# Inbreeding effects on progeny sex ratio and gender variation in the gynodioecious *Silene vulgaris* (Caryophyllaceae)

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## Summary

• In gynodioecious species, sex expression is generally determined through cytoplasmic male sterility genes interacting with nuclear restorers of the male function. With dominant restorers, there may be an excess of females in the progeny of self-fertilized compared with cross-fertilized hermaphrodites. Moreover, the effect of inbreeding on late stages of the life cycle remains poorly explored.

• Here, we used hermaphrodites of the gynodioecious *Silene vulgaris* originating from three populations located in different valleys in the Alps to investigate the effects of two generations of self- and cross-fertilization on sex ratio and gender variation.

• We detected an increase in females in the progeny of selfed compared with outcrossed hermaphrodites and inbreeding depression for female and male fertility. Male fertility correlated positively with sex ratio differences between outbred and inbred progeny, suggesting that dominant restorers are likely to influence male fertility qualitatively and quantitatively in *S. vulgaris*.

• We argue that the excess of females in the progeny of selfed compared with outcrossed hermaphrodites and inbreeding depression for gamete production may contribute to the maintenance of females in gynodioecious populations of *S. vulgaris* because purging of the genetic load is less likely to occur.

**Key words:** gynodioecy, inbreeding depression, local mate competition, partial male sterility, sex ratio, *Silene vulgaris*.

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## Introduction

Gynodioecy is the coexistence of hermaphrodites and females within plant populations (Darwin, 1877; Lewis, 1941). Inbreeding depression, the fitness reduction of inbred relative to outbred individuals (Lande & Schemske, 1985; Charlesworth & Charlesworth, 1987), is the major hypothesis explaining the maintenance of females within gynodioecious populations (see review in Charlesworth, 1999). Comparative data showed that the strength of inbreeding depression varies through a plant's life cycle and that its magnitude is generally high at late stages of the life cycle (i.e. during reproduction), regardless of the mating system (Husband & Schemske, 1996). As for self-fertilizing species, experimental investigations on gynodioecious species with intermediate self-fertilization rates found higher inbreeding depression in late than in early stages of the life cycle (Sakai *et al.*, 1997; Bailey & McCauley, 2006). These high magnitudes of late-acting inbreeding depression are thought to be the consequence of mildly deleterious alleles, which are inefficiently purged through natural selection and can result in high genetic load (Charlesworth *et al.*, 1991; Husband & Schemske, 1996; Willis, 1999a). Despite the importance of late-acting inbreeding depression, only a handful of studies have investigated its magnitude in components of male fitness

of hermaphroditic species (Willis, 1993, 1999b; Carr & Dudash, 1997; Chang & Rausher, 1999; Melser *et al.*, 1999) and it therefore deserves more investigation, including in gynodioecious species. For this sexual polymorphism, the cost of inbreeding should affect selfing hermaphrodites most strongly, whereas the impact on outcrossing females should be lower. Hence, high magnitudes of inbreeding depression should maintain the conditions favoring the outbreeding advantage of female plants.

Sex determination of gynodioecious species generally involves epistatic interactions between cytoplasmic male sterility (CMS) genes and nuclear restorers of male fertility (Koelewijn & Van Damme, 1995a,b; Charlesworth & Laporte, 1998; Byers *et al.*, 2005). The inheritance of male fertility restoration is generally a consequence of multiple restorer genes that are dominant rather than recessive (Koelewijn & Van Damme, 1995b; Charlesworth & Laporte, 1998). Thus, self-fertilization of hermaphrodites may not only result in offspring with reduced fitness but is also likely to increase the proportion of female progeny if hermaphrodites are heterozygous for dominant restorers. Emery & McCauley (2002) and Bailey & McCauley (2005) investigated introduced North American populations of the gynodioecious *Silene vulgaris* and detected a 20% increase in the proportion of females in the offspring of self-fertilized compared with cross-fertilized hermaphrodites. Their results were consistent with the action of dominant nuclear restorers. However, the magnitude of the female excess in the progeny of inbred compared with oubred hermaphrodites was larger than expected under random mating, considering a single restorer locus with one dominant allele (Emery & McCauley, 2002). A population composed of RR hermaphrodites should only produce hermaphrodites regardless of the relatedness between mates. Similarly, a population composed of Rr hermaphrodites should produce 25% females on average, whether selfed or outcrossed. Thus, the largest difference in sex ratio between selfed and outcrossed progenies would occur in a population with equal proportions of RR and Rr hermaphrodites. In this case, selfing would result in 12.5% females ($0.5 \times 0.25$) and random mating among hermaphrodites in 6.25% females ($0.5 \times 0.5 \times 0.25$). Thus, the relative excess of females in the progeny of selfed compared with outcrossed hermaphrodites should not exceed 6.25% if sex determination is controlled by a single restorer with one dominant allele restoring the male function (Emery & McCauley, 2002; Bailey & McCauley, 2005). A comparison between native and introduced populations of *S. vulgaris* could identify differences in the genetic structure of sex determination. Owing to the recent colonization history in the introduced range (McCauley *et al.*, 2003), bottlenecks could have led to an overall loss of polymorphism, and sex determination may be different from the source population(s). Furthermore, a joint estimation of the effects of selfing on sex ratio variation and male and female fertility traits would allow greater insights into the complexity of sex allocation in gynodioecious individuals.

