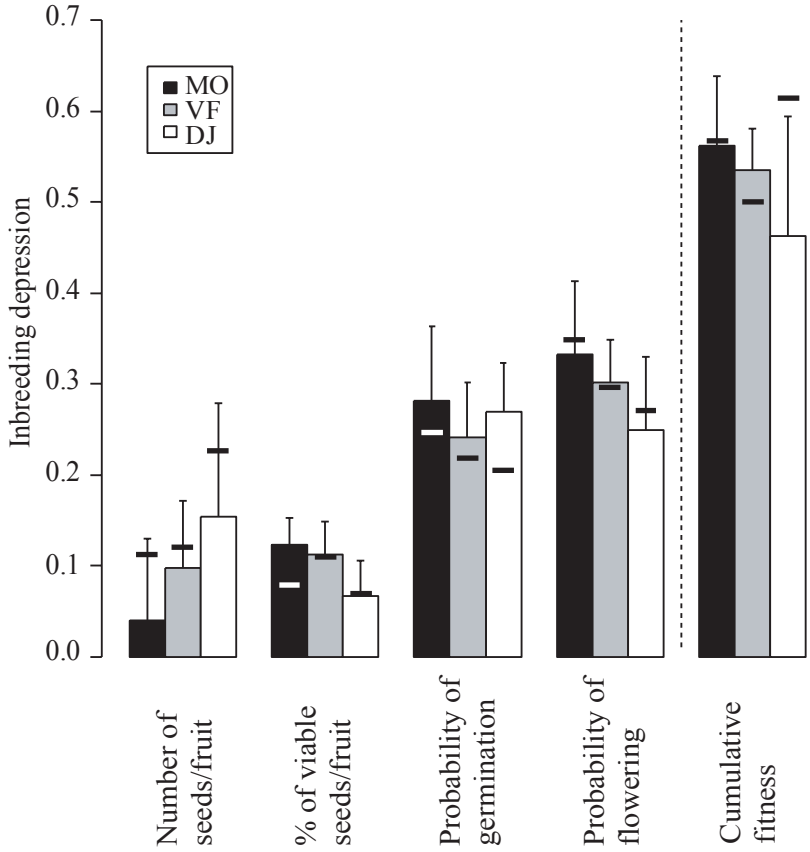


Figure 4. Mean and standard error of family inbreeding depression estimates for successive life-cycle stages and cumulative fitness estimates. Thick segments are inbreeding depression values for valley.

DISCUSSION

This study provides ample evidence for the negative effects of one generation of selfing on fitness and, a sex ratio distortion towards females in the progeny of selfed hermaphrodites. Inbreeding depression was apparent at all life-cycle stages and its magnitude increased throughout the life-cycle (i.e.: δ for "seed traits" < δ for "progeny traits"), inducing large and similar cumulative inbreeding depression estimates for each valley ($\delta_V \approx \overline{\delta}_F \approx 0.5$). Analysis of variance reveals significant family*pollination treatment interaction for most components, indicating that families respond differently to the treatments (Tab. 2). This is corroborated by a large standard error for $\overline{\delta}_F$ (Fig. 4). Though, even if genetic load differs between families, most of them respond similarly to inbreeding since mean inbreeding depression estimates for family ($\overline{\delta}_F$) and for valley (δ_V) are positive and not significantly different.

Timing and magnitude of inbreeding depression

Overwhelming evidence of inbreeding depression is detected when pollination treatments are compared with paired tests throughout all families. "Seed traits" show significant differences for number of seeds/fruit and number of aborted seeds/fruit that account for inbreeding depression. For "progeny traits", progeny issued from cross-pollination treatments was always significantly fitter than when issued from self-pollination treatments (Tab. 1). Selfed progeny was also quicker in time to germination and to flowering than outcrossed progeny. Although these traits may not be directly related to fitness, seeds that germinate more rapidly may be better at space occupancy and thus for further resource competition. Other studies on *Silene vulgaris* congruently detected inbreeding depression for the number of seeds/fruit (Pettersson, 1992b), germination rates (Jolls and Chenier, 1989) and survival to flowering (Emery and McCauley, 2002). Additionally, Pettersson (1992a) found a significant difference for the weight of seeds between selfed and outcrossed hermaphrodites. Our results showed the same trend: seeds issued from self-pollination treatments were lighter than seeds issued from cross-pollination treatments,

though this difference was only marginally significant (Tab. 1). A clear trend observed here is that overall, magnitude of inbreeding depression increases throughout the stages of the life-cycle (Fig. 4). For all valleys, differences between selfed and crossed progeny are larger at late life-cycle stages than at early ones: mean valley inbreeding depression estimates for "seed traits" ($0.08<\delta_V<0.15$) are smaller than for "progeny traits" ($0.21<\delta_V<0.35$). Thus, substantial effect of inbreeding on fitness in late life-cycle stages shows how important it is to screen as many successive life-cycle stages as possible to get good estimates of overall inbreeding depression (Husband and Schemske, 1996; Goodwillie, 2000). Moreover, it is impossible to infer inbreeding depression in late life-cycle stages from early ones since intensity of inbreeding depression was generally not correlated across traits belonging to different life-cycle stages, suggesting little of no pleiotropy and that different genes are implicated in each life-cycle stage (Rao, Widen, and Andersson, 2002).

We detected a positive correlation between inbreeding depression for number of seeds/fruit and for proportion of aborted seeds/fruit, suggesting either linkage disequilibrium between loci controlling these two traits or that one or few loci with positive pleiotropic effects control both, the amount of seed and their development into viable seeds are shared. A significant negative correlation was detected between inbreeding depression for probability of germination and probability of flowering, suggesting negative pleiotropy: favorable alleles for probability of germination are unfavorable for probability of flowering. An alternative explanation would be that some families invest preferentially in few seeds and ensure their development until maturity (i.e.: seed provisioning) whereas other families invest in many seeds but less well provisioned and thus, these seeds germinate but do not reach maturity.

Inbreeding depression and mating system inference

In this study, cumulative valley inbreeding depression estimates (δ_V) were respectively 0.57, 0.50 and 0.61 for MO, VF and DJ valley. Family inbreeding depression estimates ($\overline{\delta}_F$) were not significantly different from δ_V: $\overline{\delta}_F$ =0.56, 0.53 and 0.46 for MO, VF and DJ valley, respectively and none of these estimates differed from 0.5 (Wilcoxon Rank Sum Test, $p>0.05$). Thus, on the basis of cumulative inbreeding depression, predominant mating pattern (self- vs. cross-) cannot be inferred since 0.5 is, following Lande and Schemske (1985), the threshold value where mating system switch from predominant cross- fertilization to predominant self-fertilization. If we follow Husband and Schemske's (1996) study, we infer selfing as being predominant since the magnitude of inbreeding depression increases along the life-cycle. However this pattern was slightly different among valleys, in DJ valley, inbreeding depression for number of seeds *per* fruit was as large as for probability of flowering (Fig. 4). This would rather indicate that mating system is predominantly cross-fertilization in this valley (Husband and Schemske, 1996). Since we obtained a cumulative inbreeding depression of approximately 0.5 and some among valley differences in the relative strength of inbreeding depression across the different life-cycle stages, patterns of inbreeding depression *per se* are not sufficient to infer the predominant mating system in our *Silene vulgaris* populations. Genetic analyses of our populations are in progress and preliminary estimates of the selfing rate (calculated on the basis of F_{IS}) are similar in the three valleys: S=0.5 (Juillet et al., 2003; unpub. res.). Moreover, evidence for mixed mating system in *Silene vulgaris* already exists. Pettersson (1992) applied pollen dies and showed considerable amounts of geitonogamous pollen transfer due to the foraging behavior of pollinators. Emery & McCauley (2002) mentioned population selfing rate (estimated with allozyme markers) ranging from 0.19 to 0.61, and D. Charlesworth (unpublished manuscript) got similar estimates: selfing rates from 0.15 to 0.57. Furthermore, self-compatible gynodioecious species already suggest partially self-fertilizing populations since hermaphrodites can self and outcross whereas females can only outcross. Mixed mating could explain the levels of inbreeding

depression we found and similar patterns were found in another species. An investigation on *Schiedea adamantis* (Caryophyllaceae), a threatened gynodioecious species, corroborates our results since selfing rate was 0.5, inbreeding depression was increasing across the successive life-cycle stages and, a cumulative inbreeding depression was 0.6 (Sakai et al., 1997).

Inbreeding depression at family and valley level

For cumulative fitness and for each fitness component, valley inbreeding depression estimate (δ_V) was within the confidence interval of family level inbreeding depression ($\overline{\delta}_F$) (Fig. 4) and thus not different. One advantage of family inbreeding depression is that it allows computing variance among families which could give us hints about the genetic load. Among family difference in response to inbreeding is an important part of the variation as indicated from the family*pollination treatment interaction. Most families had higher fitness when crossed than when selfed but, few families showed the reverse pattern. There was at least one family per trait and per valley with a negative δ_F. Negative inbreeding depression, a sign of "outbreeding depression" is thought to come from co-adapted complexes of genes and/or local adaptation that can breakdown when mating with a divergent population. When considering cumulative inbreeding depression, the largest $\overline{\delta}_F$ variance and the most diverging $\overline{\delta}_F$ and δ_V are in the DJ valley. This discrepancy between $\overline{\delta}_F$ and δ_V in DJ valley arises because more families (4 versus 1 or 2 for VF and MO, respectively) display strong "outbreeding depression" (minimum δ_F in DJ valley = -0.91). Family variation in inbreeding depression can come from the number and the severity of deleterious mutations in each family and/or from the inbreeding history of each individual (Uyenoyama, Holsinger, and Waller, 1993; Charlesworth, Lyons, and Litchfield, 1994; Schultz and Willis, 1995; Fowler and Whitlock, 1999). If there is inbreeding depression, the progeny of related mates will generally be less fit than if mates are genetically more distant. Though, an individual coming from multiple generations of repeated inbreeding, for instance an individual occurring in a small population, could have purged deleterious alleles and the progeny of this individual, if selfed, could display fitness equal to outcrossing (Barrett and Charlesworth,

1991; Groom and Preuninger, 2000). Thus, if some families have purged their deleterious mutations, cumulative inbreeding depression should be lower and variation among families should increase. On the other hand, genetic drift is also larger in small populations and becomes larger than the strength of selection. Thus deleterious recessive alleles instead of being purged can become fixed in the population and will compose the fixation drift load of the population (Whitlock, 2000; Paland and Schmid, 2003). Drift load also decreases inbreeding depression estimates: since all individuals are homozygous for the fixed deleterious mutations, progeny of cross-and self-fertilization display the same fitness. Thus drift should also decrease cumulative inbreeding depression estimates but should also decrease among family variation in inbreeding depression since fixation decreases overall genetic diversity. The small size of populations within DJ valley (10 to 50 individuals versus 50 to 100 individuals for VF and ~500 individuals for MO) could account for the lowest $\overline{\delta}_F$ in comparison to the other two valleys and the large $\overline{\delta}_F$ variance (Fig. 4). However this interpretation about population size is speculative since we do not have replicates for the different population sizes within valleys. These divergences among valleys could also come from ability of valleys to cope with the new greenhouse environmental conditions.

Sex ratio distortion in inbred hermaphrodites: an effect of inbreeding depression?

An interesting outcome of this study is the greater proportion of females in the progeny of self- relative to the progeny of cross-treatments (Tab. 1; Fig. 2). Sex ratio differences between cross- and self-pollination treatments vary greatly between families. When computed as a mean throughout all individuals, we obtained a sex ratio difference of 20% between both pollination types (31% for MO and, 13% for VF and DJ). Emery and McCauley (2002) found a similar result: they obtained 27% more females in the progeny of self- compared to cross-pollination treatments and argued that the increase in female in the progeny of selfed hermaphrodites can have implications for the maintenance of inbreeding depression. Since females (issued from self-pollinations) have to outcross to set seeds, these crosses prevent further potential inbreeding (through selfing) and thus hinder the purging process (Emery and McCauley, 2002). A possible explanation for the increased proportion of females in the progeny of selfed hermaphrodites is a complex nucleo-cytoplasmic interactions for sex determinism (Charlesworth and Laporte, 1998; Emery and McCauley, 2002). Different studies have shown that in gynodioecious species, male sterility restorers tend to be dominant (Koelewijn and Van Damme, 1995; Charlesworth and Laporte, 1998; Couvet, Ronce, and Gliddon, 1998; Taylor, Olson, and McCauley, 2001; Emery and McCauley, 2002). Our results also suggest dominant restorers: if restorers were recessive, the progeny of selfed hermaphrodites would only be composed of hermaphrodites and, females could only arise in the progeny of crossed hermaphrodites. If sex determinism is due to an interaction of one CMS and its dominant nuclear restorer, the largest difference in progeny sex ratio between selfing and random mating hermaphrodites is 6.25% and thus far below our findings and those of Emery and McCauley (2002). Previous to their experiment, Emery and McCauley (2002) performed one generation of outcrossing to limit the family differences due to history of inbreeding. This manipulation could have biased the amount of females in the progeny of selfed hermaphrodites, since hermaphrodites are more likely to be heterozygous for the restorer locus after one generation of outcrossing. However, our seeds came directly from natural

populations (i.e.: they could be the progeny of self-pollinations) and could explain our lower sex ratio difference (Tab. 1; Wilcoxon Rank Sum Test, $p<0.05$). Emery and McCauley (2002) argued that nuclear restoration of male fertility should come from epistatic interactions at two or more loci.

A 4-fold increase compared to the single restorer explanation would imply very large epistatic effects over many genes. An important inbreeding depression in pollen production or fertility (Melser, Bijeveld, and Klinkhamer, 1999; Willis, 1999b; Goodwillie, 2000) is an alternative explanation for an increase in male-sterility in the progeny of selfed hermaphrodites. Furthermore the increase of inbreeding depression at later stages indicates that strong negative effects of inbreeding in the success of gametes could lead to male-sterility. While we were not able to show a positive correlation between sex ratio and family inbreeding depression estimates for cumulative fitness, still the female biased sex ratio in the progeny of selfed individuals is potentially a consequence of inbreeding, implying that a complete view of the effect of inbreeding on fitness should also include investigations of the fitness of gametes produced by selfed versus outcrossed individuals. Indeed, other studies of inbreeding depression estimates on pollen quantity and viability have been shown to be considerably high (Willis, 1993; Carr and Dudash, 1997; Melser, Bijeveld, and Klinkhamer, 1999; Willis, 1999b; Goodwillie, 2000; Hauser and Siegismund, 2000).

To conclude, we have ample evidence of large effects of inbreeding on fitness and we show that inbreeding depression increases throughout the life-cycle stages. As inbreeding depression estimates for total fitness range between 0.46 and 0.6 (a feature associated with outcrossers) but also increases along the successive life-cycle stages (a feature associated with selfers), we suggest that mating system of *S. vulgaris* is mixed. We also show that the use of family inbreeding depression estimates ($\overline{\delta}_F$) can be relevant since it is congruent with valley inbreeding depression estimates (δ_V) and gives more insights about the variation of genetic load among families.

Two consecutive generations of selfing: its effect on fitness and sex expression in the gynodioecious *Silene vulgaris* (Caryophyllaceae)

Abstract – Inbreeding depression, the reduction in fitness of inbred individuals compared to oubred ones, is one hypothesis that explains the maintenance of females within gynodioecious populations (i.e.: co-occurrence of hermaphrodites and females). Hermaphrodites can self-fertilize and suffer from inbreeding whereas females can only outcross and hence should set seeds of higher quality. Using only hermaphrodites, we investigated the effect of two consecutive generations of selfing on various fitness components across successive stages of the life-cycle. Fitness was depressed through inbreeding for many traits (i.e.: probability of germination, probability of flowering, number of flowers *per* plant, and pollen viability) demonstrating inbreeding depression. Overall inbreeding depression varied between 0.52 and 0.76 for the different valleys and was obtained by increasing inbreeding depression across the successive stages of the life-cycle. We also investigated differences in sex ratio (i.e.: proportion of hermaphrodites) between inbreeding levels since progeny sex ratio of selfed hermaphrodites has been shown to be female biased. We detected many partially male-sterile (PMS) plants (i.e.: individuals with female and hermaphroditic flowers but also hermaphrodites with differing number of viable stamens *per* flower). These PMS plants constitute a continuum between complete male sterility and hermaphroditism and could thus be relevant to disentangle between sex determinism and inbreeding depression as hypotheses for the appearance of females in the progeny of selfed hermaphrodites.

Indeed we found a female biased sex ratio in the progeny issued from selfed hermaphrodites but also a lower number of viable stamens *per* flower. Since number of viable stamens per flower can be considered as directly related to fitness we claim that inbreeding depression can partly cause the biased sex ratio.

INTRODUCTION

Gynodioecy, the co-occurrence of females (i.e.: male-sterile plants) and hermaphrodites within populations, is a widely studied breeding system in plants (Darwin, 1877; Couvet, Ronce, and Gliddon, 1998; Bailey, Delph, and Lively, 2003; Delph and Mutikainen, 2003; Shykoff et al., 2003). It has intrigued scientists since Darwin and was thought to be an intermediate step from hermaphroditism to dioecy (Charlesworth and Charlesworth, 1987; Charlesworth, 1999). The maintenance of gynodioecy requires a higher fitness of females relative to hermaphrodites, in terms of seed output (Pettersson, 1992b; Sakai et al., 1997). The larger fitness of females can come from two non-exclusive mechanisms: reallocation of resources and avoidance of inbreeding depression. Indeed, since male-sterile individuals do not need to invest resources in the male function they could produce more ovules. Also females' progeny should be fitter than hermaphrodites' progeny because females cannot self-fertilize whereas hermaphrodites do (Jolls and Chenier, 1989; Pettersson, 1992b; Mutikainen and Delph, 1998; Rankin, Weller, and Sakai, 2002). Thus inbreeding depression is one mechanism that can explain the maintenance of females within gynodioecious systems (Couvet, Bonnemaison, and Gouyon, 1986; Shykoff et al., 2003).

The way sex is inherited determines the threshold fitness advantage females must have in order to be maintained. In most gynodioecious species, sex has been shown to be inherited through nucleo-cytoplasmic interactions: a cytoplasmic male sterility (CMS) allele suppresses the male function and specific nuclear alleles restore the male function. However, if for sex determining loci, cytoplasmic or nuclear polymorphism is lost, sex determination becomes respectively nuclear or cytoplasmic (Couvet, Ronce, and Gliddon, 1998). If sex is inherited through nuclear genes, the fitness of females (i.e.: seed production) must be twice that of hermaphrodites (providing no pollen limitation) since hermaphrodites have a two-fold

advantage as they contribute via ovules and pollen to the next generation. If sex is inherited partly or totally through maternally inherited genes (i.e.: cytoplasmic or nucleo-cytoplasmic sex determinism) there only need to be a slight advantage for females' seed production (Charlesworth and Ganders, 1979).

