

GENDER VARIATION AND INBREEDING DEPRESSION IN GYNODIOECIOUS-GYMONOECIOUS *SILENE* *NUTANS* (CARYOPHYLLACEAE)

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Gynodioecy involves the stable co-occurrence of females and hermaphrodites. Its maintenance theoretically depends on differences in female and male reproductive success among gender morphs. Although many gynodioecious species also include gynomonoeious individuals that carry a mixture of female and perfect flowers, little is known about the male and female fitness of this third morph. Here, we present the first study of the reproductive system of *Silene nutans*, including females, gynomonoeious plants, and hermaphrodites. By measuring 10 floral traits in controlled conditions, we showed that females bear smaller and lighter flowers than hermaphrodites, with female and perfect flowers of gynomonoeious plants being intermediates. By measuring pollen quantity and quality, we showed that gynomonoeious plants had a lower potential male fitness than hermaphrodites at the level of both flowers and individuals. In addition, gynomonoeious plants were shown to widely vary their proportion of female flowers (0.03–0.9) and their floral traits, suggesting a quantitative restoration of male fertility. Finally, controlled pollinations showed evidence for inbreeding depression ($\delta = 0.3$) in progeny of hermaphrodites and gynomonoeious individuals, affecting both pre- and postdispersal traits; this could provide a selective advantage for females.

Keywords: gynodioecy, gynomonoeious, sex polymorphism, pollen viability, inbreeding depression, floral traits.

Introduction

Gynodioecy, one of the most common sexual polymorphisms in plants (Richards 1997), involves the stable maintenance of male sterile plants that have lost one of their sexual functions with hermaphrodites that possess both; this polymorphism has puzzled evolutionary biologists for decades. Commonly, this mating system is due to the conflicting interactions of cytoplasmic and nuclear genomes, with male sterility genes in the mitochondria, the effect of which is counteracted by nuclear alleles that restore male fertility (Saumitou-Laprade et al. 1994). Basically, sex polymorphism remains in a population as soon as a nuclear-cytoplasmic polymorphism is maintained. Theoretical studies have indicated that frequency-dependent selection could maintain such polymorphism if (1) females produce, at least marginally, more (or better) seeds than hermaphrodites and (2) carriers of nuclear restorer genes pay a fitness cost relative to those that do not (Gouyon et al. 1991; Bailey et al. 2003; Dufay et al. 2007). Understanding how such sexual polymorphism can be maintained in a given plant species thus requires a careful comparison of reproductive success among sex morphs.

A better fitness of females compared with female fitness of hermaphrodites has been reported in many gynodioecious species, matching with theoretical predictions. According to the species, such “female compensation” can be expressed as a difference in fruit set (Asikainen and Mutikainen 2003), number

of seeds (Kohn 1989), seed quality (Delph and Mutikainen 2003), or offspring survival or growth (Chang 2006). Although it has been suggested that female compensation results from reallocating resources no longer used for pollen production to other (female) fitness parameters (Barr 2004), in many species, female compensation may partially result from an avoidance of inbreeding depression for female (obligatory outcrossed) progenies (Agren and Wilson 1991; Sakai et al. 1997; Ramsey et al. 2006). Investigations of variance of seed and fruit production between females and hermaphrodites, with attention to the possible role of inbreeding depression, is thus necessary for understanding gynodioecy. To this end, many studies carried out on insect-pollinated gynodioecious species have also included a comparison of floral traits among sexual phenotypes, since differences in attraction of pollinators could give rise to a higher seed set in one of the sex categories (Talavera et al. 1996; Ramsey and Vaughton 2002).

However, the study of female reproductive success within gynodioecious species is not sufficient, since male fitness is also expected to vary among gender morphs, for several reasons. First, the cost of restorer alleles, put forward by theoretical studies, is likely to affect male reproductive success (Gouyon et al. 1991; Dufay et al. 2007). Second, many gynodioecious species include a third intermediate morph either (1) carrying flowers with nondehiscent/less numerous anthers or producing lower quantity or quality of pollen (Koelewijn and Van Damme 1996; Poot 1997; Dufay et al. 2008) or (2) carrying a mixture of female and perfect flowers (gynomonoeious individuals, as frequently described in Caryophyllaceae; Shykoff 1992; Talavera et al. 1996; Maurice 1999;

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Guitian and Medrano 2000; Lopez-Villavicencio et al. 2005). Such intermediate morphs are sometimes thought to be the result of partial restoration of male sterility (Ehlers et al. 2005) but are rarely taken into account in experimental or theoretical studies (but see Koelewijn 1996; Collin and Shykoff 2003; Lafuma and Maurice 2006; Bailey and Delph 2007). Because the dynamics of gynodioecy also depend on the fertility of intermediates relative to females and hermaphrodites, measurements of their reproductive success through both male and female function are needed, with attention to the consequence of selfing and inbreeding depression.