Indeed, sex expression is variable and individuals with intermediate sex phenotypes can be found within natural populations of gynodioecious species (Assouad & Valdeyron, 1975; Dulberg & Horovitz, 1984; Shykoff, 1988; Ågren & Wilson, 1991). A partial male-sterile (PMS) individual can grow either a mixture of hermaphroditic and female flowers (i.e. gynomonoecy) or intermediate floral phenotypes displaying variability in the number of functional anthers among flowers (Koelewijn & Van Damme, 1995b, 1996; Delph & Mutikainen, 2003). This continuum of phenotypic sex expression stresses the need for a more quantitative description of gender, as suggested by Lloyd (1980). Sex expression of PMS plants can be affected by the environment (Koelewijn & Van Damme, 1996), although evidence for a genetic basis also exists (Koelewijn & Van Damme, 1995b). Using experimental crosses, Koelewijn & Van Damme (1995b) demonstrated that restoration of male fertility was the result of numerous nuclear loci and that PMS plants were a consequence of incomplete restoration whereas hermaphrodites were completely restored at male fertility loci. Moreover, the frequency of hermaphrodites in a given progeny has been shown to correlate positively with the pollen production of those hermaphrodites, in two gynodioecious species: *Thymus vulgaris* (Gigord *et al.*, 1999) and *Plantago coronopus* (Koelewijn, 2003). Thus, if partial male sterility has a genetic basis linked to the expression of restorers, PMS individuals are more likely to occur in the progeny of self-fertilized hermaphrodites (i.e. as a result of increased homozygosity) than in the progeny of cross-fertilized hermaphrodites, and their frequency should correlate positively with the decrease in the proportion of hermaphrodites between inbred and outbred progeny.

In this study we investigate the effects of two consecutive generations of self-fertilized and cross-fertilized hermaphrodites on the qualitative and quantitative sex expression of the gynodioecious *S. vulgaris*. To assess the effects of inbreeding on female and male fertility as well as on progeny sex ratio, we sampled families of *S. vulgaris* from three different valleys in the western Swiss Alps, occurring in the native range of the species. Our investigation had four main goals: to determine the intensity of the shift in sex ratio between self- and cross-pollinated plants; to investigate whether partial male sterility is more frequent and intense in the progeny of inbred individuals; to determine the magnitude of inbreeding depression in reproductive traits, separately for female and male fitness; and to explore potential correlations between the magnitudes of inbreeding depression for reproductive traits and relative differences in the proportion of hermaphrodites between pollination treatments.

## Materials and Methods

### Study species

*Silene vulgaris* (Moench) Garke is a gynodioecious short-lived perennial herb native to Eurasia and introduced in North

 

America. Females can only cross-fertilize whereas hermaphrodites can reproduce through both cross- and self-fertilization. Since hermaphrodites are protandrous, self-fertilization mainly occurs through geitonogamy. Indeed, up to 100 flowers can be opened simultaneously within an individual and pollen dispersal through moths is known to be spatially restricted (Pettersson, 1992). Each flower bears 10 stamens, all of which produce pollen in hermaphroditic flowers and are vestigial and aborted in male-sterile individuals. PMS individuals are either composed of female and hermaphroditic flowers (i.e. gynomonecious) and/or bear flowers with variable numbers of developed stamens. Strong inbreeding and population structure, induced through limited seed and pollen dispersal, have been shown in *S. vulgaris* (Pettersson, 1992; McCauley, 1998; Taylor *et al.*, 1999).

### Field collection

In September 2000, we collected mature fruits from 200 different maternal parents of *S. vulgaris* in three different valleys of the western Swiss Alps: Mont d'Or (MO, 570°23′20.4″N, 137°24′57.6″E, altitude: 1880 m), Dent de Jaman (DJ, 546°23′52.8″N, 144°23′20.4″E, altitude: 1543 m) and Val Ferret (VF, 574°45′39.6″N, 084°50′38.4″E, altitude: 1895 m). We subsequently refer to each valley (MO, DJ and VF) as a 'population'. MO was a large population of approximately 500 individuals where females were spatially clustered and overall the proportion of hermaphrodites was 0.8. VF and DJ populations were each composed of 10 different subpopulations distributed over 2 km. The size of subpopulations varied from 10 to 50 individuals and from 50 to 100 individuals for DJ and VF, respectively. Within the DJ and VF populations, the proportion of hermaphrodites ranged from 0.8 to 1 among subpopulations.

In March 2001, we sowed seeds in a glasshouse at the University of Lausanne using Jiffy pots (diameter 5 cm; natural light conditions, 18–20°C, 50–60% relative humidity). In April 2001, we transplanted seedlings into larger pots (diameter 15 cm; substrate, 50% peat and 50% clay soil). At flowering, we randomly chose 60 hermaphrodites (20 per population) for experimental manipulation, each originating from a different hermaphroditic maternal plant in the field.

### Experimental design and plant rearing

**Generation $F_1$.** We performed self-pollinations (S) and cross-pollinations (C) on all hermaphrodites to control for maternal effects (Lynch, 1988). For each hermaphrodite (subsequently referred to as a family), we emasculated 12 flowers and enclosed each in a transparent philatelist envelope. Two flowers were not pollinated, to assess the efficiency of envelopes in preventing uncontrolled pollinations; no such flower produced seeds. Five flowers were self-pollinated (S) and five were cross-pollinated (C), each with a different pollen donor from the same population. We saturated flowers with pollen to limit the effects of different pollen loads and/or pollen viability.

In September 2001, we sowed seeds from half of the families (10 families per population) into Jiffy pots (diameter 5 cm). For each family we planted 16 seeds from each pollination treatment (S vs C). We sampled the 16 C seeds so that each different pollen donor was equally represented. In December 2001, we sowed seeds (16 S and 16 C) from the 30 remaining families (10 families per population) and supplemented plants with artificial light to set a 15 h light : 9 h dark cycle. The temperature and humidity were the same as before.

**Generation $F_2$.** In March 2003, we produced a second generation ($F_2$) through self- and cross-pollinations of S and C hermaphrodites from generation $F_1$. We randomly chose one S-hermaphrodite and one C-hermaphrodite from each family. We pollinated 15 flowers on each hermaphrodite: five self-pollinations, five cross-pollinations with C-hermaphrodites each from a different family and five cross-pollinations with a S-half sib of each C-hermaphrodite. Selfing of S- and C-hermaphrodites resulted in SS ($F \geq 0.75$) and CS ($F \geq 0.5$) progeny, respectively, whereas we denominated cross-pollinations of S and C plants as X ($F \geq 0$). The inbreeding coefficient ($F$) can be greater than or equal to 0, 0.5 or 0.75 depending on the level of inbreeding in populations sampled in the field. However, this did not alter the interpretation of our results because comparisons between inbreeding levels were subsequently assessed within families.