In this study we investigated the effect of two consecutive generations of selfing on hermaphrodites of the gynodioecious *Silene vulgaris*. This allowed us to build up three levels of inbreeding: F=0 for all cross-pollinations, F=0.5 for one generation of selfing and, F=0.75 for two successive generations of selfing. We measured different fitness components along the whole life-cycle to get accurate estimates of cumulative inbreeding depression. Indeed, studies of inbreeding depression at late life-cycle stages, such as gamete production, often found substantial inbreeding depression in these "late stages" (Willis, 1993; Melser, Bijeveld, and Klinkhamer, 1999; Willis, 1999b). Also, Husband and Schemske (1996) showed that whatever the predominant mating system is (selfing or outcrossing) inbreeding depression is always of important magnitude in late life-cycle stages. We expect a decrease in fitness with increasing inbreeding levels. Furthermore we expected synergistic epistasis, meaning that two mutations are more harmful together than expected from their separate effects (Kondrashov, 1988; Elena and Lenski, 1997) because it is thought to allow more efficient purging of deleterious alleles and thus explains the maintenance of sexual reproduction (versus asexual). Epistatic interactions can be detected when relating the log-transformed data of fitness with inbreeding levels: if this relationship is linear, fitness effects of different loci multiply and thus deleterious mutations act independently. If relationship displays a negative quadratic component showing that log of fitness declines at a greater than linear rate, synergistic epistasis is detected.

Recently, evidences for the female biased sex ratio in the progeny of selfed *S. vulgaris* hermaphrodites have been detected (Emery and McCauley, 2002). Within population cross-fertilization of hermaphrodites displayed sex ratios (i.e.: proportion of hermaphrodites) close

to one, whereas progeny sex ratio of selfed hermaphrodites was overall decreased by 20 to 27%. Emery and McCauley (2002) demonstrated that only one nucleo-cytoplasmic sex determinism complex cannot account for such a large sex ratio difference. Chapter I put forward another hypothesis. Their study showed that magnitude of inbreeding depression was increasing across the successive life-cycle stages, from probability of germination to probability of flowering. Despite the absence of correlation between inbreeding depression for cumulative fitness and progeny sex ratio difference between selfed and crossed hermaphrodites, these authors hypothesized strong negative effects of inbreeding on pollen quantity and quality, which could inhibit pollen production and lead to male-sterile individuals. Interestingly, many gynodioecious species, and among them *S. vulgaris*, can display partially male-sterile (PMS) plants where individuals can either carry female and hermaphroditic flowers (i.e.:gynomonoecy) and/or among flower differences in the number of developed (i.e.: viable) stamens *per* flower (Koelewijn and VanDamme, 1996). These PMS plants constitute a continuum between complete male sterility to hermaphroditism and could thus be relevant to disentangling the effects of sex determinism and inbreeding depression as hypotheses for the appearance of females in the progeny of selfed hermaphrodites.

Actually, some gynodioecious species, for instance *Plantago coronopus* (Koelewijn and VanDamme, 1996) do not only have strictly hermaphrodites and females but also intermediate forms, often called partially male-sterile (PMS). These partially male-sterile plants do bear either female and hermaphroditic flowers (i.e.: gynomonecy) and/or flowers with variable number of aborted stamen (Koelewijn and VanDamme, 1996) but also hermaphrodites with variable quantity of pollen grains (Gigord et al., 1999). Sex determinism is one of the most widely studied areas in gynodioecious species but has generally not been clearly determined since it seems to be complex nucleo-cytoplasmic interactions. PMS were thought to be the product of many of these nucleo-cytoplasmic interactions. Though in order to disentangle between these two (non-exclusive) hypotheses, we used the number of viable

or unviable stamen *per* flower in PMS individuals, a more quantitative way to assess sex expression, to see whether these are also affected by the level of inbreeding.

Our aim was to see whether inbreeding depression was still present in a second generation of self- and cross-fertilization and if it could affect later stages of the life-cycle (not assessed in the previous generation) such as pollen viability, number of pollen grains or mean number of viable stamen *per* flower within a PMS. Through two consecutive generations of self- and cross-fertilization of *Silene vulgaris*' hermaphrodites, we build up three levels of inbreeding ($F \geq 0$, 0.5 and 0.75) to assess whether epistasis can be detected for the expression of inbreeding depression. Since epistasis, can alter the expected linear relationship between log-transformed fitness and levels of inbreeding, we compute regression analyzes of log-transformed data on levels of inbreeding.

METHODS

Study species

Silene vulgaris is a gynodioecious herbaceous perennial plant distributed in the Holarctic. The plant flowers from June to September and can display up to 100 flowers per plant. Females are obligatory outcrossers whereas hermaphrodites can reproduce through cross-fertilization and, self-fertilization even if protandrous. Indeed, flowers at male and female reproductive stages are often simultaneously present within an individual and thus, hermaphrodites can self-fertilize through geitonogamy. A hermaphroditic flower bears 10 pollen producing stamens, and a female flower only carries aborted stamens. Partially male-sterile (PMS) individuals also occur. They can either bear female and hermaphroditic flowers (i.e.: gynomonoecy) and/or flowers with variable number of aborted stamens. Fruits can contain up to 100 seeds and these disperse through gravity. Pollen dispersal, through moths, is also

limited (Pettersson, 1992b; Taylor, Trimble, and McCauley, 1999) and implies some population structure, providing opportunities for inbred mating events (McCauley, 1998).

Sampling, growth conditions and experimental design

In September 2000, mature fruits of *S. vulgaris* were collected in three different valleys of the Western Swiss Alps: Mont d'Or (MO: 570389N/137416E, altitude: 1880m), Dent de Jaman (DJ: 546398N/144389E, altitude: 1543m) and Val Ferret (VF: 574761N/084844E, altitude: 1895m). Size of populations within valleys varied from 10 to 50 individuals for DJ, 50 to 100 individuals for VF and, MO consisted of a large population of approximately 500 individuals.

In March 2001, viable seeds from these 3 valleys were planted in a greenhouse of the University of Lausanne (Switzerland) with a temperature of 18 to 20°C and relative humidity between 50 and 60%. Seedlings and then adult plants were maintained through daily watering in natural light conditions. When flowering, plants were sexed and, 20 hermaphrodites from each valley were randomly chosen to perform controlled pollinations. Each of the 60 hermaphrodites (generation P) was the progeny of a hermaphroditic maternal plant on the field, and was self- and cross-pollinated to build the F1 generation (Fig. 1). F1 progeny was composed of 60 groups of plants that we will further call families. Each family was bearing progeny issued from self- (*S*) and cross-pollinations (*C*).

In March 2003, we build up a second generation (F2) with 3 levels of inbreeding (*F*) through self- and cross-pollinations of *S* and *C* hermaphrodites from F1 (Fig. 1). Selfing of *S* and *C* hermaphrodites resulted in *SS* ($F \geq 0.75$) and *CS* ($F \geq 0.5$) progeny, respectively and, cross-pollinations of *S* and *C* plants were denominated *X* ($F \geq 0$) (Fig. 1). Initial population structure, as is likely, would increase inbreeding coefficient (*F*), though it will affect each level of inbreeding with the same magnitude and, since inbreeding levels are subsequently compared within families, initial population structure does not need to be taken into account.

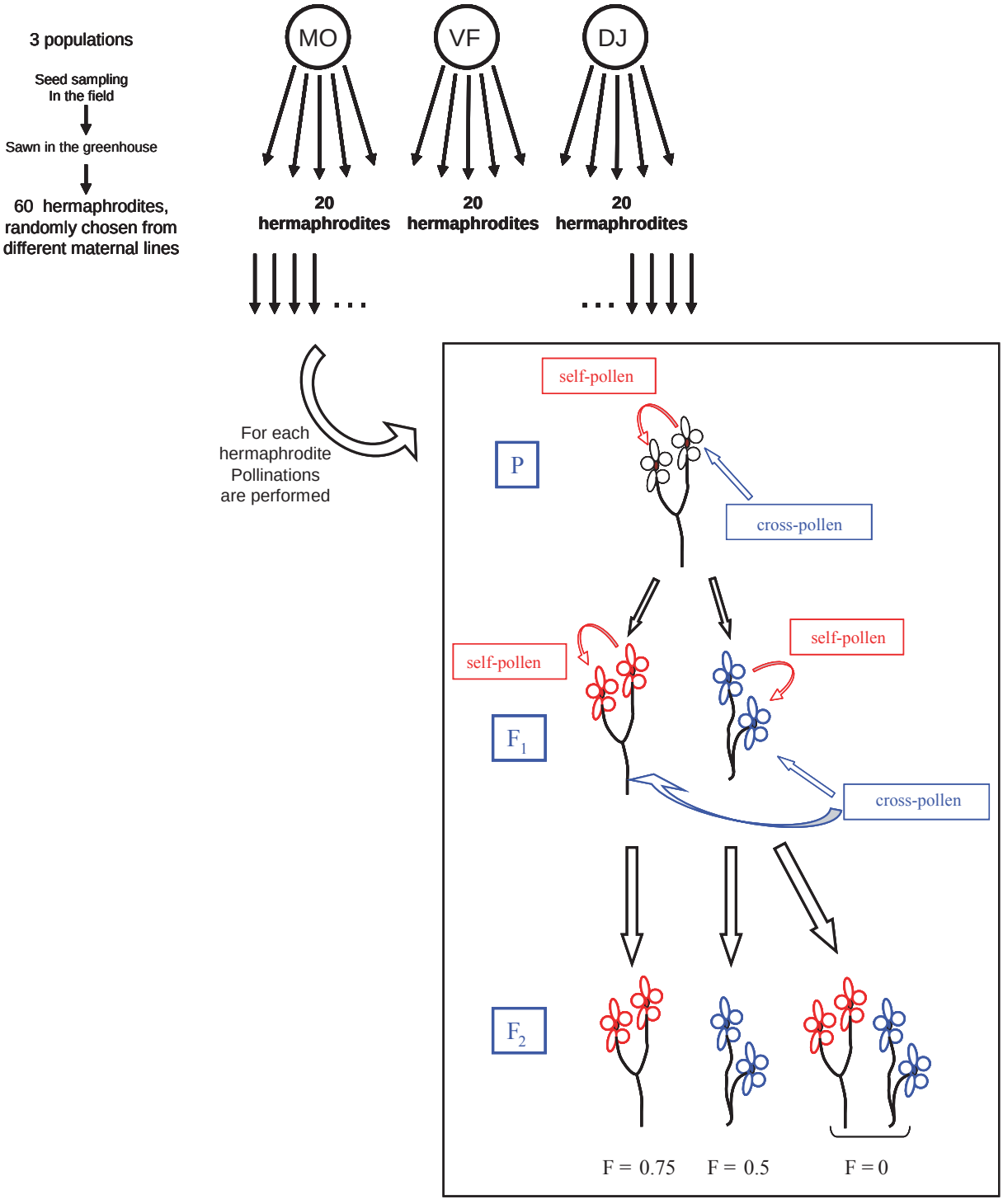


Figure 1. Scheme of sampling and design of experiment

Fitness components

Seed traits

Large effects of inbreeding depression induced the loss of some families throughout the experiment. First some *S* plants from F1 never reached flowering and thus the whole family was removed from the experiment. Secondly, to create the F2, some *S* and *C* individuals were flowered asynchronously and pollinations were not feasible. Thus, some 36 families (13 families for MO, 19 for VF and 14 for DJ) remained and allowed us to estimate fitness in "seed traits". These were estimated as fruit set, number of viable seeds *per* fruit, number of aborted seeds *per* fruit (i.e.: seeds that look dried out and that do not germinate) and mean weight of seed viable seed (estimated as the mass of all viable seeds within a fruit divided by the number of viable seeds the fruit contained).

Progeny traits

In August 2003, we planted F2 seeds. We removed families in which only one level of inbreeding (0, 0.5, and 0.75) developed into viable seeds and thus planted seeds from 30 families (8 families for MO, 10 for DJ and 12 for VF). When possible, we randomly choose 20 seeds from each inbred pollination treatment (*SS* and *CS*) and 100 to 200 seeds (i.e.: 5 to 10 different sires*20 seeds) for cross-pollinations (*X* plants). Altogether, 5'169 seeds were sown in Jiffy pots (Ø 5cm) in the greenhouse (temperature 18 to 20°C; relative humidity: 50-60%, artificial light: photoperiod, 15h of light: 9h of dark, daily watering). The date of each germinating seed was recorded and allowed us to compute probability of germination and time to germination. In November 2003, just before plants reached maturity, a sub-sample of 6 to 9 plants *per* family: 2 to 3 plants within each level of inbreeding ($F \geq 0.75$, $F \geq 0.5$, $F \geq 0$) overall 208 plants; were randomly chosen for an above ground dry biomass measurement. When harvested, each plant was dried out for 7 days at 60°C and then weighted to the nearest 1.0 mg. In early December 2003, when plants started to flower, date of first open flower was

recorded for each plant. This allowed us to compute probability of flowering as well as time to flowering.

Reproductive traits

Female fitness for reproductive traits was assessed as the number of flowers *per* plant. We counted the number of flowers on each flowering plant exactly 21 days after its first open flower. Male fitness was assessed as the number of pollen grains *per* stamen and the proportion of viable pollen grains. Pollen was also harvested within each family and each level of inbreeding. We collect two stamens *per* flower (one for estimation of pollen grain number and one for pollen viability) and stored them in 1.5µl eppendorf tubes. We sampled two flowers *per* plant and 2 to 4 plants *per* level of inbreeding (*F*). To estimate the number of pollen grains within a stamen, each collected stamen was suspended in 80µl of a counting solution (water with 20% glycerin and 20% sucrose). The suspension was homogenized for one minute in an ultrasonic bath (50 Mhz) which prevents clustering of pollen grains. Then, using a microscope (Leitz®-Diaplan), we counted pollen grains in a "Malassez" cell, which allows an aliquot of 1µl (Atlan et al., 1992; Gigord et al., 1999). Two aliquots of 1µl were simultaneously taken and, means were computed for each pollen sample. To estimate pollen viability, stamens were, immediately after harvested, placed in a solution of 20% fuchsine, 40% acetic acid, and 40% water (Atlan et al., 1992; Dafni, 1992). Microscopy was used to count the number of viable and unviable pollen grains. Just before counting, a drop of aniline blue solution was added because fuchsine solution did not help to correctly assign viable and unviable pollen grains. Viable pollen grains appeared dark blue while smaller empty grains appeared to be light blue. Pollen viability was thus estimated as the proportion of viable pollen grains within a sample.

Cumulative fitness

We computed two different coefficients of cumulative fitness: cumulative fitness A was the product of the number of seeds/fruit*probability of germination*probability of flowering and was computed to subsequently allow comparisons with generation F1 (Chapter I). The second estimate cumulative fitness B was the product of probability of germination*probability of flowering*number of flower/plant*pollen viability, which is a more accurate estimation of total fitness since more life-cycle stages are considered than in cumulative fitness A. We did not use number of seeds *per* fruit and, number of pollen grains *per* stamen to compute cumulative fitness B, because they were not significantly affected by inbreeding (see results, Tab. 1).

Sex ratio and partial male-sterility

While counting the number of flowers *per* plant, we also sexed flowering plants as hermaphrodites (H), females (F) and partially male-sterile (PMS) and computed sex ratio as the proportion of pollen bearing individuals:

$$\frac{H + PMS}{H + PMS + F}$$

The number of aborted stamens within a PMS plant was counted and allowed us to estimate a mean number of aborted stamen per flower: number of aborted stamen per plant/(number of flowers*10).

Data analyses

Analysis of variance of fitness components

For all fitness components, as well as for sex ratio and partial male-sterility, we used two models of analysis of variance to test the different descriptive variables. To compare levels of

inbreeding within families, treatment effect was tested with a repeated-measure ANOVA: family and level of inbreeding ($F \geq 0.75$, $F \geq 0.5$, $F \geq 0$) were introduced as fixed effect, in the written order for 'Type I' Sum of Squares. A second ANOVA was used to test for valley and family effects, as well as their interactions with levels of inbreeding. The valley and level of inbreeding were considered as fixed effects while family was random and nested within valleys. Since transformation of data did not help to satisfy ANOVA conditions, we performed randomization tests (1000 permutations) on the mean squares (Manly, 1997). If less than 5% of permutations gave a larger mean square than the one obtained with the observed data, the tested effect was considered as significant. The scheme of randomization was different for each descriptive variable. Levels of inbreeding were tested, with the repeated-measure ANOVA, as the within family permutations of inbreeding levels. If significant, we subsequently performed planned comparisons: outbred ($F \geq 0$) versus inbred plants ($F \geq 0.5$ and $F \geq 0.75$) and, between inbred plants ($F \geq 0.5$ versus $F \geq 0.75$) to better discriminate which level(s) of inbreeding accounted for the significant difference. Valley effect was tested by permuting the combination of family and level of inbreeding, between valleys. For family effect, we randomized families within valleys and within levels of inbreeding. The interaction family*levels of inbreeding was tested as the permutations of data within valleys and, finally valley*levels of inbreeding interaction was tested as the permutations of families between valleys combined with the permutations of inbreeding levels within families.

Similarly, for cumulative fitness B, we tested the effects of the different variance components. Estimates of cumulative fitness were computed as an average for each inbreeding level in each family. Thus, variance was distributed among valleys (random), levels of inbreeding (fixed) and their interaction. Since ANOVA conditions were not fulfilled, we computed permutations tests on mean squares (Manly, 1997). Levels of inbreeding were tested as the permutation of data within valleys. Valley effect was tested as the permutation of data within

levels of inbreeding and interaction between valley and levels of inbreeding was assessed as the random permutation of data.

Epistasis and estimates of inbreeding depression

Since we had more than two levels of inbreeding, we could test the shape of the relationship between fitness component and inbreeding levels. If the effect of homozygous loci acts in a multiplicative manner, a linear relationship is expected between the log of fitness and the inbreeding coefficient. A significant negative quadratic relationship (i.e.: resulting in a sharper decrease between $F \geq 0.5$ and $F \geq 0.75$ than between $F \geq 0$ and $F \geq 0.5$) would detect synergistic epistasis, and a significant positive quadratic relationship can be due either to diminishing epistasis (antagonistic) and/or purging of partially deleterious alleles (Dudash, Carr, and Fenster, 1997; Koelewijn, 1998). Thus, relationships between the log-transformed fitness estimates and inbreeding level were detected as the linear (f) and the nonlinear (f^2) coefficient of the quadratic regression within each population.

Since difference between inbreeding levels was significant between outbred ($F \geq 0$) and inbred individuals ($F \geq 0.5$ and $F \geq 0.75$) but not significant between both groups of inbred individuals ($F \geq 0.5$ versus $F \geq 0.75$) (see results). We pooled inbred plants together and computed classical estimates of inbreeding depression at the family ($\overline{\delta}_F$) and the valley level (δ_V):

$$\overline{\delta}_F = \frac{1}{n}\sum \frac{w_{oi} - w_{si}}{w_{\max i}} \qquad \text{and} \qquad \delta_V = \frac{\sum w_{oi} - \sum w_{si}}{\sum w_{\max i}}$$

where w_o and w_s are the fitness of outcrossed and selfed progeny, respectively and w_{max} is the maximum of both and n is the number of families (Agren and Schemske, 1993; Johnston and Schoen, 1994). The use of inbreeding depression estimates at the family level allowed us to compare the successive life-cycle stages but also F1 and F2 generation for each fitness component (Pearson or Spearman correlations).