We present the first study of the mating system of *Silene nutans*, which has been indicated in several studies to be gynodioecious-gynomonoecious (Desfeux et al. 1996; Jürgens et al. 1996, 2002) but for which no precise comparative data on females, gynomoecious plants, and hermaphrodites have been collected. We performed measurements of potential male and female fitness in plants grown in controlled conditions, and we address the following questions. (1) How does the proportion of female flowers vary among gynomoecious individuals? Does gender vary quantitatively from hermaphrodites to females in *S. nutans*? (2) How do floral traits vary with gender? In particular, do female flowers of female and gynomoecious plants differ for some floral traits, while perfect flowers of hermaphroditic and gynomoecious plants differ for others? (3) Do gynomoecious and hermaphroditic plants differ in their pollen production and pollen quality at the level of either the flower or the individual? (4) How does seed quality vary with gender? (5) Do selfed offspring suffer from inbreeding depression? If so, could the avoidance of such inbreeding depression provide a fitness advantage for females? Finally, does inbreeding depression vary between hermaphroditic and gynomoecious lineages?

Material and Methods

Study Species

Silene nutans (Caryophyllaceae) is a diploid, long-lived perennial rosette plant growing in dry, open grass communities

of hillsides. It has been described as gynomoecious-gynodioecious, with female, gynomoecious, and hermaphroditic individuals found in natural populations (Jürgens et al. 2002). Flowers are visited by a number of different insect species (Jürgens et al. 1996). Perfect flowers are protandrous, but self-pollination can occur by geitonogamy. The seeds are dispersed from an aperture at the top of the capsule by vibrations of the flower stalk.

Sex Ratio and Floral Traits

This study was carried out on a collection of *S. nutans* individuals from seven populations, four of these from Belgium and three from central France (table 1). During spring 2007, plants were placed in a greenhouse during their whole flowering period. Of these, 58 produced sufficient flowers for further study. Newly opened flowers on each of these plants were checked twice weekly so that at the end of the flowering period, each individual plant was assigned to one of these three sex categories: female (F), hermaphrodite (H), or gynomoecious (G). Additionally, a quantitative measure of gender was performed on plants from Belgian populations ($n = 44$) by recording the exact number of female and perfect flowers produced throughout the whole flowering season. This provided the proportion of female flowers for each individual plant (reaching 1 for females, 0 for hermaphrodites, and intermediate values for gynomoecious).

On each of the 58 test plants, a sample of floral buds was marked and then collected 3 d after flower opening. On females and hermaphrodites, two to three flowers were sampled; on gynomoecious plants, two to three flowers per sex category (female and perfect) were sampled for each individual plant. In this way, a total of 142 flowers were collected and measured for the following traits: flower total mass, calyx length and width, length and width of one randomly selected petal, stigma length, ovary length and width, gynoeium mass, and ovary mass.

Average floral trait values per individual plant were analyzed with a general linear model (proc GLM, SAS). For gynomoecious plants, two values were analyzed, one average

Table 1

List of Populations from Which Individual Plants Were Collected

Population	Latitude N	Longitude E	Sample size	Collected material	No. plants followed	Measures performed for this study
Central France:						
Queyras	44°46'	6°44'	65	Seeds	8	FT, CROSS
Dordogne	45°19'	0°35'	16	Seeds	2	FT, CROSS
Auvergne	44°43'	2°21'	43	Seeds	4	FT, CROSS
Belgian:						
Lefte	50°15'	4°54'	29	Rosette	11	FT, QS, POL, CROSS
Tienne	50°05'	4°40'	14	Rosette	4	FT, QS, POL
Olloye	50°04'	4°36'	30	Rosette	7	FT, QS, POL
Vireux	50°05'	4°43'	36	Rosette	22	FT, QS, POL, CROSS

Note. Data listed for each population are name and geographical coordinates, the number of individual plants either collected as seeds and grown in greenhouse or collected as rosettes and transplanted to the greenhouse, the number of individual plants that produced enough flowers for inclusion in the study, and the types of measurements performed on plants from each population: measurements of floral traits (FT), quantitative estimation of sex (QS), by recording the proportion of female flowers, measurements of pollen production (POL), and control pollination (CROSS).

value for female flowers and one for perfect flowers. Two explanatory variables were tested, population and sex, with sex being a combination of plant sex and flower sex (the sex factor thus had four levels: female flowers of female plants [FF], female flowers of gynomonoeious plants [FG], perfect flowers of gynomonoeious plants [PG], perfect flowers of hermaphroditic plants [PH]). Post hoc pairwise comparisons were performed with Tukey's tests. For GLM analyses, normality of residuals was checked (Kolmogorov-Smirnov: $P > 0.1$ for all analyses). The same analysis was run on plants from only the Belgian populations by testing for an effect of population and sex as a quantitative factor (i.e., the proportion of female flowers).