Inbreeding depression in generation $F_1$ resulted in the loss of some families during the experiment. First, some S plants (3% of families) from the $F_1$ did not reach maturity; we thus removed them from the experiment. Second, delayed flowering in S plants (compared with C plants) sometimes prevented cross-pollinations. Finally, we removed families in which only one level of inbreeding ($F$) produced viable seeds, resulting in 30 families (8, 12 and 10 for MO, VF and DJ, respectively). In August 2003, we randomly chose 20 seeds from each 0.5 and 0.75 inbreeding levels ($F$) and 100–200 seeds (i.e. five to 10 different sires × 20 seeds) for cross-pollinations ($F \geq 0$), sowed them in Jiffy pots (diameter 5 cm) and reared them as in generation $F_1$. The sample size of cross-pollinations was large to have a good representation of the different pollen donors.

### Progeny sex ratio

In generation $F_1$, we assessed the gender of each flowering plant by sexing at least 10 flowers per plant and estimated the proportion of hermaphrodites for each family and pollination treatment. In generation $F_2$, we assessed the gender of flowering individuals by screening the sex of each flower opened between the first and 21st day of flowering. We then categorized plants as: (a) hermaphrodites (H, all flowers with fully developed stamens); (b) females (F, all flowers with

undeveloped stamens); and (c) PMS (individuals with variable numbers of aborted stamens among flowers). To account for the presence or complete absence of developed and functional anthers, we estimated the proportion of pollen-bearing individuals as follows: (H + PMS)/(H + PMS + F).

### Partial male sterility, male and female fertility

In generation $F_2$, we assessed the proportion of PMS individuals in each family and pollination treatment. To obtain a more quantitative measure of the degree of partial male sterility, we estimated the mean number of developed stamens per flower as the total number of developed stamens per plant divided by the total number of flowers per plant in PMS individuals. We also computed this measure as the mean number of developed stamens per flower for all pollen-bearing individuals (H + PMS). This measure combines the proportion of PMS plants and their degree of male sterility, leading to an overall estimate of partial male sterility.

We assessed the effect of inbreeding on male fertility through estimates of the number of pollen grains per stamen and pollen viability in generation $F_2$. In each family, we sampled two stamens (one for the number of pollen grains per stamen and one for pollen viability) from two flowers per plant and two plants per level of inbreeding ($F$). We then followed the methods outlined in Atlan *et al.* (1992) and Gigord *et al.* (1999) to assess pollen quantity and quality. To estimate pollen viability, we placed stamens in a solution of 20% fuchsine, 40% acetic acid and 40% water immediately after harvest (Atlan *et al.*, 1992). Just before counting, a drop of aniline blue solution (0.1% aniline blue in phosphate buffer ($HK_2PO_4$, $H_2KPO_4$ in $H_2O$), pH 7.8) was added. Thus, viable pollen grains appeared dark blue while smaller empty grains appeared to be light blue. We used microscopy to estimate the proportion of stained pollen grains per stamen using samples of approx. 300 pollen grains (range: 94–934).

In generation $F_2$, we measured female fertility in a second flowering period, some months after the measures of male fertility. To assess the effect of inbreeding on female fertility, we counted the number of ovules per fruit in generation $F_2$. To facilitate the observation of ovules, we cross-pollinated CS and X hermaphrodites issued, respectively, from self- ($F \geq 0.5$) and cross-pollinations ($F \geq 0$). We allowed the fruits to mature for a week and then dissected them to count the number of ovules per fruit. We screened two to four hermaphrodites for each pollination treatment (CS vs X).

### Inbreeding depression

We estimated inbreeding depression $\delta$ 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 (Ågren & Schemske, 1993). This estimate of inbreeding depression varies between −1 and 1, where −1 represents a fitness of zero for outcrossed plants and 1 a fitness of zero for selfed plants. Ågren & Schemske (1993) estimated similar results to the estimation of inbreeding depression using the classical equation: $(w_o - w_s)/w_o$. We computed an inbreeding depression coefficient $\delta_F$ for each family and its mean $\bar{\delta}_F$ (family mean inbreeding depression) for each population using the following formula (Johnston & Schoen, 1994):

$$\bar{\delta}_F = \frac{1}{n} \sum \frac{w_{oi} - w_{oi}}{w_{maxi}}$$

($n$, number of families per population). Since we did not find significant fitness differences between $F \geq 0.5$ and $F \geq 0.75$, we pooled inbred plants together.

Population inbreeding depression ($\delta_P$) is a single measure of inbreeding depression per population using the mean fitness of selfed and outcrossed progeny across all families. This measure of inbreeding depression ($\delta_P$), is generally considered as a better estimate of inbreeding depression than mean family inbreeding depression ($\bar{\delta}_F$) because large differences between pollination treatments among families is more likely to bias $\bar{\delta}_F$ than $\delta_P$ (Johnston & Schoen, 1994). However, population inbreeding depression ($\delta_P$) was always within the confidence interval of mean family inbreeding depression ($\bar{\delta}_F$) for any fitness components. Thus, we subsequently only considered mean family inbreeding depression ($\bar{\delta}_F$). Moreover, family inbreeding depression estimates ($\delta_F$) allowed us to test for correlations among traits, (a) between differences in the proportion of hermaphrodites between the two generations, and (b) between reproductive traits and differences in the proportion of pollen-bearing individuals between inbred and outbred progeny.

### Statistical analysis

We used a Wilcoxon signed rank test to detect progeny sex ratio differences between pollination treatments within families and a Wilcoxon rank sum test to detect differences in female fertility (i.e. mean number of ovules per flower) in generations $F_1$ and $F_2$, respectively.