RESULTS

Evidence of inbreeding depression on fitness components

"Seed traits" where generally not affected by inbreeding levels (Tab. 1; Fig. 2). Only number of aborted seeds *per* fruit was slightly affected by inbreeding and could account for early inbreeding depression since number of aborted seeds was lower in cross-pollinated plants than in inbred plants (Tab. 1; Fig. 2c).

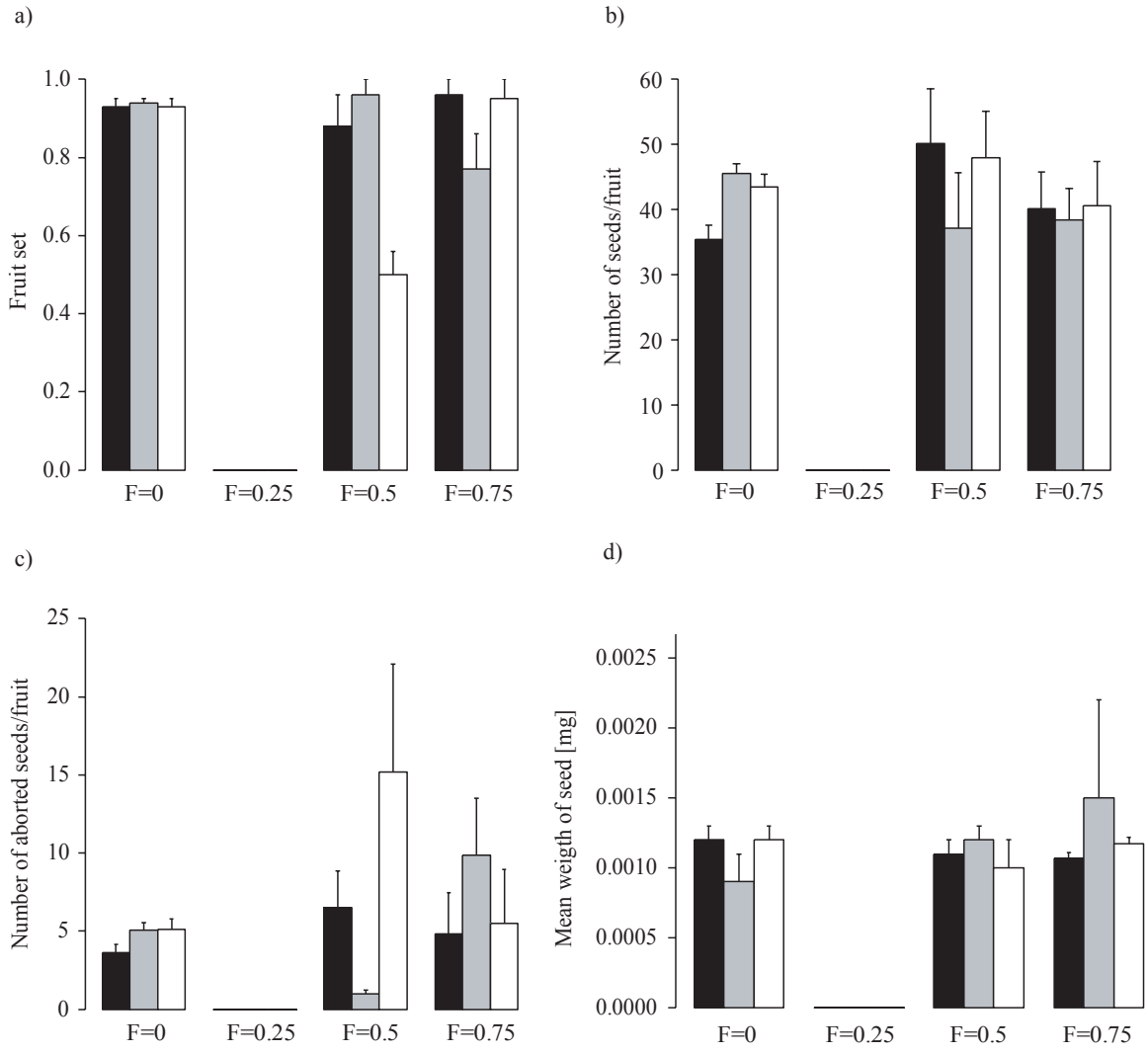


Figure 2. Relationship between seed fitness traits (+se) and levels of inbreeding (F). Fitness traits are a) Fruit set, b) Number of seeds/fruit c) Number of aborted seeds/fruit, d) Mean weigth of seeds. Bars account for the different valleys: black for MO, grey for VF and white for DJ. No significant differences among levels of inbreeding were detected for "seed traits".

Table 1. Analyzes of variance for the different estimated fitness traits. Valley and level of inbreeding are fixed effects and family, nested within valley is random.

A) Seed traits

	Fruit set			Number of seeds/fruit			Number of aborted seeds/fruit			Mean weigth of seeds [mg]		
Source of variation	df	MS	P-val	df	MS	P-val	df	MS	P-val	df	MS	P-val
Valley	2	1.69	0.007 **	2	6797	0.016 *	2	340	0.033 *	2	6.70	0.368
Levels of Inbreeding (F)	2	0.19	0.387	2	549	0.567	2	307	0.046 *	2	0.70	0.184
Family(Valley)	44	0.26	0.067 +	44	1214	0.008 **	44	92	0.240	44	6.40	0.341
Levels of Inbreeding (F)*Valley	4	0.60	0.001 ***	4	1192	0.001 ***	4	365	0.010 *	4	0.90	0.001 ***
Levels of Inbreeding (F)*Family (Valley)	68	0.18	0.832	68	751	0.499	68	148	0.145	51	0.90	0.220
Error	693	0.20		692	754		694	71		463	8.50	

* : P-val =0.05; ** : P-val=0.01; *** : P-val=0.001

B) Progeny traits

	Probability of germination			Above ground dry weight [g]			Probability of flowering		
Source of variation	df	MS	P-val	df	MS	P-val	df	MS	P-val
Valley	2	0.22	0.306	2	0.49	0.661	2	0.40	0.103
Levels of Inbreeding (F)	2	0.49	0.001 ***	2	16.01	0.001 ***	2	0.50	0.001 ***
Family(Valley)	27	0.15	0.001 ***	26	1.13	0.001 ***	27	0.17	0.002 **
Levels of Inbreeding (F)*Valley	4	0.02	0.308	4	2.66	0.644	4	0.06	0.066
Levels of Inbreeding (F)*Family (Valley)	45	0.05	0.580	44	0.62	0.620	44	0.05	0.904
Error	191	0.03		129	0.28		123	0.06	

* : P-val =0.05; ** : P-val=0.01; *** : P-val=0.001

Table 1. Analyzes of variance for the different estimated fitness traits.

C) Reproductive traits

	Number of flowers/plant				Number of pollen grains/stamen				Pollen viability			
Source of variation	df	MS	P-val		df	MS	P-val		df	MS	P-val	
Valley	2	2753	0.008	**	2	285919	0.628		2	0.08	0.138	
Levels of Inbreeding (F)	2	937	0.008	**	2	324155	0.158		2	1.42	0.001	***
Family(Valley)	27	410	0.011	*	27	413839	0.003	**	27	0.03	1.000	
Levels of Inbreeding (F)*Valley	4	647	0.002	**	4	174488	0.631		4	0.04	0.160	
Levels of Inbreeding (F)*Family (Valley)	44	188	0.852		42	73586	1.000		42	0.04	0.586	
Error	116	242			115	157508			109	0.03		

* : P-val =0.05; ** : P-val=0.01; *** : P-val=0.001

	Sex ratio				Number of aborted stamen/flower (partial male-sterile plants)			
Source of variation	df	MS	P-val		df	MS	P-val	
Valley	2	0.06	0.444		2	11.16	0.689	
Levels of Inbreeding (F)	2	0.97	0.001	***	2	128.59	0.001	***
Family(Valley)	27	0.07	0.084		27	7.00	0.228	
Levels of Inbreeding (F)*Valley	4	0.02	0.479		4	2.83	0.694	
Levels of Inbreeding (F)*Family (Valley)	44	0.10	0.023	*	44	7.53	0.240	
Error	117	0.02			116	1.73		

* : P-val =0.05; ** : P-val=0.01; *** : P-val=0.001

D) Timing traits

	Mean time to germination [days]				Mean time to flowering [days]			
Source of variation	df	MS	P-val		df	MS	P-val	
Valley	2	27.69	0.231		2	489.90	0.020	*
Levels of Inbreeding (F)	2	12.31	0.004	**	2	78.60	0.370	
Family(Valley)	27	17.43	0.001	***	27	107.60	0.013	*
Levels of Inbreeding (F)*Valley	4	1.79	0.229		4	24.30	0.014	*
Levels of Inbreeding (F)*Family (Valley)	45	3.99	0.805		44	31.10	0.999	
Error	190	3.14			117	76.40		

* : P-val =0.05; ** : P-val=0.01; *** : P-val=0.001

In contrast, "progeny traits", for instance probability of germination, above ground dry biomass and probability of flowering, were all negatively affected by inbreeding (Tab. 1; Fig. 3a,b,c). For "reproductive traits", we detected significant inbreeding depression for number of flowers *per* plant and pollen viability (Tab. 1, Fig. 3d,f) whereas number of pollen grains *per* stamen was not affected by the levels of inbreeding (Fig. 3e). Since most components directly related to fitness were negatively affected by the levels of inbreeding, cumulative fitness B also displayed significant inbreeding depression (Tab. 2; Fig.4). Traits less directly related to fitness such as time to germination showed that cross-pollinated seeds germinated earlier than self-pollinated seeds (Tab. 1, Fig. 5a) but, no timing differences between levels of inbreeding were detected for the time to flowering (Tab. 1, Fig. 5b). When subsequently performing planned comparisons, for all fitness components as well as for cumulative fitness, outcrossed plants ($F \geq 0$) were always significantly fitter than inbred plants ($F \geq 0.5$ and $F \geq 0.75$) but no significant difference was observed within inbred plants ($F \geq 0.5$ versus $F \geq 0.75$) (Fig. 3,4,5). Only the probability of germination displayed a significant difference between seeds issued from one and two successive generations of self-fertilization.

Table 2. Analyzes of covariance for cumulative fitness B.

	Cumulative fitness 2			
Source of variation	Df	Mean Sq	P-value	
Valley	2	0.497	0.972	
Levels of inbreeding (F)	1	5.889	0.001	***
Valley*Level of inbreeding (F)	2	0.080	0.720	
Residuals	70	0.161		

Considering other variance component than the level of inbreeding, we detected large variation among valleys in all "seed traits" and on number of flowers *per* plant. The significant valley effect was always associated with a significant interaction between valley and levels of inbreeding (Tab.1), indicating that when valleys differed from each other, they

also differed in their response to inbreeding levels. Family variation was also significant for some fitness components showing that intrinsic fitness value was different between families (Tab. 1).

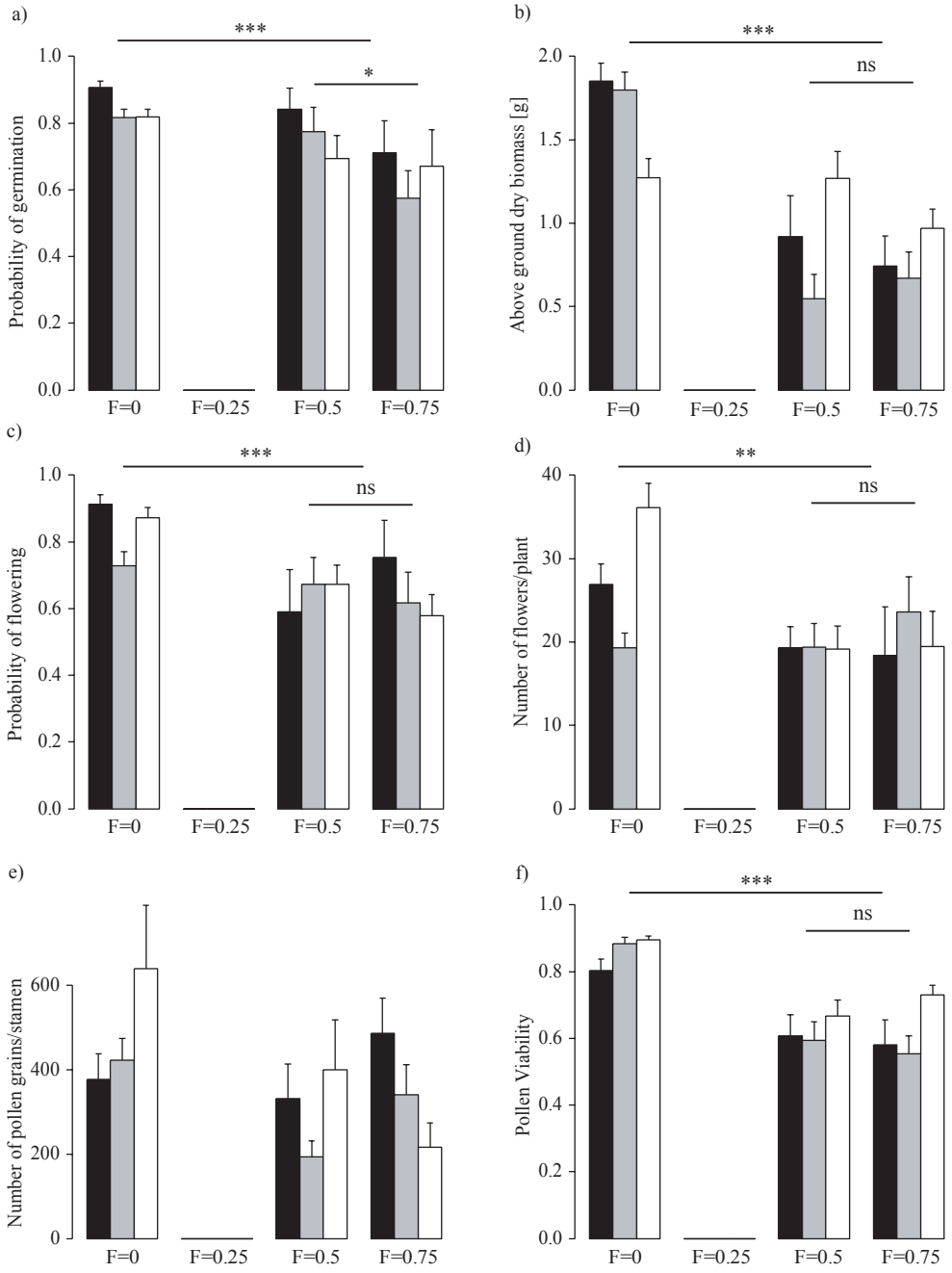


Figure 3. Relationship between fitness traits (+se) and levels of inbreeding (F). Fitness traits are a) Probability of germination, b) Above ground dry biomass c) Probability of flowering, d) Number of flowers/plant e) Number of pollen grains/stamen and f) Pollen viability. Bars account for the different valleys: black for MO, grey for VF and white for DJ. ns: $p>0.05$; *: $p<0.05$; **: $p<0.01$; ***: $p>0.001$. There was no significant difference among levels of inbreeding in number of pollen grains/stamen.

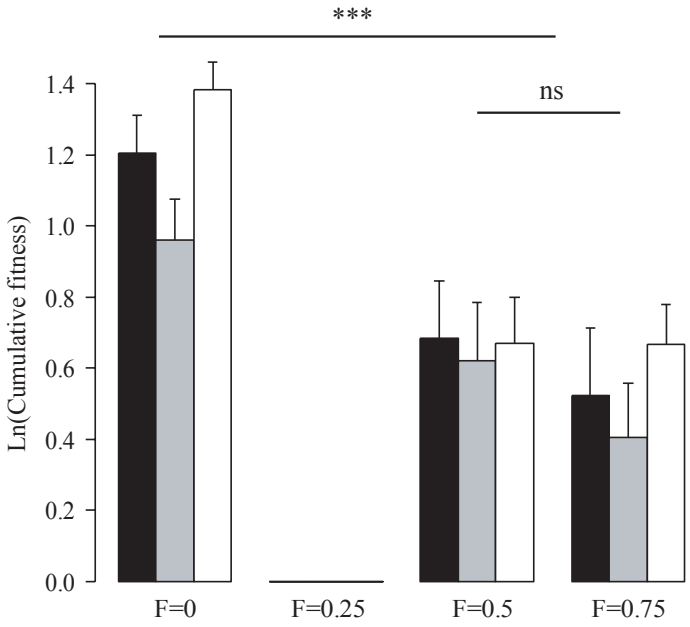


Figure 4. Relationship between log-transformed cumulative fitness traits (+se) and levels of inbreeding (F). Cumulative fitness is computed as : (probability of germination*probability of flowering*number of flowers/plant*pollen viability). Bars account for the different valleys: black for MO, grey for VF and white for DJ. ns: p>0.05; *: p<0.05; **: p<0.01; ***: p>0.001.

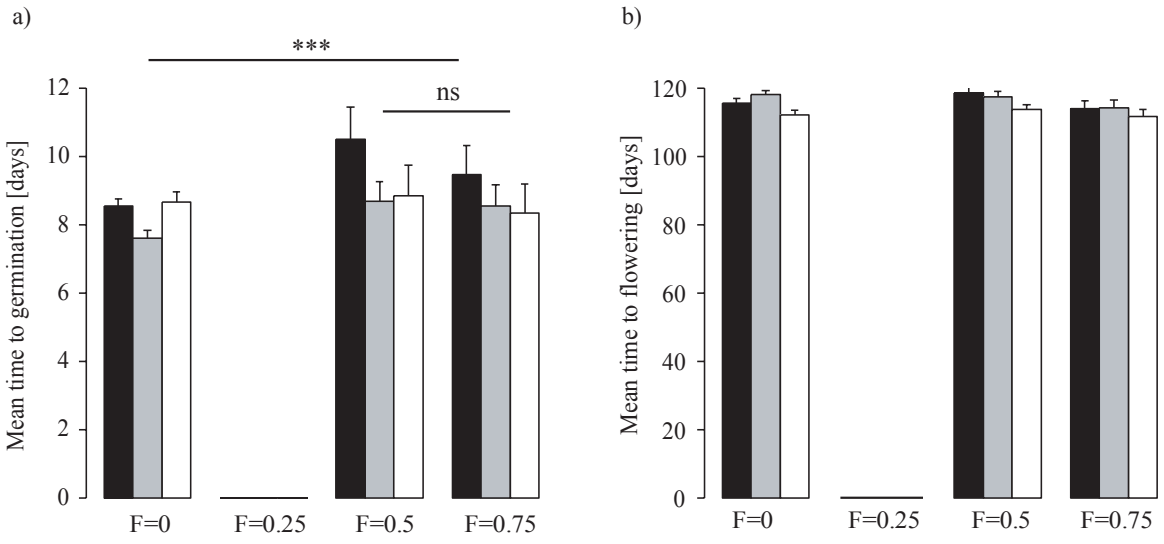


Figure 5. Relationship between a) Mean time to germination (+se), b) Mean time to flowering (+se) and levels of inbreeding (F). Bars account for the different valleys: black for MO, grey for VF and white for DJ. ns: p>0.05; *: p<0.05; **: p<0.01; ***: p>0.001. There was no significant difference among levels of inbreeding for mean time to flowering.