Pollen Quantity and Pollen Viability

Pollen production was analyzed on plants from the Belgian populations ($n = 42$; 11 gynomonoeious and 31 hermaphrodites). For each plant, two to three floral buds were chosen, and all anthers from each bud were collected and stored in ethanol at 95%. Ethanol was then evaporated and samples were placed at 56°C for 24–48 h to force anther dehiscence. One milliliter of distilled water was then added to each pollen sample and sonicated to separate pollen grains and remove them from the anthers. Tubes were then vortexed, and the number of pollen grains was estimated in 200 μL of suspension. A particle counter CASY model TT (Innovatis, Bielefeld) was used to estimate the number of pollen grains in a solution of 5 mL of pure water CASY ton for cell counter, in which the 200 μL of distilled water and pollen were diluted. Each sample was shaken to equally distribute pollen in the solution immediately before counting. The particle counter then sampled three volumes of 400 μL from the suspension and provided the result for the total 1200 μL analyzed. The number of detected particles was determined for 400 size classes ranging from 0.125 to 150 μm using the software CASY Excel 2.1. Prior observation had shown that nonviable pollen grains in *S. nutans* were of smaller size than viable pollen grains. These counts were then used to estimate both total pollen production and fraction of viable pollen grains. The total number of pollen grains was obtained from the values provided by the particle counter after correcting for the dilution ratio, that is, by multiplying all values by $5 \times 5200/1200$. The 5200/1200 factor allows estimation of the quantity of pollen grains in the 5-mL solution in which the particle counter sampled, and the 5 factor allows estimation of total pollen per flower, since only 200 μL over a total of 1 mL were used.

We analyzed the proportion of viable pollen grains, testing for two explanatory variables, population and sex (coded either as a qualitative variable [i.e., hermaphrodite vs. gynomonoeious individuals] or as a quantitative variable [i.e., the proportion of female flowers carried by each plant]), by using a logistic regression (binomial distribution, log link function, proc GENMOD, SAS) and correcting for overdispersion (dscale option, proc GENMOD, SAS). The average quantity of pollen grains per flower as well as an estimation of plant male fitness (defined as the product: number of viable pollen grains per flower \times number of perfect flowers carried by the plant) were analyzed with a general linear model (proc GLM, SAS).

Pollen viability was also directly estimated with an Alexander stain on a subsample of plants ($n = 30$) to assess the correlation between pollen grain size and viability. To cover the largest variance in pollen viability, we sampled both gynomonoeious plants ($n = 7$) and hermaphrodites ($n = 23$) from all four populations. On these plants, two additional freshly opened flowers were collected the same day as the floral buds used for particle counter analysis. Within 3 h of collection, pollen was removed from the flowers and placed on a glass slide. One drop of Alexander solution (10 mL 95% ethanol, 1 mL 1% malachite green in 95% ethanol, 5 g phenol, 5 mL 1% acid fuchsin in H_2O , 0.5 mL 1% orange G in H_2O , 2 mL glacial acetic acid, 25 mL glycerol, and 50 mL H_2O ; Alexander 1969), which stains pollen cytoplasm in purple and exine in green, was added to each pollen sample. A coverslip was used to mix and cover the pollen and Alexander mixture, after which the coverslip was sealed using clear nail varnish. Pollen samples were then examined under LM at $\times 100$ magnification. Two hundred pollen grains per sample were scored as either purple or green, and the viable proportion of pollen grains was calculated as the ratio of purple-stained pollen grains to the total number of pollen grains.