In generation $F_2$, we analyzed the proportion of pollen-bearing individuals within progenies, mean number of developed stamens per flower, number of pollen grains per stamen and pollen viability using two analyses of variance (ANOVA) models. We used a repeated-measure ANOVA to compare levels of inbreeding ($\alpha$) within families ($A$): $Y_{ijk} = \mu + A_i + \alpha_j + (\alpha A)_{ij} + \varepsilon_{ijk}$ (model 1). Family ($A$) was a random effect and level of inbreeding ($\alpha$) was a fixed effect repeated within family. We used a second mixed ANOVA with nested effects (model 2: $Y_{ijkl} = \mu + B_i + A_{ij} + \alpha_k + (\alpha B)_{ik} + (\alpha A)_{ijk} + \varepsilon_{ijkl}$) to test for the effect of population ($B$), family ($A$) and interactions with level of inbreeding ($\alpha$). We considered level of inbreeding as a fixed effect, population as random, and family as a random effect nested within population. Because transformation of the data did not meet the assumptions of

 

the ANOVA, we performed randomization tests (1000 permutations) on mean squares (Manly, 1997). If < 5% of permutations gave a larger mean square than that obtained with the observed data, the tested effect was considered significant. We conducted randomization as follows: we tested levels of inbreeding by randomizing levels of inbreeding within families (model 1). If significant, we performed the following planned comparisons: outbred ($F \geq 0$) vs inbred ($F \geq 0.5$ and $F \geq 0.75$) and between inbred plants ($F \geq 0.5$ vs $F \geq 0.75$). For the population effect, we permuted families among populations. For family effect, we randomized individuals among families within level of inbreeding for each population. We tested the interaction family × level of inbreeding as the permutation of data within populations and, finally we tested population × level of inbreeding interaction as the randomization of whole families between populations combined with the permutation of inbreeding levels within families. We performed all analyses using the statistical package R (version 2.2.1; R Development Core Team 2005).

## Results

### Progeny sex ratio

Inbreeding significantly affected the proportion of hermaphrodites in the offspring. Selfed progenies contained 18% more females than outcrossed progenies in generation $F_1$ (Wilcoxon signed rank test, d.f. = 53, $P < 0.001$). Of 54 maternal families, 12 produced only hermaphrodites and 13 had slightly fewer hermaphrodites in the progeny of cross-fertilized than in that of self-fertilized hermaphrodites. Populations were significantly different from each other, as the difference in the proportion of hermaphrodites between selfed and outcrossed progenies were 33, 9 and 13% for MO, VF and DJ, respectively (Fig. 1a; $F_{2,452} = 3.07$, $P < 0.001$). In generation $F_2$, the proportion of pollen-bearing individuals in the progeny decreased by 15% on average from cross- ($F \geq 0$) to self-fertilized progeny ($F \geq 0.5$; Table 1, Fig. 1b). Although not statistically significant, there was a further 13% decrease in the proportion of pollen-bearing individuals between offspring of one ($F \geq 0.5$) and two generations ($F \geq 0.75$) of self-pollinations (Fig. 1b).

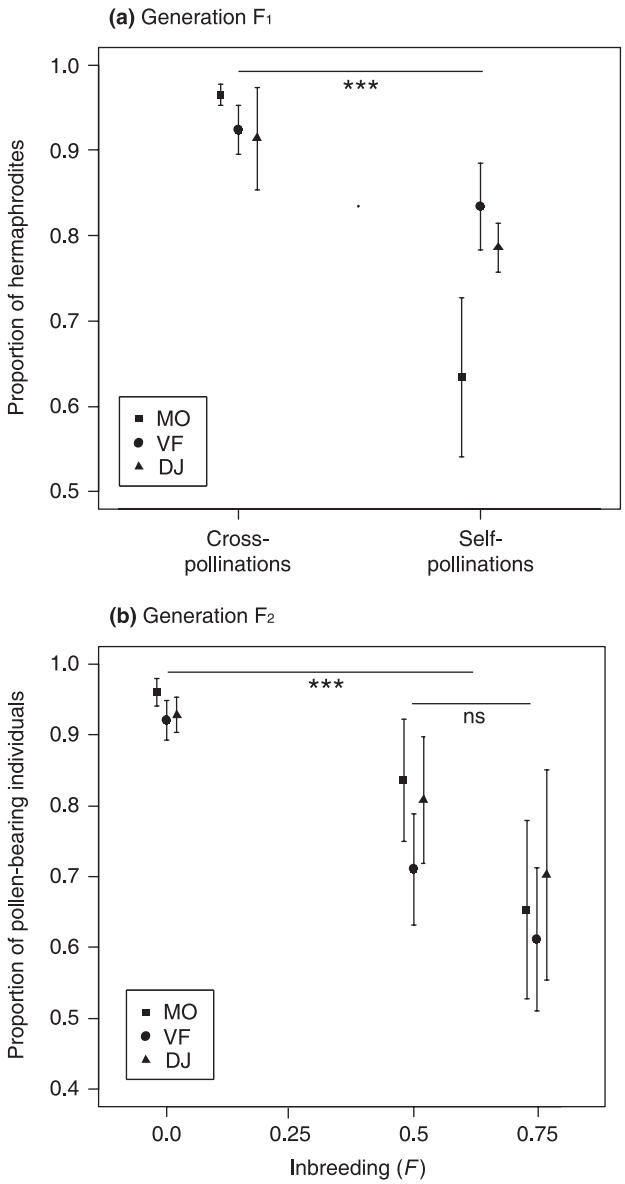


**Fig. 1** (a) Proportion of hermaphrodites (mean ± SE) for selfed and outcrossed progeny in generation $F_1$; (b) proportion of pollen-bearing individuals after two generations of self- and cross-pollinations, resulting in three levels of inbreeding (*F*) (0, 0.5 and 0.75) in three different *Silene vulgaris* populations: Mont d'Or (MO), Dent de Jaman (DJ) and Val Ferret (VF). Standard errors (SE) are estimated from the distribution of family means.