Valley and family estimates of inbreeding depression

When testing for epistasis, none of the traits displayed epistasis and thus estimates of inbreeding depression were performed for valleys and for families. In figure 6, inbreeding depression for valleys ($\delta_{V;}$ thick bars) was, for each trait, included in the confidence interval of family inbreeding depression estimate ($\overline{\delta}_F$), demonstrating that both estimates are not significantly different. Interestingly, and congruent to F1, inbreeding depression increased across the successive stages of the life-cycle (Fig. 6), leading to large estimates of inbreeding depression for cumulative fitness B: 0.52 for VF, 0.71 for MO and 0.76 for DJ. Figure 7 shows a comparison between estimates of inbreeding depression (averaged over the three valleys) for F1 and F2 generations. Inbreeding depression estimates in F1 were always larger than in F2 and thus, cumulative fitness A was lower in F2 than in F1. Moreover, this decrease in inbreeding depression between F1 and F2 seems robust since $F \geq 0.5$ and $F \geq 0.75$ were generally not significantly different and thus, were pooled together to estimate classical inbreeding depression. This could have slightly overestimated inbreeding depression in F2 because even if *CS* and *SS* displayed not significant fitness differences, *CS* plants were often slightly fitter than *SS* plants. Although inbreeding depression seems to be lower in F2, when additionally considering number of flowers per plant and pollen viability (i.e.: for cumulative fitness B) overall inbreeding depression was larger (δ=0.68) than cumulative fitness estimate in F1.

Family inbreeding depression estimates allowed among life-cycle stages comparisons but also between generation (F1 and F2) comparisons. No significant correlation was detected, neither between fitness traits within F2, nor for the number of seeds *per* fruit, probability of germination and probability of flowering when compared between generations. Congruently, cumulative fitness A in F1 was not correlated with cumulative fitness A in F2.

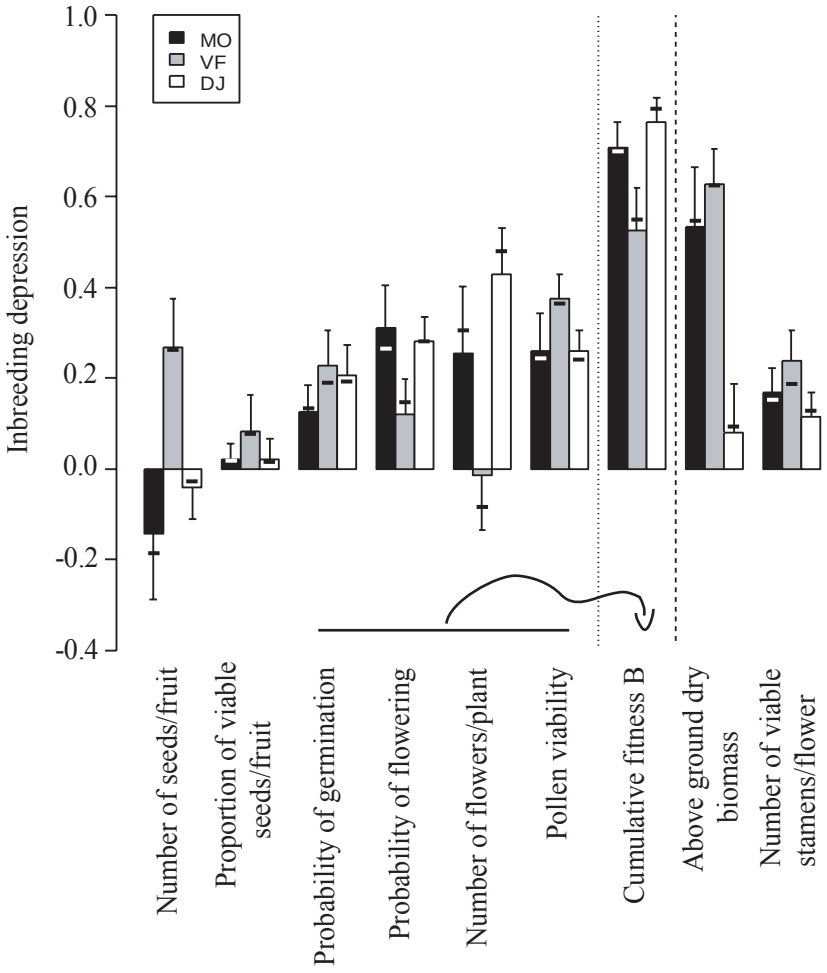


Figure 6. Mean family inbreeding depression (+se) and valley inbreeding depression (thick horizontal segments) along the life-cycle stages. Cumulative fitness B is computed as : (probability of germination*probability of flowering*number of flowers/plant*pollen viability). Bars account for the different valleys: black for MO, grey for VF and white for DJ. Inbreeding depression estimates for number of seeds/fruit are plotted, though not significantly different from zero (see Tab. 1).

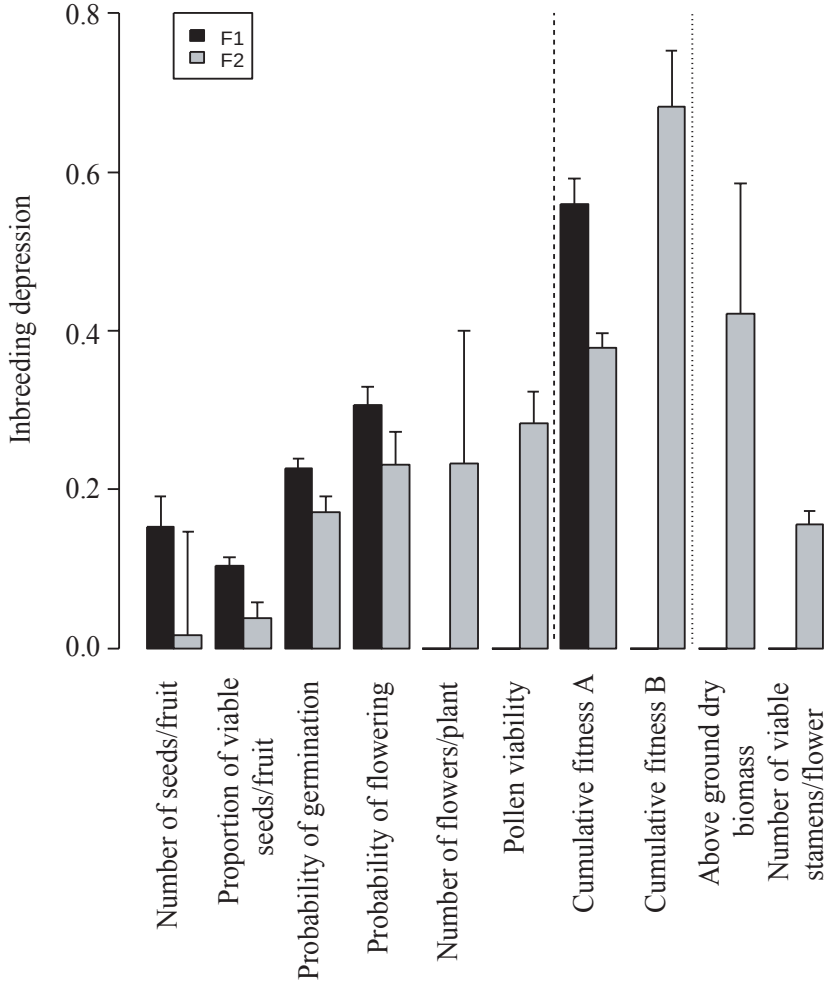


Figure 7. Mean inbreeding depression of the three valleys (+se) for genreation F1 (black) and generation F2 (grey). Cumulative fitness A is computed as : (number of seeds/fruit* probability of germination*probability of flowering). Cumulative fitness B is computed as : (probability of germination*probability of flowering*number of flowers/plant*pollen viability).

Sex ratio and partial male-sterility

Sex ratio and the number of aborted stamens *per* flower in partial male-sterile individuals were also affected by inbreeding: sex ratio (i.e.: proportion of H) was decreasing with increasing inbreeding level and, in PMS individuals, the larger the inbreeding level, the larger the number of aborted stamen *per* flower (Tab. 1, Fig. 8a,b). Though, planned comparisons

indicated large fitness difference between oubred and inbred individuals, but within inbred plants ($F \geq 0.5$ versus $F \geq 0.75$) no difference could be detected. Interestingly, family*levels of inbreeding interaction was significant for sex ratio (i.e. proportion of hermaphrodites) demonstrating that the sex ratio difference among inbreeding levels was highly variable among families (Tab. 1).

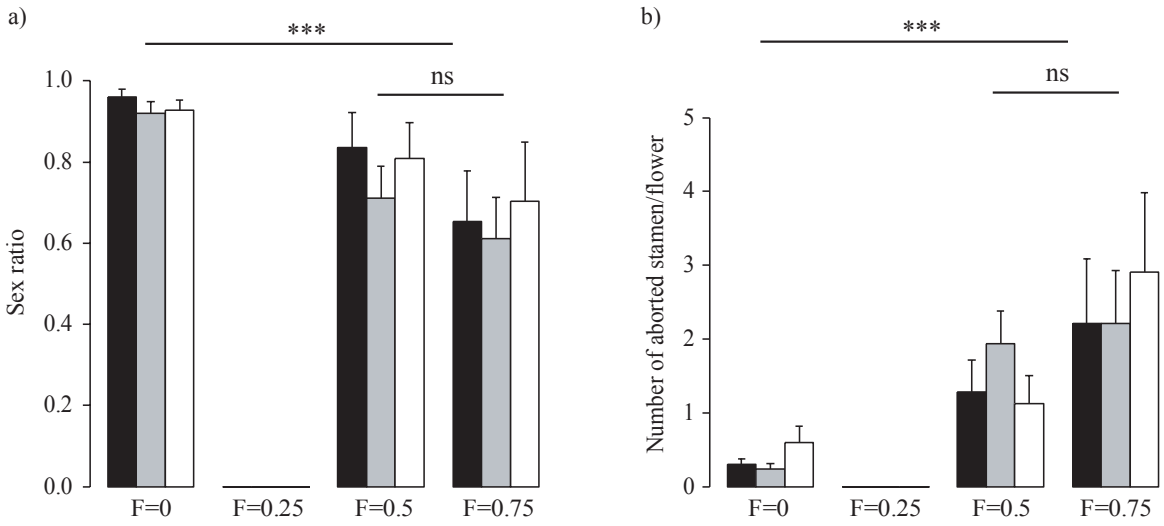


Figure 8. Relationship between a) sex ratio (+se), b) Number of aborted stamen/flower (+se) within partial male-sterile (PMS) plants and levels of inbreeding (F). Bars account for the different valleys: black for MO, grey for VF and white for DJ. ns: p>0.05; *: p<0.05; **: p<0.01; ***: p>0.001.

As with fitness traits, we computed inbreeding depression estimates for sex ratio and number of viable stamen *per* flower (i.e.: 10 – number of aborted stamen *per* flower). Since number of viable stamen *per* flower gives an estimation of the number of pollen grains produced within a flower, this trait can be considered as a fitness component. And, computing inbreeding depression estimates for sex ratio gives similar estimates to computing differences in sex ratio among inbreeding levels, since sex ratio of outbred individuals is generally 1. We further compared these two estimates with inbreeding depression estimates for fitness traits. A strong significant correlation was detected between inbreeding depression estimated for proportion of viable stamens *per* flower and sex ratio (Fig. 9: Spearman; df=27; $p<0.001$; $r=0.71$). And, inbreeding depression for proportion of viable stamens *per* flower was also positively correlated with inbreeding depression for pollen viability (Fig. 9: Pearson; df=26; $p<0.05$; $r=0.41$).

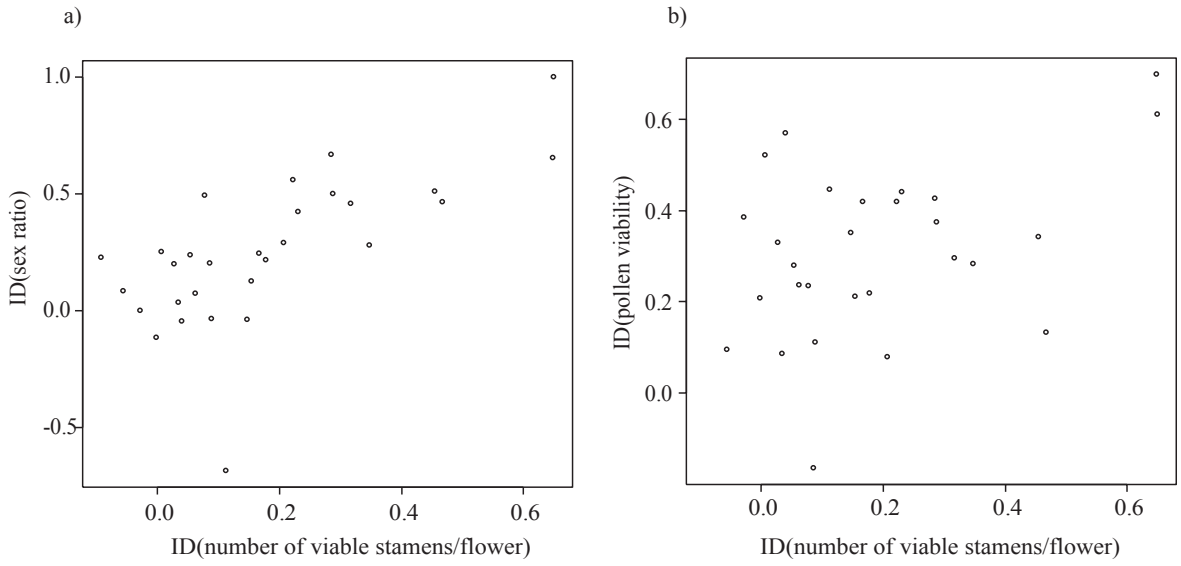


Figure 9. Correlations bewteen a) inbreeding depression for number of viable stamens/flower and inbreeding depression (i.e.: the difference of sex ratio between inbred and outbred sibships, weighted by the sex ratio of outbreds), (Spearman, df=27, $p<0.001$, $r=0.71$); and b) inbreeding depression for number of viable stamens/flower and inbreeding depression for pollen viability (Pearson, df=26, $p<0.05$, $r=0.41$).

DISCUSSION

This study shows overwhelming evidence that inbreeding depression increases along the successive stages of the life-cycle (Fig. 7). Cumulative inbreeding depression estimated from germination rates to pollen viability ranged from 0.52 to 0.76 for the different valleys. Fitness was highly depressed when comparing outbred ($F \geq 0$) and inbred individuals ($F \geq 0.5$ and $F \geq 0.75$) but no further decrease was detected between inbred plants ($F \geq 0.5$ versus $F \geq 0.75$). This indicates that purging has been going on in our experiment and, this is supported by a decrease in inbreeding depression from generation F1 to generation F2. As expected, we detected sex ratio distortion towards females in the progeny of selfed hermaphrodites. Interestingly, difference in sex ratio between outbred and inbred individuals was positively correlated with inbreeding depression for number of viable stamens *per* flower. The latter was also positively correlated with inbreeding depression for pollen viability. These two correlations indicate that restorers are closely linked to genes controlling male reproductive function. Furthermore lack of differences in sex ratio correlation between the two generations could suggest that inbreeding depression is partly affecting sex ratio.

Evidence of inbreeding depression

We detected inbreeding depression across the different life-cycle stages. Inbreeding depression was weak in "seed traits": one trait out of four (i.e.: number of aborted seeds per fruit) was affected by inbreeding. For "progeny traits" and "reproductive traits" inbreeding depression was larger since fitness estimates at five out of six traits were sharply decreasing with increasing inbreeding. Only number of pollen grains per stamen was not affected by inbreeding (Tab. 1). The number of viable stamens per flower was negatively affected by inbreeding and could count as a fitness component since number of pollen grains produced in a flower is surely closely related to the number of viable stamen within a flower. Clearly

estimates of inbreeding depression were increasing along the life-cycle stages (Fig. 6 and 7) and points out how important it is to screen many life-cycle stages to get accurate estimates (Willis, 1993; Husband and Schemske, 1996; Melser, Bijeveld, and Klinkhamer, 1999). Overall, cumulative fitness, assessed as the product of probability of germination, probability of flowering, number of flowers per plant and pollen viability (cumulative fitness B), was greatly affected by inbreeding since inbreeding depression estimates of the different valleys was 0.52 for VF, 0.72 for MO and 0.76 for DJ. These estimates of inbreeding depression for cumulative fitness suggest that cross-fertilization should be favored and could alone explain the maintenance of females within populations even under nuclear sex determinism. Indeed nuclear sex determinism requires a two-fold advantage of females relative to hermaphrodites whereas maintenance of females with cytoplasmic and nucleo-cytoplasmic allows lower relative fitness of females (Charlesworth and Ganders, 1979).

Since all levels of inbreeding were occurring within each family, we could compute estimates of inbreeding depression for each valley as well as for each family within a valley. Both estimates of inbreeding depression were always very similar (Fig. 7) showing that, although family inbreeding depression estimates were thought to be less informative (Schultz and Willis, 1995), these can allow good estimates of inbreeding depression and also give insights into the variation of inbreeding depression among the different families.

Strong purging could explain the lack of synergistic epistasis

Our results display three lines of evidence for the purging process to have occurred within our experiment. First, for each component of fitness, we showed a clear fitness decrease between outbred ($F \geq 0$) and inbred individuals ($F \geq 0.5$ and $F \geq 0.75$) but no significant fitness decease between inbreds ($F \geq 0.5$ and $F \geq 0.75$). Then, when comparing F1 and F2 generations, early life-cycle stages, for instance number of seeds *per* fruit was significantly affected by inbreeding in F1 but no more in F2. Indeed, purging is thought to be especially efficient on

early life-cycle stages (Husband and Schemske, 1996). Finally, all life-cycle stages assessed in both generations showed the same trend: inbreeding depression was larger in F1 than in F2. The difference between both generations could also come from adaptation to the greenhouse environment: in F1 it is a new environment, in F2 it is already "known". However, purging has clearly occurred since families were lost throughout the experiment (see Methods). In generation F1, selfed individuals of a few families never reached maturity, inducing among family purging. Furthermore, since selfed individuals were slower to reach maturity than outcrossed individuals, we had to use the first flowering selfed individuals to perform our F2 generation. Thus, within family purging has also occurred. Therefore, constraints of our experiment could have masked the detection of potential synergistic epistasis. Synergistic epistasis is required to explain the maintenance of sex as an adaptation allowing the purging of deleterious mutations (Kondrashov, 1988; Elena and Lenski, 1997). If there is synergistic epistasis, such that additional deleterious mutations leads to larger fitness decrease, then mean fitness of sexual populations will exceed that of asexual ones. However, no study has clearly shown this synergistic epistasis (Carr and Dudash, 1997; Dudash, Carr, and Fenster, 1997; Elena and Lenski, 1997; Fenster, Galloway, and Chao, 1997; Salathe and Ebert, 2003). Clearly, studies involving several generations of successive inbreeding could hinder the detection of synergistic epistasis since purging often becomes a confounding effect (Lynch, 1988; Carr and Dudash, 2003).