Crossing Design and Measure of Inbreeding Depression

On 42 individual plants from the collection (6 females, 5 gynomonoeious plants, and 31 hermaphrodites), six flowers were marked at the bud stage and enclosed in a mesh bag to avoid accidental pollination events. As soon as flowers opened, their stamens were cut; hand-pollinations were performed once stigmas became receptive. On gynomonoeious and hermaphroditic plants, three flowers were self-pollinated and three others were cross-pollinated; on female plants, all flowers were cross-pollinated. Self-pollinations were performed with pollen collected from other flowers of the same plant; cross-pollinations were made using a mixture of pollen from three hermaphrodites from the same population. Few gynomonoeious plants were included in this experiment, since the sexual phenotype could be assigned with certainty only at the end of the flowering season, once the crossing experiment had already been performed.

At fruit maturity, seeds were collected and counted. A sample of 100 seeds per mother plant and cross type (inbred vs. outcrossed) was constituted and weighted. Each group of 100 seeds was then randomly split into two lots of 50 seeds, from which the total mass was also measured. Each lot of 50 seeds was randomly assigned to group 1 or 2 (defining different growth conditions at seedling stage). Seeds were then sown in Petri dishes on Wattman paper; germination rate and mortality at early stage were monitored for each lot of 50 seeds. Average seed mass was analyzed with an ANOVA (proc GLM, SAS); rate of germination was analyzed using a logistic regression (binomial distribution, log link function, proc GENMOD, SAS), correcting for overdispersion (dscale option, proc GENMOD, SAS).

After 12 d, 10 seedlings per lot were randomly selected, transplanted into a soil mix (3/4 compost; 1/4 perlite), and placed at 20°C with daily moistening to minimize stress of transplantation. After 2 wk, seedlings were spread into two growth conditions and followed for growth and survival in the green-

house; for each mother plant and cross type, two lots of seedlings were followed, one for each growth condition. In condition 1, temperature was set between 21° and 25°C with daily watering. In condition 2, temperature mostly followed natural conditions, and daily temperature ranged between 15° and 25°C; watering occurred every 5 d only (allowing time for the soil to dry between two consecutive watering events). At 8 wk, seedlings were collected, and their dry mass was measured and analyzed with an ANOVA, testing for an effect of the mother plant, its gender, its population, growth conditions, and cross type (proc GLM, SAS).

Finally, for each lot of seeds (per mother plant, cross type, and growth conditions), a multiplicative measure of cumulative offspring quality was computed as proportion of germination \times proportion of seedling survival \times mean dry mass. For each growth condition, we thus calculated the relative performance of inbred versus outcrossed offspring as follows: $\delta = (W_O - W_I)/W_{\max}$, with W_O and W_I being the cumulative offspring quality of outcrossed and inbred offspring, respectively, and W_{\max} being the larger of the two first values.

Results

Sex Ratio and Comparison of Floral Traits among Sex Types

Among the 58 individual plants followed for their floral traits, 7 were purely females, 17 were gynomonoeious, and 34 were hermaphrodites. Among all floral traits, only flower mass, petal length, and petal width significantly depended on sex. All other variables depended either on population only or on neither of these factors (table 2). Flower mass, petal length, and petal width of hermaphrodites (PH) were significantly higher than for flowers carried by females (FF), with female and perfect flowers from gynomonoeious individuals being statistically intermediate (table 3).

To better understand the status of flowers carried by gynomonoeious plants, we then focused on the Belgian populations on which the exact number of flowers as well as the proportion of female flowers per plant had been monitored. Flower number varied from 7 to 48; it differed only marginally among populations ($F_{3,42} = 2.27$, $P = 0.09$) but did not differ among sex morphs ($F_{2,43} = 0.03$, $P > 0.1$). Among plants from Belgian populations, 14 were gynomonoeious, with the proportion of female flowers varying from 0.03 to 0.9 (mean proportion = 0.33, SD = 0.28; fig. 1). Using the proportion of female flowers per plant, we could thus test for an effect of quantitative estimate of gender on floral traits. For these analyses, because very few female flowers could be measured for their floral traits, we chose to reduce our data set to perfect flowers ($n = 41$, 14 gynomonoeious and 27 hermaphrodites). We found a significantly negative effect of the proportion of female flowers on calyx length, petal length, and petal width (table 4). Similar results hold when our analysis was focused on gynomonoeious plants: a significantly negative effect of the proportion of female flowers was found for calyx length ($F_{1,13} = 8.03$, $P = 0.017$) and petal length ($F_{1,13} = 11.76$, $P = 0.006$). These results suggest that perfect flowers carried by “female-biased” gynomonoeious plants tend to be smaller.