### Partial male sterility, male and female fertility

Partially male-sterile progeny was more common in self- than in cross-pollinations. The mean (± SE) proportion of partial male steriles (PMS) was 24.6 (± 2.9), 39.3 (± 3.9) and 50.4 (± 5.8) for $F \geq 0$, $F \geq 0.5$ and $F \geq 0.75$, respectively. Within PMS individuals the degree of male sterility (i.e. proportion of undeveloped stamens) increased with inbreeding (13 and 8% between $F \geq 0$ and $F \geq 0.5$ and between $F \geq 0.5$ and $F \geq 0.75$, respectively). Considering all pollen-bearing individuals (H + PMS), the proportion of developed stamens per flower decreased significantly by 10% between outcrossed progeny ($F \geq 0$) and one generation of self-fertilized progeny ($F \geq 0.5$) (Table 1, Fig. 2a). There was an 11% further decrease for offspring issued from two generations of self-pollination; however, this difference was not significant (Fig. 2a). Inbreeding depression ($\bar{\delta}_F \pm$ SE) for mean number of developed stamens per flower on pollen-bearing individuals was 0.18 ± 0.04.

The number of pollen grains per stamen was significantly different among families but was not affected by the level of inbreeding (Table 1). By contrast, pollen viability was significantly affected by the level of inbreeding (*F*). Pollen viability

**Table 1** Analyses of variance for the proportion of pollen-bearing individuals, mean number of developed stamens per flower, number of pollen grains per stamen and pollen viability after two consecutive generations of self- and cross-pollinations in *Silene vulgaris*

| | Proportion of pollen-bearing individuals | | | Mean number of developed stamens per flower | | | No. of pollen grains per stamen | | | Pollen viability | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Source of variation | d.f. | *MS* | *P* | d.f. | *MS* | *P* | d.f. | *MS* | *P* | d.f. | *MS* | *P* |
| Population | 2 | 0.06 | 0.444 | 2 | 0.89 | 0.689 | 2 | 285 919 | 0.628 | 2 | 0.08 | 0.138 |
| Levels of inbreeding (*F*) | 2 | 0.97 | 0.001*** | 2 | 49.94 | 0.001*** | 2 | 324 155 | 0.158 | 2 | 1.42 | 0.001*** |
| Family (population) | 27 | 0.07 | 0.084 | 27 | 2.12 | 0.216 | 27 | 413 839 | 0.003** | 27 | 0.03 | 1.000 |
| Levels of inbreeding (*F*) × population | 4 | 0.02 | 0.479 | 4 | 2.21 | 0.694 | 4 | 174 488 | 0.631 | 4 | 0.04 | 0.160 |
| Levels of inbreeding (*F*) × family (population) | 44 | 0.10 | 0.023* | 44 | 3.00 | 0.250 | 42 | 73 586 | 1.000 | 42 | 0.04 | 0.586 |
| Error | 117 | 0.02 | | 115 | 1.26 | | 115 | 157 508 | | 109 | 0.03 | |

Population and levels of inbreeding (*F*) are random and fixed effects, respectively; family, nested within population is random.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

decreased by 27% between cross-fertilized progeny ($F \geq 0$) and one generation of self-fertilized progeny ($F \geq 0.5$; Table 1, Fig. 2b). No significant difference in pollen viability could be detected between inbred offspring after one ($F \geq 0.5$) and two ($F \geq 0.75$) generations of self-fertilization (Fig. 2b). Averaged over the three populations, inbreeding depression ($\bar{\delta}_F \pm$ SE) for pollen viability was $0.31 \pm 0.04$.

The number of ovules per fruit in the progeny after one generation of self-pollination was significantly lower than the number of ovules produced by outcrossed progeny (one-sided Wilcoxon rank sum test, d.f. = 34, $P < 0.05$; Fig. 3). Averaged over the three populations, inbreeding depression ($\bar{\delta}_F \pm$ SE) for the number of ovules per fruit was $0.11 \pm 0.10$.

### Among traits correlations

Family inbreeding depression estimates ($\delta_F$) allowed us to test for genetic correlations among traits. There was a positive correlation between inbreeding depression for mean number of developed stamens per flower and the difference in the proportion of pollen-bearing plants between cross- and self-fertilized progeny (Fig. 4a; Spearman, d.f. = 27, $r = 0.7$, $P < 0.001$). The proportion of pollen-bearing individuals in the progeny of cross-fertilized hermaphrodites was between 0.85 and 1 in 26 out of 28 families (Fig. 1b) and the proportion of viable stamens per flower was equal or higher than 90% in 25 out of 28 families (Fig. 2a). Removing these few families with 'unusual' values for the proportion of pollen-bearing individuals in outbred progeny (i.e. lowest data point in Fig. 4a) did not change the results of the correlation. Thus, the correlation between inbreeding depression for mean number of developed stamens per flower and the difference in the proportion of pollen-bearing plants between cross- and self-fertilized progeny (Fig. 4a) is mainly the result of the correlation between the mean number of developed stamens per flower and the proportion of pollen-bearing individuals in the progeny of self-fertilization (Pearson, d.f. = 27; $r = 0.81$; $P < 0.001$).

We also detected a positive correlation between inbreeding depression for mean number of developed stamens per flower and inbreeding depression for pollen viability (Fig. 4b; Pearson, d.f. = 26, $r = 0.41$, $P < 0.05$). However, this correlation was no longer significant (Pearson, d.f. = 24, $r = 0.08$, $P = 0.7$) when removing the two families with the largest magnitudes of inbreeding depression for both number of developed stamens per flower and pollen viability (Fig. 4b).

## Discussion

We demonstrated that self- vs cross-fertilization of hermaphrodites in the gynodioecious *S. vulgaris* had three major effects: (i) there were more females in inbred progeny compared with outbred progeny; (ii) the proportion of PMS individuals as well as the degree of partial male sterility increased with levels of inbreeding; and (iii) both female and male fertility decreased with inbreeding. Furthermore, we showed that inbreeding depression for male reproductive traits co-vary with the decrease in the proportion of pollen-bearing plants between cross- and self-fertilized progenies. Even under intense inbreeding, the femaleness resulting from inbreeding is likely to prevent purging. Thus, the effect of inbreeding on sex ratio and the large genetic load associated to male fertility should maintain the conditions favoring females in small populations.