Evidence of inbreeding depression in male reproductive functions suggests that sex ratio can be affected by inbreeding

Sex ratio has been shown to be affected by the nature of pollination: selfing hermaphrodites decrease its progeny sex ratio (i.e.: sex ratio is more female biased). Emery and McCauley (2002) demonstrated that more than one nucleo-cytoplasmic complex and possible epistatic interaction between them are needed to explain the sex ratio difference they obtained between

cross- and self-fertilization of hermaphrodites. We detected similar estimates of sex ratio differences between selfed and outcrossed individuals of the same family (Chap. 1). Since inbreeding depression in late life-cycle stages, for instance male reproductive traits like pollen quantity and quality, have been observed in different species (Willis, 1993; Melser, Bijeveld, and Klinkhamer, 1999; Willis, 1999a) we claimed that in addition to complex sex determinism systems inbreeding depression could partly explain the lower sex ratio in the progeny of selfed hermaphrodites. In this study, we found a positive correlation between the difference of sex ratio between inbred and outbred individuals and inbreeding depression for the number of viable stamen *per* flower in partial male-sterile individuals (Fig. 9). Thus, when sex ratio difference between outbred and inbred individuals was large, differences in number of viable stamen *per* flower was also large between outbred and inbred PMS. Conversely, when progeny of selfed hermaphrodites were nearly all hermaphrodites (i.e. low difference in sex ratio), PMS displayed only few aborted stamen *per* flower in inbred as well as in outbred individuals. A second positive correlation was found between inbreeding depression estimates for pollen viability and number of viable stamen *per* flower, demonstrating that families that were not affected by inbreeding for pollen viability also produced PMS with more viable stamens *per* flower (i.e.: more pollen) within inbred groups. The close associations within male reproductive functions (pollen quantity and quality) and, between male reproductive function and sex ratio suggest either the presence of linkage disequilibrium between male fertility restorers and genes involved in male reproductive functions, or that the same nuclear genes act both on sex determinism and male reproductive function through pleiotropic effects. The latter would imply that the large proportions of females in the progeny of selfed individuals are at least partly an expression of inbreeding depression. This was partly supported by a lack of correlation of sex ratio differences between generation F1 and F2 but also by the weak decrease in sex ratio between $F \geq 0.5$ and $F \geq 0.75$, even if not significant. Only a few studies have jointly explored male reproductive functions and sex ratio in

gynodioecious species (Gigord et al., 1999; Koelewijn, 2003) but only through cross-fertilization between and within populations. Gigord et al. (1999) detected positive correlations between sex ratio and reproductive functions for hermaphrodites but and no such correlations for females. These authors suggested that restorer genes are involved in both sex determinism and efficiency of resource allocation to reproductive functions. Koelewijn (2003) detected that sex ratio was positively correlated to investment into pollen whereas it was negatively related investment in ovules, and suggested that nuclear genes that act on the restoration of male function also act on the efficiency of reproductive function by changing sex-allocation pattern of hermaphrodites. In *Plantago coronopus*, partial male-sterile individuals with labile sex expression had varying sex allocation with changing environment: PMS became more male-sterile when temperature was increasing (Koelewijn and VanDamme, 1996). Here we report PMS becoming more male-sterile with increasing inbreeding level. If inbred individuals are less efficient in nutrient acquisition, they could have less energy to produce large amounts of viable pollen grains.

In conclusion, we detected large amounts of inbreeding depression which increase throughout the successive life-cycles stages. We were not able to detect an expected synergistic epistasis and suggest that the occurrence of purging in our experiment could have masked the expression of synergistic epistasis. Thus experiment aiming to detect epistasis should be very careful in trying to limit within experiment purging. Finally, we found very interesting correlations between sex ratio and male reproductive functions which suggest that the appearance of females in the progeny of selfed hermaphrodites could partly come from large inbreeding depression in reproductive traits.

Is inbreeding affecting the potential pollinator attractiveness of *Silene vulgaris (*Caryophyllaceae)?

Abstract – Inbreeding depression, the reduction in fitness of inbred individuals compared to outbred ones, is widely studied since it is thought as the major force explaining the evolution of mating systems towards enhanced outcrossing. In this study we focus on visual cues that can play a role in pollinator attraction. Through two generations of self- and cross-fertilization of *Silene vulgaris* hermaphrodites, we experimentally created three levels of inbreeding ($F \geq$ 0, 0.5 and 0.75) and tested whether fitness traits such as flower size, floral display and fluctuating asymmetry could be influenced by inbreeding. Our results showed that flower size and floral display were affected by inbreeding since both traits decrease with increasing inbreeding, while fluctuating asymmetry did not increasing with increasing level of inbreeding, as expected. Hence, visual cues for pollinator attractiveness like flower size and floral display could act as sexual selective traits enhancing reproductive success of outbred individuals.

INTRODUCTION

Although hermaphroditism seems to be the rule in flowering plants, other mating systems such as dioecy, monoecy or gynodioecy have evolved to enhance outcrossing. The major selective force that drive mating systems to evolve against selfing is inbreeding depression; the fitness reduction of inbred individuals relative to oubred ones (Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987; Keller and Waller, 2002). Gynodioecy is the co-occurrence of females and hermaphrodites within the same species and commonly within the same population. The maintenance of this mating system requires a greater fitness of females compared to hermaphrodites. Since hermaphrodites are able to self-fertilize, their progeny has on average a lower fitness than females' progeny (Jolls and Chenier, 1989; Pettersson, 1992b; Mutikainen and Delph, 1998; Rankin, Weller, and Sakai, 2002) and inbreeding depression is therefore one of the mechanisms that explains the maintenance of females (Couvet, Bonnemaison, and Gouyon, 1986; Shykoff et al., 2003).

Studies of inbreeding depression in gynodioecious species have shown that it can be important: estimates of cumulative inbreeding depression are of 0.39 for *Plantago coronopus* (Plantaginaceae) (Koelewijn, 1998), vary from 0.43 to 0.7 for *Schiedea menziesii* (Caryophyllaceae) according to the year of experimentation (Rankin, Weller, and Sakai, 2002) and from 0.4 to 0.6 for different populations of *Silene vulgaris* (Chapter I).To obtain good estimates of overall inbreeding depression, these experiments were usually performed on successive life-cycle stages (number of seeds, germination, and survival to maturity). Furthermore, it was shown that magnitude of inbreeding depression is often of important in late life-cycle stages like reproductive stages whatever the reproductive system is (Husband and Schemske, 1996). In the study species *S. vulgaris*, experimental investigations showed that inbreeding depression increases along the successive life-cycle stages (Emery and

McCauley, 2002). However, within the few inbred plants that survive to flowering stages, estimates of inbreeding depression in late stages such as fertility of gametes and potential pollinator attractiveness have been more rarely assessed (Barrett and Charlesworth, 1991; Melser, Bijeveld, and Klinkhamer, 1999; Willis, 1999b; Andersson and Waldmann, 2002).

Since different degrees of attractiveness can influence pollinator movements, patterns of mating within populations can be nonrandom and traits affecting pollinator attraction can therefore be considered as sexual selective traits (Deplh, 1996). Flowering plants have different strategies to attract pollinators: flowers can produce scents, rewards such as nectar and/or pollen, but can also use visual cues. Visual cues known to influence pollinator attractiveness are colors, floral display: the number of flowers/plant (Vaughton and Ramsey, 1998), flower size (Deplh, 1996; Kobayashi, Inoue, and Kato, 1997; Vaughton and Ramsey, 1998; Eckhart, 1999; Kawagoe and Suzuki, 2002) and fluctuating asymmetry: deviation from bilateral or radial symmetry (Moller and Eriksson, 1994; Moller and Sorci, 1998; Moller, 2000). In this study we focused on the effect of inbreeding on visual cues for pollinator attractiveness and focused on fluctuating asymmetry, flower size and floral display.

Fluctuating asymmetry (FA) is an estimation of development instability. It has been shown to depend on environmental factors such as nutrients, pathogens, competition, and on genetic factors such as hybridization and inbreeding (Siikamäki and Lammi, 1998; Moller and Shykoff, 1999; Siikamäki, 1999; Waldmann, 1999, 2001). It is therefore thought to provide estimates of individual vigor or capacity to cope with an environment given its genetic background. Fluctuating asymmetry can play a role in pollinator attractiveness because symmetrical flowers have been shown to be more visited than asymmetric ones (Moller and Eriksson, 1994; Moller and Sorci, 1998; Moller, 2000). The study of Moller and Sorci (1998) has shown that with artificial flowers, insects from different orders (Diptera, Hymenotera and Coleoptera) are more attracted by flowers when symmetric than when asymmetric.

Flower size is also known to affect pollinator behavior: large flowers are more attractive to pollinators than small flowers. This phenomenon has been shown in a variety of species and especially studied in dioecious and gynodioecious species where sexual dimorphism is common (*reviewed in* Delph, 1996 *and* Eckhart, 1999). For instance, large male flowers of *Wurmbea dioica* received 53% more visits per plant than smaller female flowers when floral display was equal in both sexes (Vaughton and Ramsey, 1998). The increased visitation rate of male flowers should increase pollen load on insects and should therefore increase male and female reproductive success. Indeed, a larger number of visits induce more pollen removal and male reproductive success could be increased if pollen is subsequently deposited on a female flower. Evidence for increased male reproductive success has been shown in the hermaphroditic plant *Campanula punctata* (Campanulaceae) (Kobayashi, Inoue, and Kato, 1997). These authors set up artificial populations. Within each population they placed 3 pollen donors with different corolla sizes and with genotypes that could be discriminated with their five allozymes. A paternity analysis demonstrated that large flowers sired more seeds than small flowers (Kobayashi, Inoue, and Kato, 1997). Female reproductive success should also be increased through large pollen loads by providing enough pollen to fertilize all ovules and by enhancing pollen competition within the style which may subsequently increase progeny vigor (Delph, Weinig, and Sullivan, 1998).

Although, large floral display can increase pollinator attraction (Vaughton and Ramsey, 1998; Barrett, 2003; Karron et al., 2004), producing many flowers can induce large costs. In hermaphrodites particularly large floral display increases within-plant movements of pollinators thereby increasing selfing through geitonogamous pollen transfer and, decreasing the amount of exported pollen (i.e.: pollen discounting) (De Jong, Waser, and Klinkhamer, 1993; Harder and Barrett, 1995; Karron et al., 2004). Thus, a balance between benefits to attract pollinators and limited outcrossing opportunities should somehow limit the number of flowers occurring on a plant. Furthermore, producing numerous large flowers can be very

energy consuming and it is thought that a trade-off between flower size and floral display should exist (Morgan, 1998; Sato and Yahara, 1999; Worley et al., 2000). This outcome has not been clearly shown and environmental variation is thought to obscure the trade-off between flower size and floral display (Worley et al., 2000). Indeed, good environments can mask a potential trade-off until resources become limiting.

Our aim is to test whether inbreeding can affect a) fluctuating asymmetry, b) flower size, and c) floral display and hence d) flowering size could potentially affect pollinators' attractiveness. For this purpose we sampled seeds from natural populations located in three valleys within the western Swiss Alps. Two consecutive generations of self- and cross-fertilization of *Silene vulgaris*' hermaphrodites, enabled us to build up three levels of inbreeding: $F \geq 0$ for cross-pollinations, $F \geq 0$ 0.5 after one generation of selfing and, $F \geq 0.75$ after two consecutive generations of selfing. We predict that fluctuating asymmetry increases with inbreeding levels whereas flower size and floral display decrease with increasing inbreeding. We also tested for a trade-off between flower size and floral display. Since trade-offs are thought to be better revealed when resources are limiting we expect that inbred individuals, having a lower capacity to uptake nutrients, are more likely to show this trade-off between flower size and number.

METHODS

Study species

Silene vulgaris is a self-compatible gynodioecious perennial weedy plant distributed in the Holarctic. The plant is flowering from June to September and can display up to 100 flowers per plant. Female and hermaphrodite morphs can reproduce through cross-fertilization while hermaphrodites can also self-fertilize through geitonogamy, because flowers at male and female reproductive stages are often simultaneously present within an individual. In *S.*

vulgaris, as in most gynodioecious species, females have smaller flowers than hermaphrodites (Deplh, 1996; Eckhart, 1999; Williams, Kuchenreuther, and Drew, 2000; Collin et al., 2002). Seeds are disperses through gravity and pollen dispersal, through moths, is limited (Pettersson, 1992b; Taylor, Trimble, and McCauley, 1999). This induces some population structure and provides opportunities for inbred mating events (McCauley, 1998).

Sampling

In September 2000, mature fruits of *S. vulgaris* were collected in three different valleys of the Western Swiss Alps: Mont d'Or (MO: 570389N/137416E, altitude: 1880m), Dent de Jaman (DJ: 546398N/144389E, altitude: 1543m) and Val Ferret (VF: 574761N/084844E, altitude: 1895m). Size of populations within valleys varied from 10 to 50 individuals for DJ, 50 to 100 individuals for VF and, MO consisted of a large population of approximately 500 individuals.

Growth conditions and design of experiment

In March 2001, viable seeds from these 3 valleys were planted in a greenhouse of the University of Lausanne (Switzerland) with controlled environmental conditions (temperature 18 to 20°C; relative humidity: 50-60%). Seedlings and then, adult plants were maintained through daily watering in natural light conditions. When flowering, plants were sexed and, 20 hermaphrodites from each valley were randomly chosen to perform controlled pollinations. Each of the 3*20 hermaphrodites (generation P) was the progeny of a hermaphroditic maternal plant on the field, and was self- and cross-pollinated to build the F1 generation (Fig. 1). F1 progeny was thus composed of 60 groups of plants that we call families in the following. Each family was bearing progeny issued from self- (*S*) and cross-pollinations (*C*). A second generation (F2) was created by self- and cross-pollinations of *S* and *C* hermaphrodites from the F1 generation (Fig. 1). *S* and *C* hermaphrodites were respectively coded *SS* and *CS* when selfed and, cross-pollinations of *S* and *C* plants were denominated *X*.

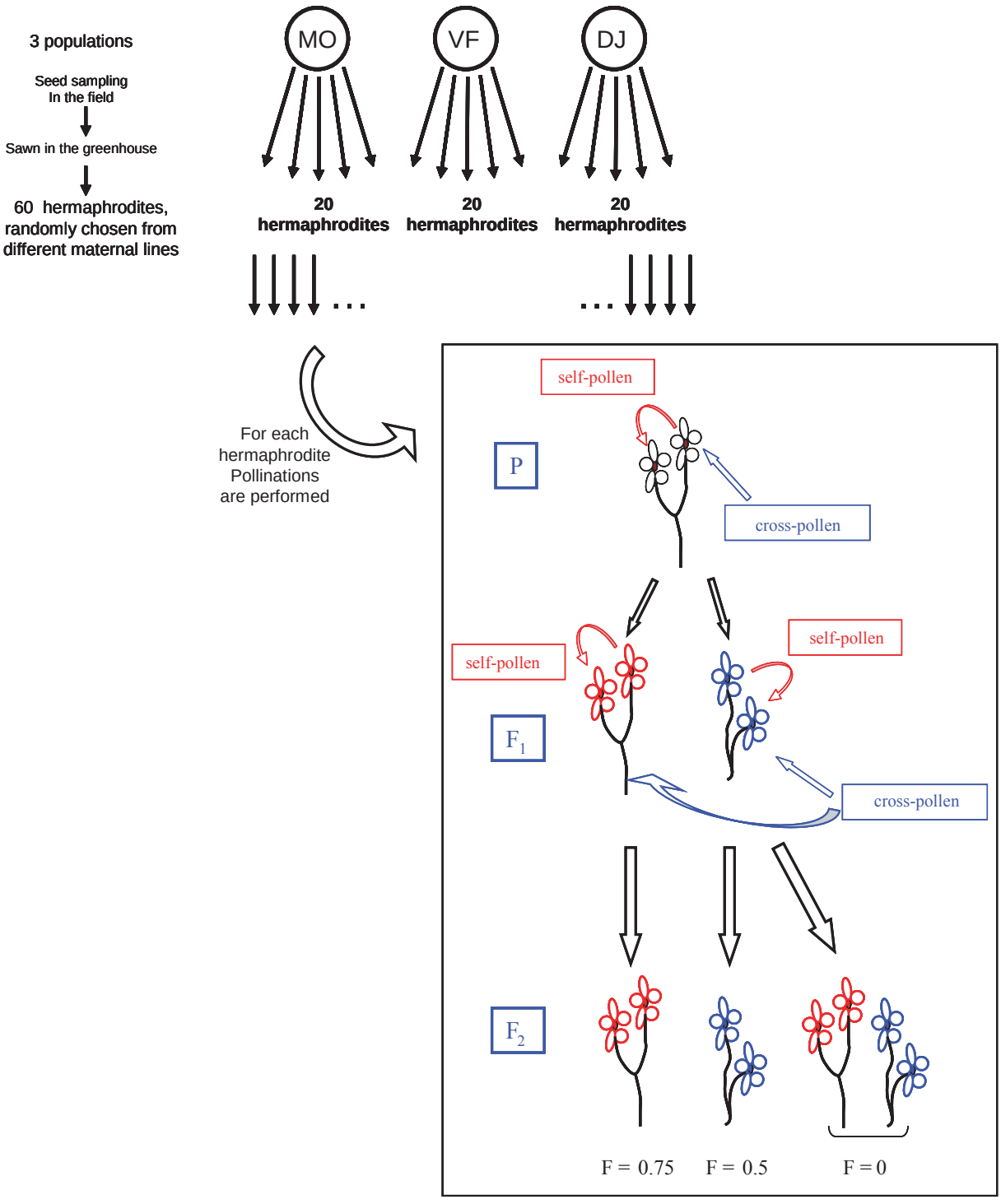


Figure 1. Scheme of sampling and design of experiment

As some pollination failed and some families were lost through large effects of inbreeding, we finally had 30 remaining families (8 families for MO, 10 for DJ and 12 for VF). The F2 generation allowed us to obtain three levels of inbreeding (*F*): *X* plants had an inbreeding coefficient close to 0. *CS* plants, originating from one generation of selfing, had an inbreeding coefficient (*F*) around 0.5 and *SS* individuals, coming from two consecutive generations of selfing had an *F* of approximately 0.75. Relatedness between seeds in generation P is possible according to the initial population structure. Thus initial population structure could be taken into account to reevaluate the real inbreeding coefficient (*F*) but, since we further compare inbreeding levels within families, original population structure does not to be considered.

For each family, 2 individuals were randomly chosen for each level of inbreeding and 2 flowers on each individual. In total 312 flowers were analyzed. To ensure that flowers were all in the same development stage: when the flower opened (day 1: first day of anthesis), they were marked with a small string for identification and then harvested two days later (at day 3) between 10:30am and 2:00pm. For each flower, all petals were removed by cutting them at the point where they come out of the calyx (apparent parts of petals) and three of those were randomly chosen and placed between two glass slides. We further took pictures of the fixed petals with the help of a digital camera (Nikon Coolpix). In order to calibrate the pictures, a reference scale was photographed before each bound of petal pictures (i.e.: every day). With the UTHSCSA ImageTool 3.00 software by Wilcox et al. (1995; http://ddsdx.uthscsa.edu/dig/itdesc.html) we drew a line along the photographed reference scale which gave us the number of pixels on the line and thus allowed us to transform areas from pixel to square millimeters. For each glass slide, two petals were analyzed with ImageTool 3.00. Since petals of *S. vulgaris* are bifid, the area of each lobe was measured by following the edge of each lobe and drawing a right angle to the point were the two lobes separate (Fig. 2).