Table 2

ANOVA of Floral Traits on Flowers Carried by Female, Gynomonoeious, and Hermaphroditic Individual Plants

Variable and source of variation	df	MS	F	P
Flower mass:				
Population	6	.00031	1.89	.09
Sex	3	.00049	3.01	.038
Error	52	.00016		
Calyx length:				
Population	6	.6351	.70	.6495
Sex	3	1.0945	1.21	.3156
Error	52	.9051		
Calyx width:				
Population	6	.4549	4.43	.0011
Sex	3	.0718	.70	.5564
Error	52	.1026		
Petal length:				
Population	6	1.7962	1.50	.1954
Sex	3	18.1495	15.19	<.0001
Error	52	1.1946		
Petal width:				
Population	6	.2481	1.86	.1051
Sex	3	.7422	5.57	.0022
Error	52	.13324		
Stigma length:				
Population	6	19.0979	1.21	.3149
Sex	3	3.0796	.20	.8990
Error	52	15.7501		
Ovary length:				
Population	6	2.7534	12.33	<.0001
Sex	3	.3828	1.71	.1754
Error	52	.2233		
Ovary width:				
Population	6	.0671	1.10	.3764
Sex	3	.1056	1.73	.1730
Error	52	.0612		
Ovary mass:				
Population	6	.000016	1.78	.1215
Sex	3	.000003	.36	.7808
Error	52	.000009		
Gynoecium mass:				
Population	6	.000007	.59	.7370
Sex	3	.000004	.36	.7793
Error	52	.000012		

Note. Sex is a combination of flower sex and plant sex (with four levels because gynomonoeious plants carry both female and hermaphroditic flowers).

Pollen Quantity and Viability

Pollen grain size, as estimated by the particle counter, was highly variable both within and among individual plants. Two major size classes could be defined: from 30 to 40 μm (small pollen grains) and from 40 to 60 μm (large pollen grains). On 30 individual plants, both the proportion of large pollen grains, estimated with the particle counter, and the proportion of viable pollen grains, estimated with Alexander stain, were recorded. These two variables were positively correlated ($R^2 = 0.8$; fig. 2). Thus, we assumed that the proportion of large pollen grains was a reliable estimation of the proportion of viable pollen grains.

Table 3

Results of Multiple Pairwise Comparisons of Floral Traits among Sex Categories for Analyses That Found a Significant Sex Effect

Variable	FF	FG	PG	PH
Flower mass (g)	.0484 ^A	.0539 ^{AB}	.0613 ^{AB}	.0645 ^B
Petal length (cm)	7.10 ^A	9.19 ^B	10.14 ^{BC}	10.58 ^C
Petal width (cm)	1.397 ^A	1.530 ^{AB}	1.763 ^{AB}	1.916 ^B

Note. FF = female flowers of female plants; FG = female flowers of gynomonoeious plants; PG = perfect flowers of gynomonoeious plants; PH = perfect flowers of hermaphrodites. Numbers are average values (least squares means, PROC GLM) for each sex category. Letters indicate categories significantly different from one another (results of post hoc Tukey's test; $P < 0.05$).

In the 42 plants that were monitored for their pollen production, neither population nor plant sex had an effect on pollen quantity produced per flower (proc GLM, $P > 0.1$). However, the proportion of viable pollen grains did vary according to both population ($\chi^2_{4,36} = 14.24$, $P = 0.0066$) and plant sex (hermaphrodites vs. gynomonoeious individuals: $\chi^2_{1,36} = 6.28$, $P = 0.0122$), with hermaphroditic plants producing a higher proportion of viable pollen grains than gynomonoeious ones. Similarly, when coding plant sex as a quantitative variable (the proportion of female flowers carried by the plant), we found a significant effect of both population ($\chi^2_{4,36} = 11.76$, $P = 0.0192$) and sex ($\chi^2_{1,36} = 6.79$, $P = 0.0091$), with the proportion of female flowers being negatively correlated with the proportion of viable pollen grains at the flower level. Finally, we calculated an estimate of plant potential male fitness by multiplying the number of viable pollen grains produced per flower by the number of perfect flowers and found a marginally higher estimate of male fitness for hermaphrodites compared with gynomonoeious

plants ($25,082 \pm 20,086$ vs. $12,715 \pm 10,836$ viable pollen grains; proc GLM; $F_{1,36} = 3.68$, $P = 0.0629$).