### Progeny sex ratio

Females should be present in the progeny of self-fertilized hermaphrodites if restorer genes are dominant and polymorphic. Relative to cross-fertilized progenies, there were 18 and 15% increases in the proportion of females in the progeny of self-fertilized hermaphrodites in generations $F_1$ and $F_2$, respectively (Fig. 1). Our results are in accordance with previous studies conducted over a single generation in North American populations of *S. vulgaris* (Emery & McCauley, 2002; Bailey

 

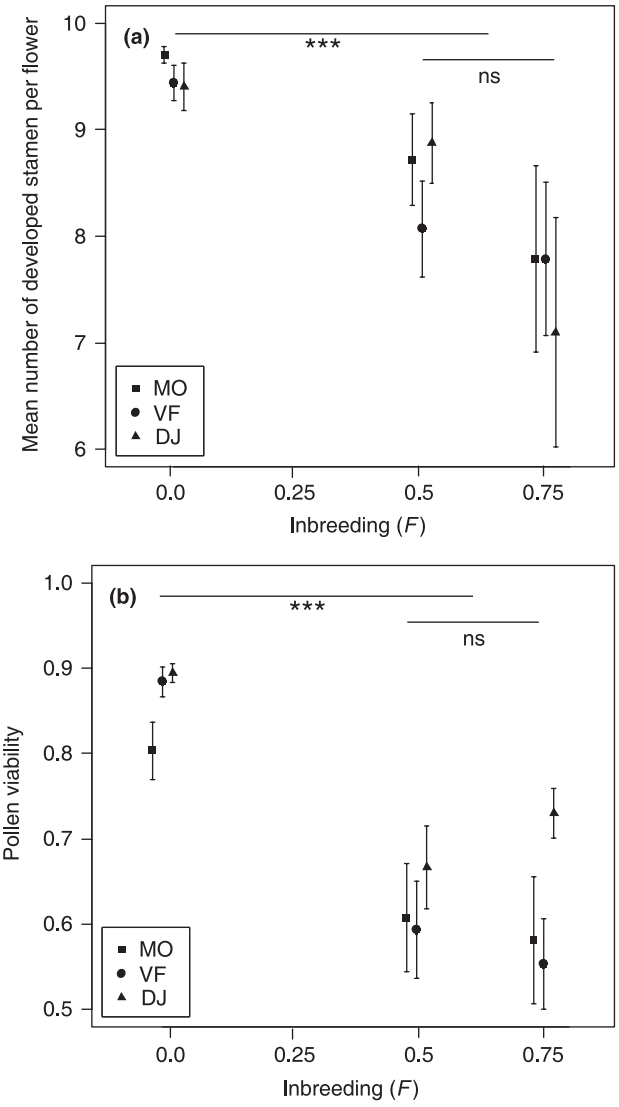


**Fig. 2** (a) Mean number of viable stamens per flower (mean ± SE); (b) pollen viability (mean ± SE), for all nonfemale plants after two generations of self- and cross-pollinations resulting in three inbreeding levels (*F*) (0, 0.5 and 0.75) in three different *Silene vulgaris* populations: Mont d'Or (MO), Dent de Jaman (DJ) and Val Ferret (VF). Standard errors (SE) are estimated from the distribution of family means.

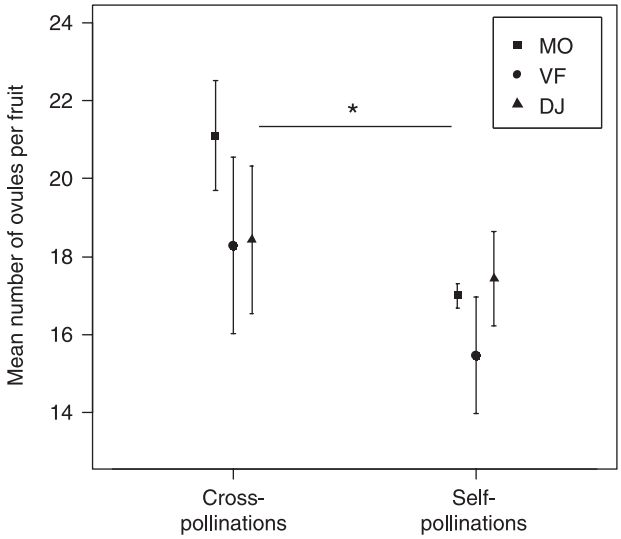


**Fig. 3** Mean number of ovules per fruit (mean ± SE) of the progeny of self- ($F \geq 0.5$) and cross-pollinations ($F \geq 0$) in generation $F_2$ in three different *Silene vulgaris* populations: Mont d'Or (MO), Dent de Jaman (DJ) and Val Ferret (VF). Standard errors (SE) are estimated from the distribution of family means.

& McCauley, 2005). These authors found between 20 and 30% fewer hermaphrodites in the progeny of selfed compared with outcrossed hermaphrodites. Thus, the female bias following self-fertilization was slightly larger in the species' introduced range than in its native range. However, this difference was not significant (Wilcoxon rank sum test, d.f. = 7, $P = 0.26$) and a larger population survey is needed to understand better the mechanisms behind sex-ratio distortion in *S. vulgaris*. As in the studies of Emery & McCauley (2002) and Bailey & McCauley (2005), our results show that the intensity of the female bias following self-fertilization was larger than expected if caused by a single locus with one dominant nuclear restorer. Differential survival to flowering between females and hermaphrodites of inbred progenies could account for this female bias; however, no gender-specific markers are available to investigate this hypothesis. Furthermore, sex determination in *S. vulgaris* is known to be complex and to involve multiple genes (Charlesworth & Laporte, 1998; Taylor *et al.*, 2001). Thus, the excess of females in the progeny of self-compared with outcrossed hermaphrodites detected in different *S. vulgaris* populations is likely to come from multiple dominant restorers interacting epistatically in both the native and the introduced range of the species.