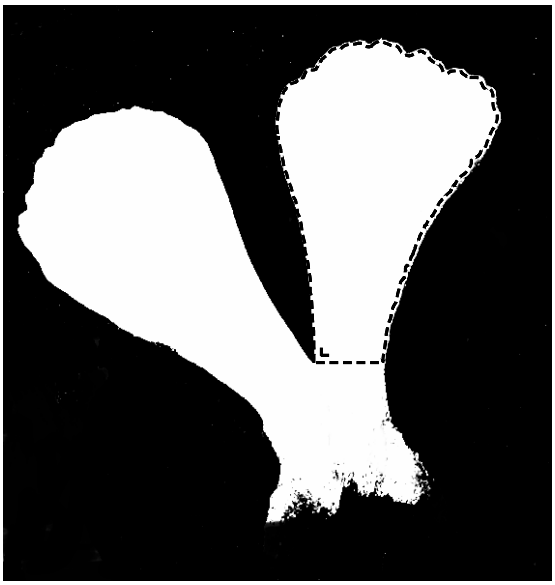


Figure 2. Area measured from a bifid petal of *S. vulgaris*; each of both lobes were measured in the same way, by following the edge of the petal and cutting it with a right angle at the point the lobes separate.

Measures of attractiveness

Fitness estimates per plant were assessed as 1) Flower size: the sum of the area of both lobes, averaged for the two measured petals within a flower and for both flowers on a plant. 2) Fluctuating asymmetry was estimated as the absolute difference between the areas of the two lobes divided by flower size. We standardized the difference of area between both lobes by flower size because fluctuating asymmetry was positively correlated to flower size ($r=0.52$; $p<0.001$) (Palmer and Strobeck, 1986). Estimates of fluctuating asymmetry were also averaged for the 4 petals measures within a plant. 3) Floral display was estimated as the number of flowers/plant 3 weeks after the plant had started flowering (i.e.: first open flower). We also computed 4) Flowering size which was the product of flower size and floral display.

Data analysis

Two models of analysis of variance were used to test the different explanatory variables. To compare levels of inbreeding within families, treatment effect was tested with a repeated-measure ANOVA: family and level of inbreeding ($F \geq 0.75$, $F \geq 0.5$, $F \geq 0$ or respectively *SS*,

CS, *X*) in the written order for 'Type I' Sum of Squares. A second ANOVA was used to test for valley and family effect, as well as interactions with levels of inbreeding. The valley and level of inbreeding were considered as fixed effects while family was random and nested within valleys. Since transformation of data did not help to satisfy ANOVA conditions, we performed randomization tests (1000 permutations) on the mean squares (Manly, 1997). If less than 5% of permutations gave a larger mean square than the one obtained with the observed data, the tested effect was considered as significant. The scheme of randomization was different for each explanatory variable. Levels of inbreeding were tested, with the repeated-measure ANOVA, as the within family permutations of inbreeding levels. If significant, we subsequently performed planned comparisons: outbred ($F \geq 0$) versus inbred plants ($F \geq 0.5$ and $F \geq 0.75$) and, between inbred plants ($F \geq 0.5$ versus $F \geq 0.75$) to better discriminate which level(s) of inbreeding accounted for the significant difference. For valley effect, all levels of inbreeding within a family were combined as a block and, blocks were permuted between valleys. For family effect, we randomized families within levels of inbreeding for each valley. The interaction family*levels of inbreeding was tested as the permutations of data within valleys and, finally valley*levels of inbreeding interaction was tested as the permutations of whole families between valleys combined with the permutations of inbreeding levels within families.

Finally, trade-off between floral display and flower size was analyzed using an analysis of covariance (ANCOVA). The model tested variation of floral display which was explained by valley, inbreeding levels (introduced as a factor) and the covariate flower size. Log-transformations of data were performed to satisfy ANCOVA conditions. With this model, evidence of a trade-off would be detected if the covariate is significant.

RESULTS

Effects of inbreeding levels and valleys

The level of inbreeding affected significantly flower size, floral display and flowering size whereas fluctuating asymmetry of petals was not affected by the level of inbreeding of individual plants (Tab.1), even if MO valley seems to display the expected predictions: the larger the level of inbreeding the larger fluctuating asymmetry (Fig. 3). Outcrossed plants ($F \geq 0$) displayed significantly more flowers per plant, larger flowers and overall plant size than inbred plants ($F \geq 0.5$ and $F \geq 0.75$) (Fig. 3a,c,d). However none of the measures of attractiveness were significantly different between the two levels of inbreeding ($F \geq 0.5$ and $F \geq 0.75$) within inbred plants. Even if there was no significant difference between $F \geq 0.5$ and $F \geq 0.75$, figure 3a shows a trend towards a decrease in flower size with increasing level of inbreeding for each valley, thus the more inbred the plant the smaller the flowers.

Level of inbreeding*valley interaction was significant for flower size, floral display and flowering size (Tab.1). Indeed, the relative decrease in floral display and plant size between inbreeding levels was different for all three valleys (Fig. 3). Intrinsic differences between valleys for flower size, floral display and plant size were detected by significant valley effect (Tab. 1). Finally, family effect was affecting significantly flower size, indicating that flower size was varying among families independently of the level of inbreeding (Tab. 1).

Table 1. Analyzes of variance for the different estimated fitness traits. Valley and level of inbreeding are fixed effects and family, nested within valley is random.

	Flower size [mm^2]				Fluctuating asymmetry				Floral display [# of flowers]				Flowering size [cm^2]			
Source of variation	Df	MS	P-value		Df	MS	P-value		Df	MS	P-value		Df	MS	P-value	
Valley	2	764.5	0.042	*	2	0.88	0.757		2	4799.5	0.001	***	2	1124.6	0.001	***
Level of inbreeding (*F*)	2	1690.0	0.001	***	2	3.48	0.225		2	3696.8	0.002	**	2	1284.8	0.001	***
Family(Valley)	27	187.0	0.002	**	27	3.40	0.183		27	335.2	0.676		27	53.8	0.393	
Level of inbreeding (*F*)*Valley	4	15.8	0.020	*	4	4.21	0.750		4	2539.5	0.001	***	4	445.6	0.001	***
Level of inbreeding (*F*)*Family (Valley)	44	93.8	0.852		44	2.50	0.751		44	579.1	0.107		44	82.3	0.337	
Error	78	61.7			78	2.78			75	256.2			75	28.3		

Notes: MS for Level of inbreeding (*F*) was tested with a repeated measure ANOVA to enable within family comparisons (see Methods)
(* : P-value = 0.05; **: P-value = 0.01; ***: P-value = 0.001)

Table 2. Analysis of covariance of floral display with flower size as covariate.

	Floral display			
Source of variation	Df	MS	F-value	P-value
Valley	2	6.15	9.47	***
Level of inbreeding (*F*)	2	4.55	7.00	**
Log(Flower size)	1	0.47	0.72	
Valley*Level of inbreeding (*F*)	4	2.47	3.81	**
Valley*Log(Flower size)	2	1.08	1.66	
Level of inbreeding (F)*Log(Flower wize)	2	0.80	1.23	
Valley* Level of inbreeding (*F*)*Log(Flower si	4	0.81	1.25	
Residuals	137	0.65		

Note: (* : P-value < 0.05; **: P-value < 0.01; ***: P-value < 0.001)

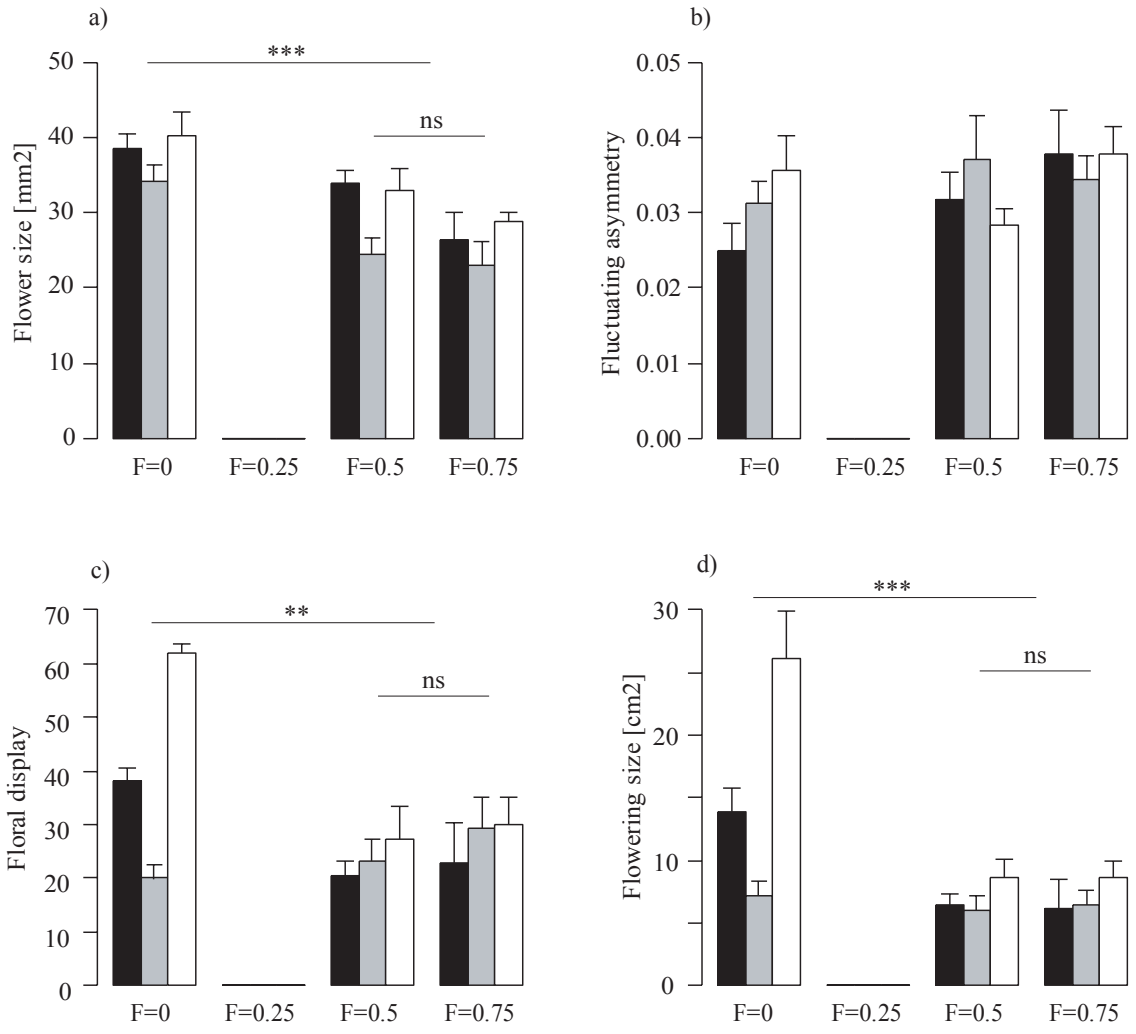


Figure 3. Relationship between traits related to fitness (+se) and levels of inbreeding (F). Traits are a) Flower size, b) Fluctuating asymmetry, c) Floral display and d) Flowering size. Bars account for the different valleys: black for MO, grey for VF and white for DJ. ns: $p>0.05$; **: $p<0.01$; ***: $p>0.001$

Flower size – floral display trade-off

We found no evidence for a trade-off between flower size and floral display as indicated by the non-significant effect of the covariate (Tab. 2). However, MO valley displayed a trend towards negative slopes when computing a regression between floral display and flower size. And, interestingly VF and DJ valleys, the relationship between floral display and flower size were positive when inbreeding coefficient (*F*) was 0 and became negative when $F \geq 0.75$, indicating that a trade-off might have been detected with larger sample size (Fig. 4).

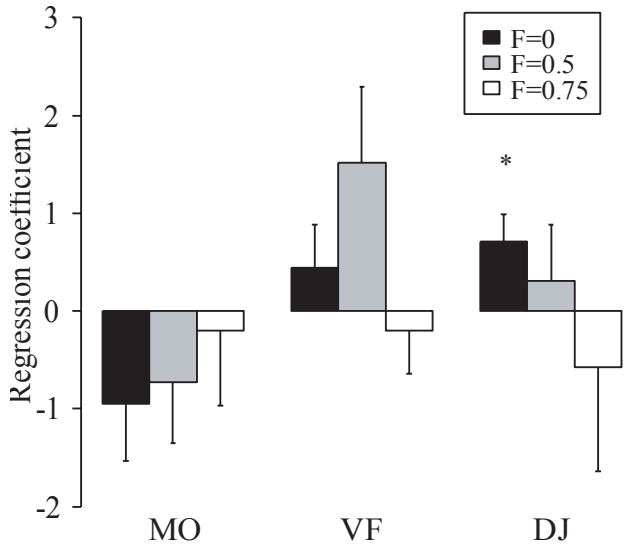


Figure 4. Coefficient of regression analysis between floral display and flower size. All coefficients are not significantly different from zero appart from *: $p<0.05$

DISCUSSION

Few studies have explored the occurrence of inbreeding depression in late life-cycle stages of flowering plants and, for instance traits that can affect pollinator attractiveness. Inbreeding depression was detected in pollen quantity and viability (Melser, Bijeveld, and Klinkhamer, 1999; Willis, 1999a) and on number of flowers per plant (Dudash, Carr, and Fenster, 1997; Good-Avila and Stephenson, 2003) but were rather considered as fertility advantages than as potential attractive cues for pollinators. Our study reports evidence for inbreeding depression in visual cues for pollinator attractiveness and thus for traits thought to be involved in sexual selection (Eckhart, 1999). Flower size, floral display and therefore overall flowering size were reduced with inbreeding whereas fluctuating asymmetry was not affected. Thus no inbreeding depression was detected for development instability of flowers (Tab. 1, Fig. 3). We did not detect a trade-off between floral display and flower size (Tab.2).

Evidence of inbreeding depression for traits affecting pollinator attractiveness

Flower size was larger for plants issued from cross-fertilization than for plants issued from one or two generations of self-fertilization. There was also a trend for further decrease as inbreeding level increases: flower size was smaller when plants were issued from two consecutive generations of selfing than when coming from one generation of self-fertilization (Fig. 3a). A similar trend was observed in the single other study looking at the effect of inbreeding on flower size (Andersson and Waldmann, 2002). Inbreeding depression expressed in flower size could decrease the reproductive success of inbred individuals, since pollinator visitation rate are known to decrease with flower size (Kobayashi, Inoue, and Kato, 1997). In gynodioecious species, females display generally smaller flowers than hermaphrodites and pollinator visits have been shown to be more frequent for hermaphrodites than for females

(Shykoff, 1992; Deplh, 1996; Eckhart, 1999; Shykoff et al., 2003). Similarly, many dioecious species display larger male flower that are more frequently visited by pollinators than female flowers. The larger male flower size is generally explained by the Bateman's principle (Eckhart, 1999), which states that male reproductive success depends more on visitation rates than female reproductive success which is thought to be limited by the amount of available resources for seed maturation (Vaughton and Ramsey, 1998). Nectar production could also be enhanced in larger outbred flowers. Indeed, large hermaphroditic flowers of *S. vulgaris* are known to produce nectar with higher sugar content than the smaller female flowers (Jolls, Chenier, and Hatley, 1994). Moreover, in the previous F1 generation, we tested for differences in the amount and concentration of nectar production. We did not detect a difference between inbred and outbred plants for nectar quantity but quality of nectar (i.e.: sugar concentration) was significantly larger in outbred than in inbred individuals for MO valley (Paired T-test, df=6, p=0.014) (unpublished results). However, flower size was not jointly measured and we do not know whether it was positively correlated with nectar quality or not.

The negative effect of inbreeding on floral display was larger than for flower size. There was a sharp decrease between totally outbred plants and inbred ones and, no difference was detected between $F \geq 0.5$ and $F \geq 0.75$. Large floral displays advertise more potential rewards to pollinators and have been shown to increase visitation rates of pollinators (Vaughton and Ramsey, 1998; Karron et al., 2004). Since producing many flowers should also increase the number of potentially produced seeds, large floral displays can contribute to enhanced female reproductive success (Barrett, 2003). For hermaphrodites, floral display should be under a trade-off between the fitness gains arising through pollinator attraction and, the costs due to the large amount of self-fertilization occurring through geitonogamous pollen transfer (Karron et al., 2004), and the associated low amount of pollen exported (a phenomenon known as pollen discounting) (Harder and Barrett, 1995; Barrett, 2003).

Fluctuating asymmetry is known to influence pollinator behavior (Moller and Sorci, 1998; Moller, 2000) and to be affected by environmental and genetic factors (Siikamäki and Lammi, 1998; Moller and Shykoff, 1999; Siikamäki, 1999; Waldmann, 1999, 2001). Since experiments herein were performed in a greenhouse with controlled conditions, we expected no environmental effects but a positive relationship between inbreeding level and fluctuating asymmetry. This was not the case, only MO valley showed such a trend (Fig. 3b). Waldmann (1999) showed, in *Silene diclins*, that inbred individuals displayed more fluctuating asymmetry for petal area and petal length than individuals issued from crosses between unrelated plants belonging to the same species. Since fitness also generally decreases with inbreeding, it has been claimed that fluctuating asymmetry is a measure of individual quality. However, studies that, in parallel with fluctuating asymmetry of flowers, assessed fitness components did not manage to show an association between fitness and fluctuating asymmetry (Andalo, Bazin, and Shykoff, 2000; Siikamäki, Lammi, and Mustajäravi, 2002), indicating that fluctuating asymmetry was not consistently related to individual quality. These controversial data could explain why, in the current study, we did not detect inbreeding effects on fluctuating asymmetry (as our measures were repeatable). Alternatively, since inbreeding depression and fluctuating asymmetry are known to be stronger in harsh conditions, one could suggest that sufficient resources could have buffered any development instability problems associated with inbreeding. Thus, planting seeds in more stringent environment could have enhanced fluctuating asymmetry and could help to detect some inbreeding depression.

Trade-off between flower size and floral display

Analysis of covariance showed no evidence for a trade-off between floral display and flower size. However, when performing regression analysis between floral display and flower size we detected trends for a trade-off in MO valley. This trend was also detected in DJ and VF

valleys when inbreeding level was 0.75 (Fig. 4). This goes towards our predictions in which trade-off, if existing, should rather be apparent when plants are inbred and likely to be less efficient than outbred individuals in terms of resource acquisition. Only one study managed to show such a trade-off where some individuals had attractive large flowers but fewer flowers than other individuals displaying more but smaller flowers (Sato and Yahara, 1999). However, experimental investigations combining measures of flower size and floral display were generally unsuccessful in detecting a trade-off (Morgan, 1998; Worley et al., 2000). Lack of concordance with theoretical expectations (Brunet, 1992) was accounted for as coming from a large variability in the environment masking possible trade-offs between flower size and number (Morgan, 1998; Worley et al., 2000). Morgan's study (1998), conducted on *Claytonia virginica*, also put forward the fact that perennial species (such as *S. vulgaris*) could sacrifice the reproductive phase for future growth or survivorship and that experimental investigations for trade-off between flower size and floral display should rather be performed on annual species.