Seed and Offspring Quality

Average seed mass was calculated for each lot of 100 seeds. On this data set, we tested for an effect of (1) population of the mother plant and (2) cross type (with five levels) that included both sex of the mother plant and the breeding treatment (self- vs. cross-pollination). We found an effect of population ($F_{5,68} = 16.47$, $P < 0.0001$) but no cross type effect ($P > 0.1$). On the contrary, the rate of germination did not depend on population (proc GENMOD: $\chi^2_{5,68} = 8.47$, $P > 0.1$) but did depend on cross type ($\chi^2_{4,68} = 11.41$, $P = 0.023$). Contrast analyses revealed that crossed seeds of hermaphrodites had a higher germination rate than selfed seeds of both gynomonoeious plants and hermaphrodites (proc GENMOD, $P < 0.05$). No other cross type effect was found to affect growth of offspring (proc GENMOD, $P > 0.1$). In particular, no difference was found between seeds produced by females and those produced by other sex morphs. Therefore, we reduced our data set to include only progenies produced by gynomonoeious plants and hermaphrodites and focused on the comparison between outcrossed and inbred offspring.

Focusing on gynomonoeious and hermaphroditic plants, paired t -tests were performed to compare average seed mass and germination rate between inbred and outcrossed seeds within each maternal offspring. This revealed a larger seed mass for outcrossed seeds compared with inbred seeds (0.45 vs. 0.41 g, respectively; $t = 2.47$, $P = 0.018$, $n = 36$). Seeds produced by cross-pollination also showed a higher germination rate (0.91 vs. 0.81 for cross and selfed, respectively; $t = 4.02$, $P = 0.0003$, $n = 36$). Germination rate was also analyzed with a logistic regression (without performing pairwise comparison within each maternal offspring): while neither

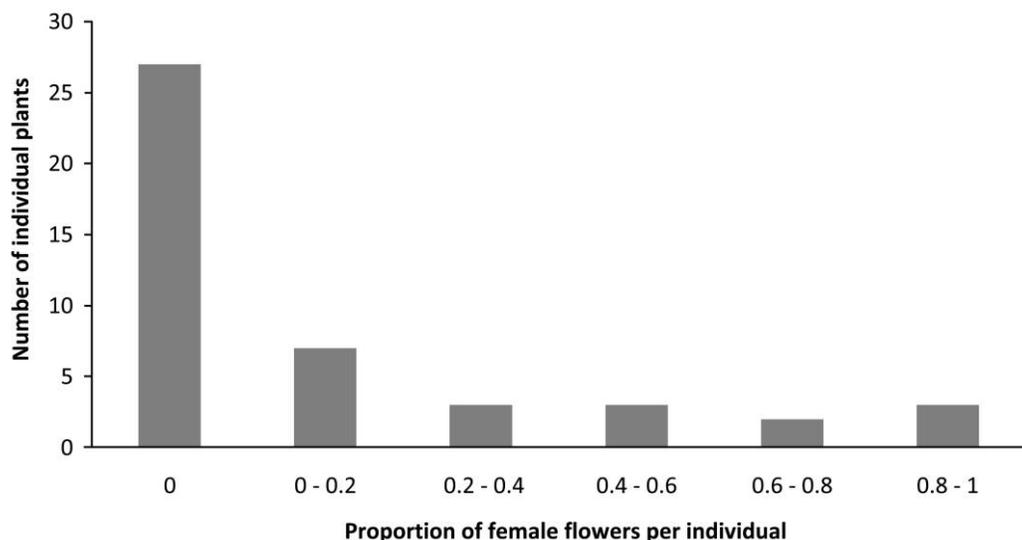


Fig. 1 Gender variation among individual plants from Belgian populations. Plants that carried no female flowers were purely hermaphroditic. Other individual plants were either gynomonoeious or females. Three individual plants had from 80% to 100% female flowers; they included two females (100% female flowers) and one gynomonoeious plant that carried 90% of female flowers.

Table 4

ANOVA of Floral Traits Measured on Hermaphroditic Flowers Carried by Both Hermaphrodites and Gynomonoecious Plants from Belgian Populations

Variable and source of variation	df	MS	F	P
Flower mass:				
Population	3	.00014	1.03	.3890
Female flowers (%)	1	.00013	.96	.3343
Error	36	.00013		
Calyx length:				
Population	3	.1889	.25	.8605
Female flowers (%)	1	4.0376	5.35	.0265
Error	36	.7546		
Calyx width:				
Population	3	.0988	.90	.4519
Female flowers (%)	1	.0197	.18	.6740
Error	36	.1101		
Petal length:				
Population	3	2.3260	2.10	.1172
Female flowers (%)	1	12.0039	10.84	.0022
Error	36	1.1071		
Petal width:				
Population	3	.0309	.33	.8027
Female flowers (%)	1	.3703	3.97	.0540
Error	36	.0933		
Stigma length:				
Population	3	10.2064	.57	.6145
Female flowers (%)	1	16.4119	.91	.3468
Error	36	18.0595		
Ovary length:				
Population	3	3.3116	12.87	<.0001
Female flowers (%)	1	.0433	.17	.6848
Error	36	.2582		
Ovary width:				
Population	3	.0840	1.32	.2821
Female flowers (%)	1	.0467	.74	.3968
Error	36	.0635		
Ovary mass:				
Population	3	.000029	3.35	.0297
Female flowers (%)	1	.000002	.19	.6633
Error	36	.000009		
Gynoecium mass:				
Population	3	.000015	1.22	.3161
Female flowers (%)	1	.000003	.24	.6285
Error	36	.000012		