## Partial male sterility, male and female fertility

Partial male sterility represents a continuum between complete male-sterile and hermaphroditic individuals and is known to have some genetic basis (Koelewijn & Van Damme, 1995b; Widén & Widén, 1999). The degree of male sterility can also be a consequence of phenotypic plasticity since it is influenced by environmental conditions (Koelewijn & Van Damme, 1996; Widén & Widén, 1999). For example, PMS individuals are more female-like with increasing temperatures in *Plantago coronopus* (Koelewijn & Van Damme, 1996). In our study, partial male sterility was more frequent and pronounced with increasing levels of inbreeding (Fig. 2a). Thus, our data support a genetic basis for partial male sterility in *S. vulgaris*. Two hypotheses could explain this pattern: (a) heteroplasmy, the occurrence of genetically different mitochondria bearing different CMS factors within individuals (Städler & Delph, 2002; McCauley *et al.*, 2005); (b) incomplete restoration of male fertility (Koelewijn & Van Damme, 1995b; Gigord

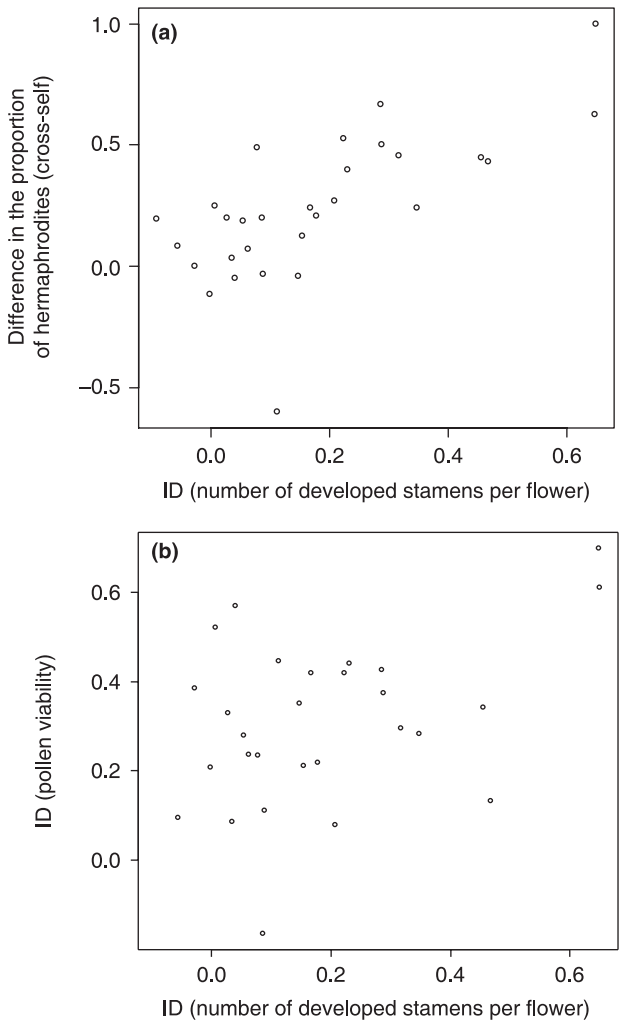


**Fig. 4** Correlations between (a) inbreeding depression (ID) for number of viable stamens per flower and the difference in the proportion of hermaphrodites between cross- and self-treatments in *Silene vulgaris* (Spearman, d.f. = 27, $P < 0.001$, $r = 0.7$); and (b) inbreeding depression for number of viable stamens per flower and inbreeding depression for pollen viability in *S. vulgaris* (Pearson, d.f. = 26, $P < 0.05$, $r = 0.41$).

*et al.*, 1999). The first hypothesis can be ruled out as partial male sterility caused by heteroplasmy should not depend on the level of inbreeding.

From their experiments, Koelewijn & Van Damme (1995b, 1996) concluded that the more nuclear genes involved in restoration of male fertility, the larger the probability of finding partially male-sterile plants. In this study, we detected an increase in partial male sterility with inbreeding. The probability of being homozygous (rr) for the recessive (i.e. non-restorer) alleles at restorer loci increases with inbreeding and might explain the prevalence of PMS individuals in inbred progenies. Moreover, we found a positive correlation between inbreeding depression for the proportion of developed stamens per flower and the difference in the proportion of pollen-bearing individuals between self- and cross-fertilized progeny (Fig. 4a). This correlation could come from the inbreeding history of hermaphroditic parents obtained from the seeds collected in natural populations. For instance, more outbred individuals than inbred individuals might be more likely to be heterozygous for restorers. These families would have large differences in sex ratio and large inbreeding depression for male fertility, while families derived from inbred hermaphrodites would have low values for both types of traits. However, when removing the effect of inbreeding history and assessing a correlation using only the selfed and outcrossed progeny from C-hermaphrodites there was still a positive correlation (Spearman, d.f. = 26, $r = 0.51$, $P < 0.01$). Therefore this correlation is more likely to be due either to linkage disequilibrium between restorer loci and genes responsible for stamen development (bearing deleterious alleles) or pleiotropic effects of restorers affecting both sex ratio and the development of stamens. Whether caused by the action of restorers themselves or by genes closely linked carrying genetic load, incomplete restoration of male fertility is likely to account for the increased frequency of partial male sterility in inbred progenies of *S. vulgaris*.