Differences between levels of inbreeding and valleys – ongoing purging and drift

Analyzes of variance and further pairwise comparisons showed that valleys were different from each other, and also responded differently to the inbreeding level. The largest difference was for floral display and flowering size, where plants from VF valley showed a slight increase for these traits with increasing inbreeding coefficient, whereas plants from DJ and MO showed a decrease with increasing levels of inbreeding. Two mechanisms could account for the fact that inbreeding did not affect floral display in VF valley. First, several generations of recurrent inbred matings in conjunction with strong inbreeding depression could have purged deleterious alleles from this valley (Barrett and Charlesworth, 1991). Secondly, random genetic drift, especially important in populations of small size, could have driven deleterious alleles to fixation. And, if the deleterious alleles are fixed within the valley no

signs of inbreeding depression can be detected when matings are performed within populations. An argument in favor of drift load is that (outbred and inbred) individuals from VF valley have a similar fitness to inbred individuals belonging to DJ and MO valley (Fig. 3c). For flower size, VF valley always had smaller flowers than the two other valleys whatever the inbreeding level is (Fig.3a). Drift could also have fixed some deleterious alleles on certain loci determining flower size in VF valley, but fixation is not complete at all loci since inbreeding depression was detected.

Comparisons between the different levels of inbreeding showed that measured traits displayed a strong and significant difference between totally outbred and inbred plants. But no difference was detected among the two classes of inbred individuals. The lack of further fitness decrease, with increasing inbreeding can be interpreted as an evidence for purging. It could come from our experimental design because families which were mostly affected by inbreeding depression in F1 generation could not be used for F2 generation since selfed individuals failed to reach maturity. Moreover, as inbreeding depression is increasing along the life-cycle in *S. vulgaris* (Emery and McCauley, 2002), in order to successfully create F2 we needed to ensure the availability of mature plants and used early flowering selfed F1 within families. Thus purging has also been going on within families since we probably selected the fittest selfed individuals from F1. Therefore double artificial purging, between and within families, could explain the absence of fitness decrease between individuals issued from one and two generations of consecutive selfing.

Sexual selection and the maintenance of gynodioecy

Flower size, floral display and thus overall plant size are used as visual cues for pollinator attractiveness and show here that they are negatively affected by inbreeding. Thus, inbred individuals should be less attractive to pollinators. This can be seen as ongoing sexual selection since pollinators will visit plants with large flower size and number, likely outbred.

Thus, mating success of inbred individuals should be lower than for outbred individuals and inbred progeny should only have a minor contribution to the genetic pool of the next generation, thereby enhancing the purging process. However, moths (Noctuideae, Geometrideae) known to be efficient pollinators of *S. vulgaris* also use flowers as oviposition substrate for the development of their larvae which predate developing fruits (Pettersson, 1992a; Collin et al., 2002). Furthermore, these pollinators are also the vectors of *Microbotryum violaceum* a fungus that sterilize infected plants. Since pollinators are more attracted by large plant size (i.e.: the combination of flower size and floral display) predation should be larger on plants displaying large and numerous flowers. Furthermore, since large floral display increases within plant movements of pollinators, and therefore selfing rate (Karron et al., 2004) and pollen discounting (De Jong, Waser, and Klinkhamer, 1993; Harder and Barrett, 1995), it should also negatively affect reproductive success. Increased selfing rate of hermaphrodites will decrease their overall fitness by reducing male and female reproductive success and could contribute to the maintenance of gynodioecy. Thus, the joint occurrence of higher selfing rate and larger parasitic infections in outbred plants with large floral display could therefore allow inbred plants to still contribute and transmit their deleterious alleles to the next generation. Purging would thus be partly prevented and this could contribute to the maintenance of the intensity of inbreeding depression, thereby maintaining gynodioecy.

Our study showed evidence for strong inbreeding depression in late life-cycle stages. Flower size, floral display and overall plant size where larger when measured on outbred than on inbred plants. Thus, pollinators being more attracted by progeny issued from cross-pollinations should increase their mating success. To ensure attractiveness and potential enhanced seed set of large plant size (i.e.: flower size and floral display) in *S. vulgaris*, direct estimates of pollinator visitation rates should be assessed with different plant sizes. Such an experimental investigation should be done in conjunction with estimates of within plant

selfing rates and predation risks, in order to have a clearer view of how these interacting forces could shape the evolution of a mating system like gynodioecy.

Evidence for cryptic incompatibility in Silene vulgaris (Caryophyllaceae)

Abstract - In several angiosperms, prezygotic incompatibility have evolved to counteract the negative effects of inbreeding (i.e.: inbreeding depression). Here we report evidence for the existence of 'cryptic incompatibility' (i.e.: differential pollen tube growth rate of different donors) in the self-compatible gynodioecious Silene vulgaris. Pollen tube growth rates of Self-, Close- and Far-pollen are compared within the same individual. Results show that, for hermaphrodite-recipients, self-pollen grows at lower rates than outcross-pollen. Moreover, outcross pollen coming from distant donors (>11m) tended to perform better than outcross-pollen from neighboring donors (<5m). This likely reduces the proportion of inbred progeny within a single capsule. Because post-pollination sexual selection can occur through pollen competition between different pollen donors, enhanced fitness for female function could arise through increased offspring quality.

INTRODUCTION

Many plants have evolved mechanisms to promote outcrossing in order to resist the deleterious effect of increased homozygosity when selfing (Charlesworth and Charlesworth, 1987; Keller and Waller, 2002; Barrett, 2003). However, selfing has several advantages because it makes reproductive success less dependant on external conditions such as stressful environment and/or on population size in the face of metapopulation processes like colonization events or bottlenecks. Theoretical work by Lande and Schemske (1985) showed that mixed mating system are unstable. However, recent experimental and theoretical studies have shown circumstances under which partial self-fertilizing populations can be stable. For instance, spatial heterogeneity, temporal fluctuations (Cheptou and Mathias, 2001) and/or population structure with density dependence (Ronfort and Couvet, 1995) can stabilize mixed mating systems. Nevertheless, pre-zygotic and post-zygotic mechanisms may have evolved in order to counteract the negative effects of selfing, even in partially selfing species.

Mechanisms for avoiding inbreeding can be classified as pre- or post-zygotic. Two efficient pre-zygotic mechanisms, the gametophytic and sporophytic incompatibility, exist in plants. They involve recognition between pollen and pistil of the mates through expression of incompatibility alleles (Barrett, 2002, 2003; Bernasconi et al., 2004). Separation of sexes such as dioecy can also be considered as a pre-zygotic barrier, thought to have evolved from hermaphroditism as a consequence of inbreeding depression (Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987; Waser and Price, 1993). Inbreeding depression at very early life-stages, for instance seed abortion is sometimes considered as a post-zygotic isolation mechanism (Mahy and Jacquemart, 1999). Clearly, reproductive barriers that act at pre-zygotic stages give a selective advantage in comparison to post-zygotic barriers, because they allow ovules not to be fertilized by close relatives, leaving them free for fecundation

(Waser and Price, 1991b) and thereby avoiding seed discounting (Johnston, 1998; Herlihy and Eckert, 2002).

In the gynodioecious *Silene vulgaris*, a strong pre-zygotic barrier such as self-incompatibility does not exist, since any pair of mates can produce a developing fruit and viable seeds. Nevertheless, preventing inbreeding is possible through decreased performance of pollen coming from relatives. Competition among pollen tubes occurs when the amount of pollen deposited on the stigma is larger than the number of ovules that can be fertilized (Walsh and Charlesworth, 1992; Delph and Havens, 1998). Thus, when different types of pollen are present on the same style, differences in performance are likely to lead to differential seed-siring success (Snow and Spira, 1991a; Snow and Spira, 1991b; Johnston, 1998; Aronen et al., 2002). In self-compatible species, differences in pollen performance can emerge as the consequence of less-compatible pollen growing slower than more-compatible-pollen (Weller and Ornduff, 1989; Walsh and Charlesworth, 1992; Baker and Shore, 1995). Differences in pollen performance among different pollen donors can be detected only when the donors are compared within the same individual, because interactions with the stylar environment can alter the outcome of pollen competition and thus, there is not an absolute value for compatibility. Such a system is called 'cryptic incompatibility', and is expressed in relative terms ('less-'or 'more-'compatible pollen (Cruzan and Barrett, 1993; Manicacci and Barrett, 1996).

Silene vulgaris is a gynodioecious species, meaning that hermaphrodites and females occur at the same time in the same population. Individuals of both sexes can reproduce through outcrossing. Self-fertilization of the hermaphroditic flowers is prevented because they are protandrous. Although fertilization within the same flower is not possible, hermaphrodite individuals can self by geitonogamy (fertilization between different flowers of the same individual), because flowers in the male and in the female phase can be found simultaneously within the same plant. In addition, bi-parental inbreeding (breeding with a close relative) is

likely to occur for both sexes. In this species, fitness disadvantages for selfed individuals as compared to outcrossed have been found at different life stages (Jolls and Chenier, 1989; Pettersson, 1992b; Emery and McCauley, 2002), suggesting that selfed individuals can suffer from inbreeding depression.

Since *S. vulgaris* suffers from inbreeding depression but shows no signs (other than the presence of females) of pre-zygotic incompatibility, we predict that cryptic incompatibilities should occur in this species. We test this prediction by quantifying *in vivo* pollen germination and pollen tube growth rate as a function of the relatedness between pollen donors and pollen recipient. In other terms, we ask whether cryptic incompatibility has evolved in *S. vulgaris*. For the present study, a Swiss population of 83 individuals, distributed in 5 patches, was transplanted in the greenhouse. We investigated whether cryptic compatibility occurs in hermaphrodites of *S. vulgaris*, by comparing the *in vivo* pollen tube growth rate of different donor plants within the same individuals. Pollination were carried out with (a) self-pollen, (b) pollen coming from a neighboring individual within the same patch (crossing distance < 5m), and (c) pollen coming from an individual belonging to another patch (crossing distance > 11m). We predict that pollen performance is negatively correlated to the genetic relatedness between the mates, quantified by their geographical distance. A pre-requisite is that neighboring individuals are more related than distant individuals. To estimate genetic distances, all individuals were genotyped using four highly polymorphic microsatellite loci (Juillet et al., 2003).

MATERIALS AND METHODS

Study species

Silene vulgaris (Moench) Garke (Caryophyllaceae) is a gynodioecious perennial weedy plant widely distributed and native to Europe. It has spread to other parts of the world : North America, Asia and North Africa (Dulberg and Horovitz, 1984). In Switzerland, where the present study was conducted, populations of *S. vulgaris* can be found at low altitudes along roadsides as well as in mountain grasslands, up to 2500m. Populations are generally constituted of female and hermaphrodite individuals. In natural populations of the study region, the 3-styled flowers are usually pollinated by nocturnal moths, principally Noctuidae (Pettersson, 1991; unpublished observations). Moths often forage within restricted areas, most of the pollen is transported within an area of 5m and a lot of within-plants pollen transfers also occurs, inducing self-fertilization of hermaphrodites (Pettersson, 1992b). The resulting fruit is a capsule containing up to 100 seeds, which are dispersed principally through gravity. Population structure is therefore induced through the limited dispersal of pollen and seeds (McCauley, 1998).

Study population, sampling, and growth conditions

The population considered in this study, located in Les Mosses (Switzerland, 138035N/573746E) at an altitude of 1430m, was surveyed in August 2002. The population consisted of 83 adult flowering plants, patchily distributed within an area of approximately 3000m^2. We defined patches so that all individuals within a patch were separated by less than 5 meters, whereas individuals from different patches were separated by at least 11 meters and at most 85 meters. The larger patch was subdivided into two patches (PR1 and PR2) because a small stream passed through. Thus, we had five resulting patches between which number of

individuals, sex ratio and selfing rate differed markedly (Tab. 1). In August 2002, stems from each individual in the population were transplanted in a greenhouse of the University of Lausanne and kept in controlled conditions (a light:dark photoperiod of 15:9 hours, temperature: 18 to 20° C, relative humidity: 50 to 60%). After a few days new stems started growing and old stems (those grown in the field) were excised. This procedure minimizes differences in fitness among individuals due to variable growth conditions in the field.

Table 1. Characteristics of the population used in this study. We recorded the number of hermaphrodites (H) and females (F) and also sex ratio as the proportion of females (F/(F+H)). We also recorded individuals in the vegetative state for which sex could not be determined (Undet). Total number of plants in each patch was the sum of females, hermaphrodites and undetermined individuals (Total). Selfing rate was estimated from inbreeding coefficient FIS.

Patch	**Total**	**Undet**	**H**	**F**	**Sex ratio F/(H+F)**	**Selfing rate**
Route	23	4	2	17	0.90	0.479
Sapin	15	6	8	1	0.11	0.492
Enclos	9	4	4	1	0.20	0.473
PR1	7	2	3	2	0.40	0.745
PR2	29	8	18	3	0.14	0.521
All patches	**83**	**24**	**35**	**24**	**0.41**	**0.523**

Microsatellite analysis

For each individual, DNA was extracted from dry leaves using FastDNA kit (Qbiogene®). Genetic analysis were conducted using four highly polymorphic microsatellite loci, developed for *S. vulgaris* (Juillet et al., 2003). Population structure was analyzed by computing f and θ (Weir and Cockerham, 1984), analogous to Wright's FIS and FST , with Fstat 2.9.3.2 (Goudet, 1995, 2001). This program was used to test the significance of both fixation indices (Goudet, 1995; Goudet et al., 1996). Assuming that the inbreeding coefficient is entirely due to selfing,

an average selfing rate was inferred from the value of F_{IS} using the classical result $S = 2 F_{IS} / (1+F_{IS})$ (Hartl and Clark, 1997).

Pollination experiment

Experimental design and procedure - The experiment was carried out on twenty-one hermaphrodites; each individual was used as recipient for three types of pollen: self-pollen (Self), pollen from a plant belonging to the same patch as the recipient (Close) and pollen from a plant belonging to another patch (Far). The three treatments (Self, Close and Far) were applied to each recipient by hand-pollination of one flower with one type of pollen. Since the three styles of each flower were pollinated with the same pollen, we computed a mean (over the three styles) for each flower on each recipient to avoid psoeudoreplication. Only small amounts of pollen (5 to 80 pollen grains) were deposited on each stigma to facilitate the measurements; a large pollen load deposition would result in numerous pollen tubes and make their discrimination difficult.

Flowers used as recipients were emasculated immediately after anthesis, before styles grew out of the calyx. After emasculation, single flowers were bagged with transparent philatelist envelops for 4 days until stigma had expanded. Hermaphroditic flowers become receptive on day 4 (1 day after the anthers have wilted) and stay receptive for about 3 days. Thus, we pollinated flowers the first day of stigma receptivity. For the "Close treatment", the closest available pollen producing neighbor in the field was chosen when flowers were mature for pollination. All Close-pollen donors were within the same patch as the recipient plant, in a range 0.5 to 5 meters around the recipient. For the "Far" treatment, a sire was chosen haphazardly among the flowering plants belonging to a different patch.

Four hours after pollination, styles were cut with a small portion of the ovary and fixed in FAA solution (40% formaldehyde, glacial acetic acid and 70% ethanol at the ratios 1:1:18) for 24h, and stored for two months in 70% ethanol at 4°C. Styles were then softened for 3.5h

in 4N NaOH and then washed overnight in tap water. After washing, styles were stained 1.5ml of 0.1% aniline blue in phosphate buffer (HK_2PO_4, H_2KPO_4 in H_2O) pH 7.8 for 4h (Martin, 1959), and finally gently squashed (by pressure) on a microscopy slide and examined by epifluorescence microscopy (Leitz®-Diaplan microscope with DAPI filter and Leica®-300F digital camera).

The work of Aizen et al. (1990) on *Dianthus chinensis* (Caryophyllaceae) suggested that in some species the growth of pollen tubes should be scaled to the length of the style. We therefore measured the length of each style, using an ocular micrometer, to account for this and we also counted pollen grains attached to stigma (PG) in order to control for variation in pollen loads, since large pollen loads can increase the pollen germination rate and therefore can strongly influence the pollen-germination and -growth rates (Waser and Price, 1991a). For each style, we measured the number of pollen tubes that have reached a section (S_1) placed 4mm below the tip of the style and we took the same measure for a second section (S_2), closer to the ovary, placed 8mm below the tip of the style (Fig. 1).

Figure 1: Measuring procedure

Pollen grains attached to stigma were counted directly on microscope, in order to control for different pollen loads. The length of the style was estimated as the length of the stylar support tissue measured with ocular micrometer. Sections S_1 and S_2 (respectively 4mm and 8mm below the stigma) were 0.9mm large and included the whole width of the style. We recorded the number of pollen tubes in each of the two sections to estimate relative pollen performances among the treatments Self, Close and Far. Measures were made using a Leitz®-Diaplan microscope with DAPI filter and a Leica®-300F digital camera.
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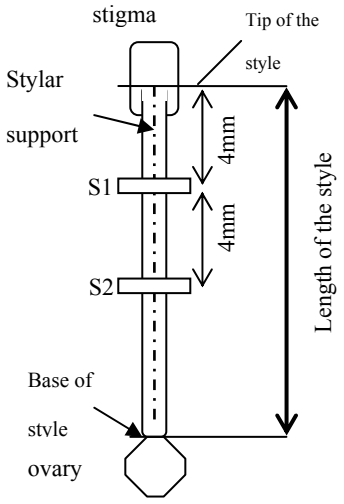

Counting pollen tubes – We counted individual pollen tubes in each section (S_1 and S_2). When pollen tubes were not distinguishable, the number of pollen tubes was estimated from the number of callose plugs. The number of callose plugs in a given section has been reported to be a sensitive indicator of pollen tube number going through the section (Weller and Ornduff, 1989; Snow and Spira, 1991a; Snow and Spira, 1991b).

Measurements made on preparations in which individual pollen tubes were distinguishable indicate that the callose plug *per* pollen tube ratio (CP/PT) was constant (1.03±0.03). Thus, the number of callose plugs (CP) in a given section (S) can be used to estimate the number of pollen tubes (PT) by the linear proportion $PT_S = \alpha\ CP_S$. The coefficient α was estimated by linear regression using the number of callose plugs as the independent variable and the number of pollen tubes as the dependent variable. The intercept was removed from the regression to assume proportionality, so that no pollen tubes were counted if callose plugs were totally absent. Regressions made on sections S_1 and S_2 separately did not differ significantly. Thus, a single regression was used to estimate the number of pollen tubes from the number of callose plugs ($\alpha = 0.972 \pm 0.028$, $p<10^{-6}$; $R^2=0.93$).

Measures of pollen performance - Two estimations of pollen tube growth were assessed : "early pollen tube growth" was estimated as the ratio between the number of pollen tubes in S_1 and number of pollen grains attached to the stigma $PTG_1 = PT_{S1}/PG$. And "late pollen tube growth" was estimated as the ratio of the number of pollen tubes between the two sections: $PTG_2 = PT_{S2}/PT_{S1}$.