Note. Both population and quantitative gender (proportion of female flowers) were tested on each trait.

population nor sex affected germination rate, both breeding treatment (self vs. cross) and average seed mass simultaneously affected this variable (proc GENMOD, cross type effect: $\chi^2_{1,69} = 7.92$, $P = 0.0049$; average seed mass: $\chi^2_{1,69} = 8.18$, $P = 0.0042$). When average seed mass was included in the model, breeding treatment still significantly affected germination rate, suggesting that differences in germination rate between cross types were not only because of a difference in seed mass.

Dry biomass of seedlings after 8 wk did not depend on the sex of their mother (gynomonoecious plant vs. hermaphrodite) but did depend on the mother's identity (nested in population \times maternal sex), population, breeding treatment,

and growth conditions (table 5). Overall, seedling from selfed seeds averaged 76% of the mass of the average for the outcrossed treatments, and seedlings that grew in environment 1 (warmer temperature and regular water supply) produced 25% more biomass than those in environment 2. No significant interaction was found between the main factors.

The relative performance of inbred versus outcrossed offspring δ , based on the cumulative offspring quality for each maternal plant, was 0.31 on average, with strong variation among families ($SD = 0.41$), and was significantly different from 0 ($t = 5.18$, $P < 0.0001$, $n = 48$). It did not depend on growth conditions, population, or gender of the mother plant (gynomonoecious individual vs. hermaphrodite: $P > 0.1$).

Discussion

To our knowledge, this is the first study of sex polymorphism in *Silene nutans*. *Silene nutans* had been described as gynodioecious in studies carrying out pollination biology within the *Silene* genus and was sometimes presented as gynodioecious-gynomonoecious (Desfeux et al. 1996; Jürgens et al. 1996, 2002). However, other studies that focused on maternal choice (Hauser and Siegmund 2000) and flowering phenology (Hauser and Weidema 2000) of *S. nutans* populations in Denmark and Sweden did not mention any gender variation within the species. When sex polymorphism is not the primary aim of the survey, many studies do not necessarily report the occurrence of females in populations and even less likely of gynomonoecious plants. Future studies should thus investigate whether sex polymorphism effectively varies across the species' range, as it has been observed in many other gynodioecious species (Thompson and Tarayre 2000; Asikainen and Mutikainen 2003; Nilsson and Agren 2006; Dufay et al. 2009). This work carried out on plants from Belgian and French populations revealed that a sex polymorphism occurred within both regions and that gynomonoecious individuals were always more frequent than females (29% vs. 12% in this study). These results are similar to those found in other *Silene* species or other so-called gynodioecious species belonging to the Caryophyllaceae family (Shykoff 1992; Maurice 1999; Guitian and Medrano 2000).

Comparison of Females and Hermaphrodites

As a result of low female frequency in populations, our data set included very few females. Several floral traits were nevertheless compared between females and hermaphrodites, revealing smaller petals and lower mass in flowers of female plants. This is similar to results found in many insect-pollinated gynodioecious species (Puterbaugh et al. 1997; Williams et al. 2000; Ramsey and Vaughton 2002; Caruso et al. 2003; Chang 2006), including several *Silene* species and other Caryophyllaceae (*Silene stockenii*: Talavera et al. 1996; *Gypsophila repens*: Lopez-Villavenciado et al. 2003; *Silene italica*: Lafuma and Maurice 2006). Petal size is thought to be an important trait for pollinator attraction and is consequently often considered as a typically "male trait" strongly selected for in hermaphrodites (or males in dioecious species) compared with females (Queller 1983). Thus, future studies should measure