Inbreeding also affected more quantitative measures of male fertility: pollen viability of inbred progenies was lower than that of outbred progenies, resulting in an inbreeding depression of 0.31 (Fig. 2b). Even though most plants are hermaphrodites and contribute, on average, half their genes through the male function, most studies have overlooked the effect of inbreeding on pollen production and viability (see Charlesworth & Charlesworth, 1987; Husband & Schemske, 1996). However, inbreeding depression for pollen viability ranges from 0.1 (Mayer *et al.*, 1996) in *Collinsia heterophylla* to 0.30 (Carr & Dudash, 1997; Willis, 1999a) in *Mimulus guttatus*. Pollen competition experiments, which might be related to pollen viability, showed that pollen from inbred plants sired fewer seeds than pollen from outcrossed plants (Jóhannsson *et al.*, 1998; Melser *et al.*, 1999). Furthermore, comparisons of magnitudes of inbreeding depression for male and female functions showed inconsistent results. Chang & Rausher (1999) found larger inbreeding depression for the female than for the male function, whereas Carr & Dudash (1997) found that inbreeding affected the male function much more than the female function. Thus, the magnitude of inbreeding depression for male fertility cannot be inferred from measures on the female function. Our data showed that the magnitude of inbreeding depression for the mean number of ovules produced per flower ($\delta = 0.11$) was lower than that for pollen viability ($\delta = 0.31$). This difference in the magnitude of inbreeding depression between male and female fertility could be partly related to the way we assessed female fertility. Since male and female fertility were assessed in the first and second flowering periods, respectively, mortality between these two flowering periods may have underestimated inbreeding depression for female fertility. Indeed, selfed

 

progenies died between the two flowering periods in 30% of families. An experiment assessing female and male fertility simultaneously on the same set of plants would allow this possible methodological bias to be ruled out.

The studies of Gigord *et al.* (1999) and Koelewijn (2003) found correlations between the proportion of hermaphrodites within families and investment into ovule and pollen production of hermaphrodites, suggesting direct or indirect effects of restorer genes in resource allocation to reproductive functions. We explored potential correlations between sex allocation patterns and differences in the proportion of hermaphrodites between inbred and outbred offspring. Inbreeding depression for the number of ovules per flower correlated neither with sex ratio differences nor with inbreeding depression for male fertility (i.e. proportion of developed stamens per flower or pollen viability). On the other hand, pollen viability correlated positively with inbreeding depression for the proportion of developed stamens per flower (Fig. 4b). Although indirectly linked to sex ratio variation, pollen viability may partly result from the expression of restorers or loci linked to restorers. Thus, restorer genes (or a complex of genes associated to restorers) are likely to affect sex allocation but only through the male function by direct or indirect effects on the proportion of developed stamens and pollen viability.

### Inbreeding, sex-ratio adjustment and the maintenance of gynodioecy

The magnitude of late-acting inbreeding depression (i.e. at reproductive stages) is generally large, regardless of the mating system (Husband & Schemske, 1996; Sakai *et al.*, 1997; Bailey & McCauley, 2006). However, the effects of inbreeding on late traits, in particular on components of male fertility, are often not assessed (but see Melser *et al.*, 1999; Willis, 1999a,b). Here, we show that inbreeding affected both male and female fertility. Unlike inbreeding depression for female fertility ($\delta = 0.11$), the magnitude of inbreeding depression for male fertility ($\delta$-values for proportion of developed stamen per flower and pollen viability were 0.18 and 0.31, respectively) was high and larger than for earlier traits (i.e. seed production) assessed in the same experiment (Glaettli & Goudet, 2006). Several authors argued that the effects of numerous mildly deleterious alleles (rather than a few lethal ones) could explain the high magnitudes of inbreeding depression observed in late life-cycle stages (Charlesworth *et al.*, 1991; Willis, 1999a,b). These minor alleles are difficult to purge and thus are likely to maintain high magnitudes of inbreeding depression in traits occurring late in the life cycle. If selfing is high during one generation, the decrease in the proportion of hermaphrodites in inbred progeny is likely to increase the frequency of outcrossing in the next generation. High rates of selfing could occur in a small, recently colonized population. This 'sex-ratio adjustment' could further decrease the efficiency of purging the genetic load.

The previous discussion is focused on proximal causes and consequences of selfing on sex ratio. But our data can also be interpreted in an evolutionary context, as it might be advantageous for a selfing hermaphrodite to invest more in its female function. Sex-ratio adjustments by the parents are commonplace in animals (reviewed in West *et al.*, 2005), and have recently received attention in the plant kingdom (Pannell, 2001; Lopez & Domínguez, 2003; De Jong & Klinkhamer, 2005). Sex-ratio adjustments can occur when related males compete for the same female (local mate competition; Hamilton, 1967) or when related females compete for resources (local resource competition). In plants, local mate competition occurs when related pollen grains compete for the same ovules (De Jong & Klinkhamer, 2005). The consequence of local mate competition is that an individual will bias the sex ratio of its offspring in favor of females if the offspring are likely to mate with each other. In an extreme case, where a single fertilized female is alone in a patch, it is adaptive if she produces just enough sons to fertilize all her daughters since the number of ovules is the limiting factor to maximize her fitness. If a dichogamous hermaphrodite colonizing a new patch could use selfing as a cue to its degree of isolation then it would be advantageous to produce more females in its progeny. In this metapopulation context, hermaphroditism and the ability to self is favored through reproductive assurance, while increased investment in the female function is favored through local mate competition. A similar argument has been used by Pannell (2001) to explain the evolution of androdioecy. However, another factor needs to be considered: a female-biased patch may experience a greater risk of extinction as a result of pollen limitation (McCauley & Brock, 1998; Taylor *et al.*, 1999; Vila & Garciá, 2006). Therefore, the strength of selection for an increased proportion of females in the progeny of selfed hermaphrodites will depend on: (a) the extent to which hermaphrodites are found in isolation; (b) the fitness of females resulting from self-fertilization (resulting from inbreeding and pollen limitation); and (c) the effects of biparental inbreeding on progeny sex ratio. Clearly, these ideas need to be investigated. However, as both inbreeding depression and conceivably local mate competition drive the sex ratio in self-fertilized hermaphrodites towards a female-biased sex allocation, one could argue that, in a metapopulation where local extinction and recolonization are frequent, the maintenance of females in gynodioecious plant populations might be facilitated.

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