Statistical analyses

Pollen performance (PTG_1 and PTG_2) was analyzed with repeated-measure ANOVAs (PTG for –Self, Close, Far - pollen were used as repeats within a recipient). Boxcox transformation (λ=-5.5) was applied to PTG_2 to enforce normality assumptions. Contrasts between pairs of treatments in PTG_2 were further analyzed using Tukey Honestly Significant (HSD) differences as implemented in S-plus. All statistical analysis were performed using S-plus© 2000.

RESULTS

Microsatellite analysis and population structure

Microsatellite analysis indicates a fairly large overall inbreeding coefficient (F_{IS} = 0.315) and low structure among patches (F_{ST} = 0.013). Nevertheless, both fixation indices were found to be strongly significant ($p < 0.005$), indicating non-random mating in the population at large. The average selfing rate (S) in the population was estimated (from F_{IS}) to be 52.3%. Selfing rates per patch are presented in table 1. The selfing rate was not significantly correlated with sex ratio where many females reduce opportunities for selfing (Spearman correlation, N=5, p=0.95).

Pollen tube growth experiment

Pollen tubes reaching section 1 and 2 were not significantly correlated with the length of the style (Kendall's correlation test for S_1: r = -0.052, n=179, p=0.3; and for S_2: r = 0.034, n=179, p=0.51). Thus, style length had no significant effect on pollen tube growth.

For early pollen tube growth (PTG_1), the proportion of pollen grains with pollen tubes reaching S_1 after 4h of growth was 27.9% (± 33%, n=18) for Self-pollen, 42% (± 34%, n=15) for Close-pollen and 52.6% (± 22%, n=17) for Far-pollen. Thus, as expected, Self-pollen

performed worse than Close-pollen and Far-pollen performed better than Close-pollen, though differences were not significant (Fig. 2a). Indeed, an analysis of variance on PTG_1 (Tab. 2) revealed a significant effect for the recipient (p=0.007), but only a marginal effect of the pollination treatment (p=0.070).

Late pollen tube growth (PTG_2) is an estimation of the proportion of pollen tubes that grow through S_1 to reached S_2: for Self-pollen this proportion was 6.1% (±13.5%, n=18) and 11.3% (±13.7%, n=15) for Close-pollen. In this case, pollen tubes from Far-pollen 23.4% (±17.4%, n=17) performed considerably better that the latter two (Fig. 2b). Analysis of variance for boxcox transformed PTG_2 showed no significant difference among recipient (p=0.95) but a significant treatment effect (p=0.028, Tab. 2). Tukey HSD tests showed a significant difference between Self- and Far-pollen treatments (p<0.05; Fig. 2b).

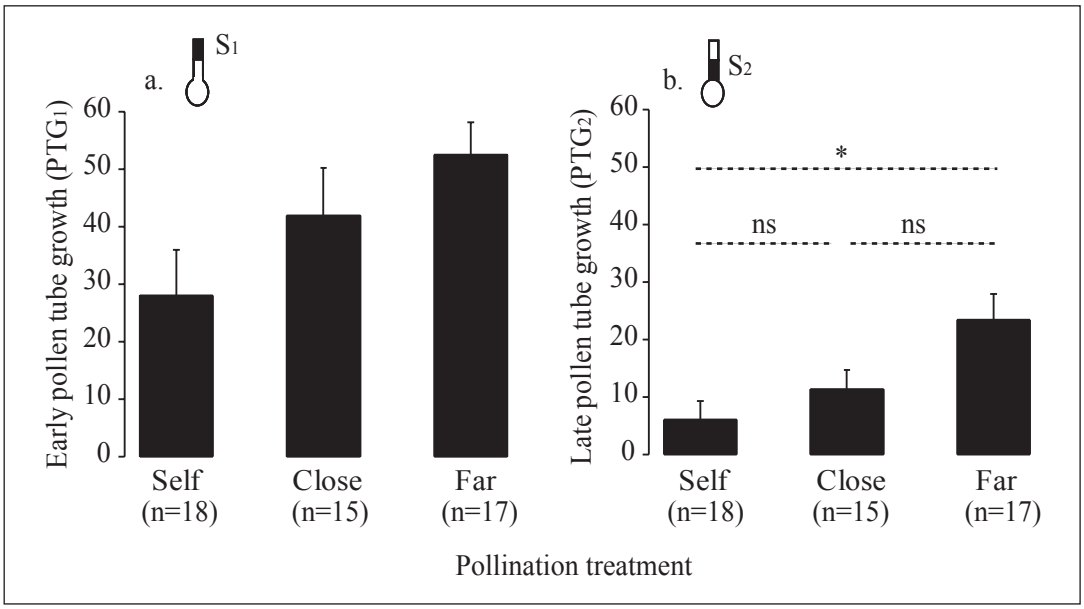

Figure 2. Observed means (±SE) of pollen tube growth rate measured in stylar sections S1 (a.) and S2 (b.). Pollination treatment was compared either with Self, Close or Far Pollen. Tukey HSD test was applied for contrasts between pairs of treatments (dotted lines) * : p<0.05, ns: p>0.05. ANOVAs are presented in tab. 2.

Table 2. Analysis of variance on pollen performance measures PTG_1 and boxcox transformed PTG_2. Recipient and pollination treatment were treated as fixed effects for a repeated-measure ANOVA. Distribution of the residuals does not differ significantly from the normal distribution (Kolmogorov-Smirnov test, $p>0.3$). ‡ $p<0.10$; * $p<0.05$; ** $p<0.01$.

	PTG_1			PTG_2		
Source	df	SS	F	df	SS	F
Recipient	20	2.845	2.769 **	20	0.095	0.494
Treatment	2	0.301	2.934 ‡	2	0.079	4.106 *
Error	27	1.387		27	0.261	

DISCUSSION

Our results show "cryptic incompatibility" (i.e.: differential performance of pollen from different donors) in hermaphrodites of *Silene vulgaris*. Pollen tube growth rate was significantly lower for Self- than for Far-pollen donors when observed close to the ovary. Moreover, we observed a trend towards better growth of Close- versus Self-pollen, and of Far versus Close-pollen (Fig. 2). This indicates that distance between mates influences pollen tube growth rate. Since pollen tube growth rate has been shown to correlate with the probability of fertilization in a number of species (Snow and Spira, 1991a; Pasonen et al., 1999) we predict that distant individuals should have larger siring success than nearby individuals when pollen of different origins arrive simultaneously on the stigma. Since significant population structure was found between patches, pollen performance likely depends on the genetic dissimilarity between mates and can thus act as a prezygotic barrier to prevent inbreeding (selfing and bi-parental inbreeding).

Microsatellite analysis and population structure

We found a significant large amount of heterozygote deficit due to non-random mating and some population structure among patches in the study population. Significance of F_{ST} indicates that individuals co-occurring within a patch are more closely related to each other than individuals belonging to different patches. And, similarly the significant F_{IS} indicates that inbreeding is substantial in these patches. By assuming that heterozygote deficit is entirely due to geitonogamous pollen transfer, selfing rate is estimated at 52.3%. Observations of pollinators foraging behavior (Pettersson, 1991, 1992b) showed restricted pollen movement in *S. vulgaris*, thereby inducing selfing through geitonogamy as well as mating with close relatives. This was demonstrated by Pettersson (1992), who used dye particles to estimate pollen movements in *S. vulgaris* and found that most particles were deposited on recipients within 1 meter around the plant. Similarly, fruit set in hermaphrodites was not altered with increasing distances of isolation between mates, whereas females fruit set was decreasing (Taylor, Trimble, and McCauley, 1999), again suggesting that hermaphrodites can compensate by selfing. As biparental inbreeding also seems to occur (Pettersson, 1992b) a selfing rate of 52.3% is probably somehow overestimated. Nevertheless, the high and significant F_{IS} implies that inbreeding occurs in this natural population. Thus, since inbred *S. vulgaris* plants suffer from inbreeding depression (Jolls and Chenier, 1989; Pettersson, 1992b; Emery and McCauley, 2002), mechanisms should evolve to prevent self-fertilization and/or biparental inbreeding.

Evidence for cryptic incompatibility

Our *in vivo* measures of pollen tube growth indicate that in hermaphrodite recipients, Far-pollen grows at a higher rate than Close-pollen, and both outperform Self-pollen (fig. 2). Thus, when different types of pollen are deposited on a stigma, differences in pollen tube

growth rate are likely to emerge among pollen donors, with respect to their geographical distance to the recipient plant. Such a relationship was shown in *Delphinium nelsonii* (Ranunculaceae) (Waser and Price, 1991a), however in this species an optimal crossing distance for pollen tube growth rate was detected (10m-pollen outperformed 1m- and 100m). Pollen tube growth rate in the clonal *Alstroemeria aurea* (Alstroemeriaceae) also showed significant positive relationship with geographical crossing distance (Souto, Aizen, and Premoli, 2002). Therein, genetic dissimilarity between ramets increased with increasing crossing distance. In our study population, neighboring individuals are genetically more similar than distant individuals, as demonstrated by the significant FST.

Even if our study indicates a continuum for increased pollen tube growth from Self- to Far-pollen, Tukey tests on PTG_2 only detected significant differences between Self- and Far-pollen, indicating that selfing is lowered by slower growth of self pollen tube through the style of the recipient flowers. Though, if only self-pollen is available, for example in a population where sex ratio is strongly biased towards females and pollen availability is limited, ovules can still be sired through self-fertilization, because pollen tube growth is lowered but not prevented. Slower pollen tube growth of self-pollen compared to outcross-pollen has been found in *Hibiscus moschetus* (Malvaceae) (Snow and Spira, 1991a) and in *Dianthus chinensis* (Caryophyllaceae) (Aizen, Searcy, and Mulcahy, 1990) . Similar results were found by Hauser and Siegismund (2002), who analyzed seed siring success of different pollen donors in *Silene nutans* (Caryophyllaceae). They showed that self-pollen fathered significantly less seeds than cross-pollen.

Multiple paternity was shown in natural populations of *Ipomopsis aggregata* (Campbell, 1998) and in other species (Bernasconi, 2003). In *S. vulgaris*, simultaneous deposition of multiple-donor pollen is likely to occur, and multiple paternities within a single fruit seems to be frequent, as suggested by a preliminary paternity analysis (from a nearby population), in which we identified an average of 4.8 different sires within single free-pollinated capsules of

different individuals (N=18, unpublished results). In the light of our results, we can suggest that, when pollen loads from multiple-donor are deposited, sexual selection through pollen competition could significantly bias the proportion of pollen received from different donors and their relative seed siring success. Experiments where equal amounts of pollen from two (i.e.: self vs. outcross) or more donors were simultaneously deposited in excess on the same stigma documented non-random seed siring success (Snow and Spira, 1991a; Pasonen et al., 1999; Aronen et al., 2002). Seed siring success correlated positively with *in vitro* pollen vigor in *Picea abies* (Aronen et al., 2002), as well as with *in vivo* pollen growth tube in *Betula pendula* (Pasonen et al., 1999) and *Hibiscus moscheutos* (Snow and Spira, 1991a). However, other studies failed to show a positive relationship between pollen tube growth and seed siring success (Melser, Rademaker, and Klinkhamer, 1997), suggesting that pollen tube growth may not be the only factor controlling for seed paternity. Indeed, many sources of variation could break down the efficiency of vigorous pollen grains to sire seeds. Interactions between different types of pollen growing on the same style, interaction between the pollen and the style and post-zygotic events such as selective seed abortion have been shown to influence the outcome of pollen competition (Cruzan, 1990; Herrero and Hormaza, 1996; Hormaza and Herrero, 1996). In the present study, early pollen tube growth (PTG_1) was significantly affected by the identity of the recipient plant (Tab. 2). This could come from an interaction between the pollen and the stylar environment. Indeed, for pollen availability reasons, some pollen donors were used for different recipients within a pollination treatment and they often showed large variation in pollen germination success among the different recipients. Alternatively, the significant recipient effect in early pollen tube growth could come from either stigma receptivity and/or pollen viability (Chapter I and unpublished results). However, neither stigma receptivity, nor pollen viability seemed to account for the significant recipient effect in PTG1, since all Close- and Far- pollination treatments displayed at least 5% pollen grain germination and since some pollen were used for several different recipients within

pollination treatments and variation for pollen germination was important (variance ranging from 0.02 to 0.4 for one pollen donor within a pollination treatment). Thus, we think that interaction between pollen donor and recipient accounts for the recipient effect in early pollen tube growth (Tab. 2).

Since direct competition was not performed in this experiment, interaction between different sires within the same style could not be detected. However there is evidence for variance in sire quality: if pollen comes from a sporophyte growing in a favorable environment, pollen is better provisioned (with nutritive elements) to achieve its growth through the style (Delph, Johannsson, and Stephenson, 1997). Also the level of heterozygosity in the pollen-producing parent has been shown to affect pollen performance through pollen provisioning (Johannsson, Gates, and Stephenson, 1998; Stephenson et al., 2001).

While partial separation of sexes and protandry have probably evolved as a response to the negative effects of selfing and/or the loss of outcrossing opportunities for pollen that has served for self-fertilizations; i.e.: pollen discounting (Harder and Barrett, 1995; Barrett, 2003), it is also likely that "cryptic incompatibility" has evolved in this species to avoid inbreeding depression caused by geitonogamy or by mating with close relatives. This interpretation is also supported by a work on the closely-related hermaphroditic species *Silene nutans* (Hauser and Siegismund, 2000).

Cryptic incompatibility will have two main effects. First, in the presence of foreign pollen, it makes geitonogamous reproduction less likely because self-pollen is outperformed by outcross-pollen. Second, it also reduces the possibility for bi-parental inbreeding because distant pollen-donors are favored with respect to neighboring pollen-donors. With respect to population structure this should translate into lower selfing rate, lower overall inbreeding coefficient, and higher gene flow among distant individuals (i.e. lower structure among patches - F_{ST}). Genetic analysis partially supports these results, as structure among patches has been found to be low, suggesting that the gene flow among them is important. From the

individuals' point of view, this discrimination among pollen donors lowers the average homozygosity in its offspring and thus is likely to be advantageous, especially for hermaphrodites. Moreover, in *S. vulgaris*, the ovules that are fertilized first are probably given an advantage by better resource provisioning by the maternal parent (Delph, Weinig, and Sullivan, 1998). These authors showed that the first ovules to be fertilized gave rise to seeds with better germination and developed into more vigorous progeny, independently of the pollen donor. Preferential provisioning of ovules that are fertilized first could further favor outcross pollen because, in addition to the lower inbreeding depression in the offspring and the higher probability to father seeds, these foreign pollens will be found to be in better provisioned seeds. Even if we showed that "cryptic incompatibility" and inbreeding depression exist in *S. vulgaris*, inbreeding is substantial in this population. This demonstrates that advantage of faster foreign pollen growth within the style is not sufficient to eliminate geitonogamous selfing, induced by the pollinators' behavior.

In conclusion, the results presented here suggest the existence of "weak" pre-zygotic barrier acting against inbreeding in *S. vulgaris*. This has implications for the species' mating system since effective gene flow can be affected. Sexual selection through pollen competition could compensate for large amounts of deposited self-pollen; by enhancing the chances that outcross-pollen achieves fertilization. However, that strict self-incompatibility has not evolved in *S. vulgaris* is probably because it is a weedy plant that needs the advantage of selfing to survive metapopulation processes.

CONCLUSIONS

Gynodioecy has been considered as an intermediate step along the pathway from hermaphroditism to dioecy (Darwin, 1877; Charlesworth, 1999). However, a phylogenetic analysis of the genus *Silene*, containing hermaphrodites, gynodioecious and dioecious species suggested that instead of hermaphroditism, gynodioecy is the ancestral mating system in the genus (Desfeux et al., 1996) and thus dioecy and hermaphroditism have evolved from gynodioecy. Hermaphrodite species *S. gallica* and *S. conica*, which derived from ancestral gynodioecy, are highly autogamous. Theoretically, reversion from gynodioecy to hermaphroditism is not surprising since conditions for the maintenance of females are rather stringent (Gouyon, Vichot, and Van Damme, 1991). Since these two hermaphroditic species are highly autogamous, one could argue that purging of the genetic load could have lowered inbreeding depression and thus brought gynodioecy to switch to hermaphroditism. Indeed, the occurrence of inbreeding depression in gynodioecious species is seen as one of the mechanisms that maintains females (Couvet, Bonnemaison, and Gouyon, 1986; Frank, 1989). This thesis deals with the intensity of inbreeding depression in the gynodioecious plant *Silene vulgaris*. The different investigations of this thesis give also insights into the way inbreeding depression is maintained in *S. vulgaris*. The maintenance of inbreeding depression can come from (1) large mutation rates that maintain large genetic load even if purging occurs (Agren and Schemske, 1993; Charlesworth, Lyons, and Litchfield, 1994), (2) overdominance because under this genetic mechanism purging of the genetic load is impossible (Lynch and Walsh, 1998; Keller and Waller, 2002) and (3) any feature that enhance cross-fertilization and thus hinder the purging process to take place. This thesis gives evidence for 3rd point just mentioned. I will now describe these mechanisms:

First, “cryptic incompatibility”, the differential performance of pollen, was higher when mates were not related than when self-pollination was performed. This mechanism allows cross-

pollen to be the first one to reach ovules and thus increases potentially the chance of cross-fertilization when different pollen types (self- and cross-pollen) are simultaneously deposited on the stigma (Delph, Weinig, and Sullivan, 1998; Aronen et al., 2002). Therefore, "cryptic incompatibility" being a mechanism promoting cross-fertilization, potential purging cannot occur since many successive generations are required for purging to be efficient.

Secondly, our estimates of inbreeding depression were as large as 0.76 because we assessed them in many different traits in the successive life-cycle stages. Indeed, inbreeding depression was increasing from "seed traits" to "reproductive traits". This pattern of the distribution of inbreeding depression across the successive stages of the life-cycle could also reduce the amount of purging. Indeed, a quite large amount of inbred individuals reached maturity and thus, still had opportunities to reproduce and to transmit the deleterious genes they bear to the next generation; hindering purging.

Thirdly, floral display (i.e.: the number of flowers *per* plant) and flower size are known to play a role in attracting pollinators. The larger the floral display and the size of flowers, the more pollinators are attracted. This of course would decrease the mating success of inbred individuals with fewer small flowers. However, large floral display increases the within plant pollinator movements and thus outbred plants are likely to produce more self-fertilized offspring than an inbred plant. Moreover, pollinators of *S. vulgaris* do also use flowers for oviposition of their eggs and the developing larvae induces large costs to the plant since it not only eat the fruit it was laid in but once the fruit eaten the larvae goes on and feeds on other developing fruits of the plant. Thus seeds from outbred plants with large and numerous flowers are more likely to be eaten than small inbred plants. This could favor the reproduction of inbred plant and again would prevent any potential purging process.

Finally, we showed that the offspring of self-fertilized hermaphrodites displays a female biased sex ratio and since females are obligate outcrossers, subsequent selfing and thereby purging are, as pointed out by Emery and McCauley (2002) prevented.

Thus, for sure, *Silene vulgaris* has been a good and thoughtful model to explore and to understand the evolution of mating systems and their interplay with inbreeding depression but also with ecological processes maintaining opportunities for self-fertilization.

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