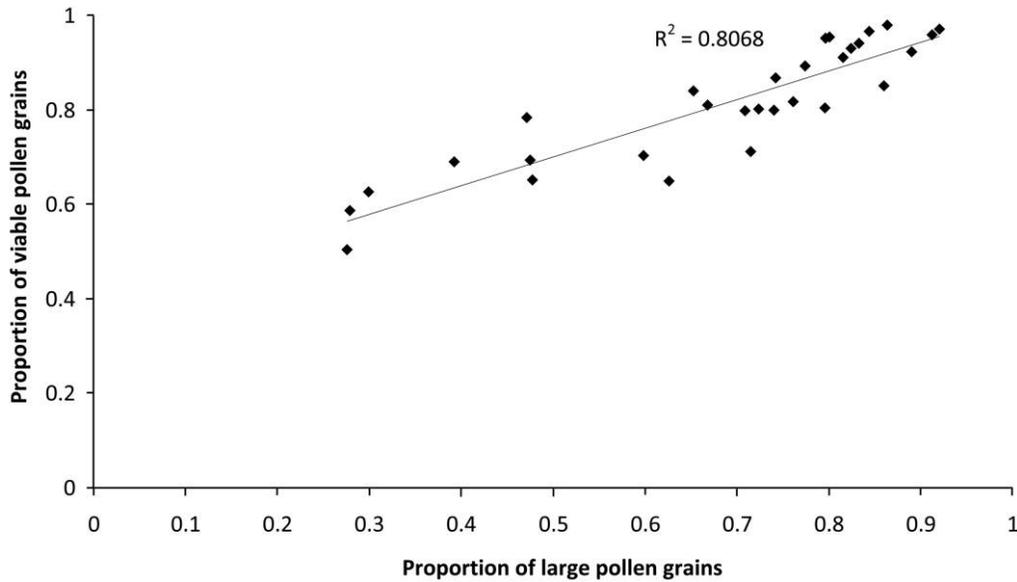


Fig. 2 Correlation between the proportion of large pollen grains, obtained with the particle counter (X-axis), and the proportion of viable pollen grains, from observation with Alexander stain (Y-axis). This analysis was carried out on 30 individual plants (comprising both hermaphrodites and gynomonoecious individuals), from which both measures were taken from flowers that opened at the beginning of the flowering season.

fruit set and seed set in natural populations of *S. nutans* in order to investigate whether these differences in flower size lead to a decrease in pollinator attraction and in seed set for female individuals.

Nonetheless, in many gynodioecious species, females have been found to compensate for the loss of their male reproductive function by increasing their female reproductive success commonly in terms of seed set, fruit set, or seed quality (Kohn 1989; Asikainen and Mutikainen 2003; Delph and Mutikainen 2003; Chang 2006). Such female advantage can result either from resource allocation from male to female function or from an avoidance of self-pollination and associated inbreeding depression (Agren and Wilson 1991; Sakai et al. 1997; Barr 2004; Ramsey et al. 2006). In *S. nutans*, although females produced smaller and lighter flowers and could subsequently benefit from higher resources to invest in

seed production, no such female advantage was detected in terms of seed mass, germination rate, or offspring quality. It is, however, difficult to interpret these results because of the reduced statistical power of our analyses due to the small number of included females. Future works will attempt to enlarge the sample size and investigate the possible differences in both seed number and seed quality between females and other sex morphs, with special attention on the effect of the breeding treatment. Because this study showed evidence for strong inbreeding depression, one should expect a female advantage to be found when comparing obligatory outcrossed female progenies and inbred offspring from hermaphrodites and gynomonoecious individuals. Moreover, because theory predicts sex ratio in populations to be correlated with the magnitude of female advantage (Bailey et al. 2003; Dufay et al. 2007), the overall low frequency of females in *S. nutans* could

Table 5

Results of ANOVA (PROC GLM) of Dry Mass on 8-wk-old Offspring Produced by Hermaphrodites and Gynomonoecious Plants in Two Different Growth Conditions

Source of variation	df	MS	F	P	Effect
Mother (population × gender)	25	.31	10.89	<.0001	
Growth conditions	1	1.40	49.60	<.0001	Condition 1 > condition 2
Breeding treatment	1	1.21	42.75	<.0001	Cross > self
Population	4	.24	1.08	.3858	
Maternal gender	1	.02	.10	.7542	
Population × maternal gender	1	.34	1.89	.1798	
Error	747	.02			

Note. Condition 1: temperature between 21° and 25°C with regular water supply. Condition 2: temperature between 15° and 25°C with water supply every 5 d only. Both the identity of the mother plant, nested in population × maternal gender, and population were coded as random factors. Interactions between main factors were not found significant.

actually be due to a low magnitude female advantage. If so, this would have reduced the probability of detection of any statistical difference between females and hermaphrodites, in particular on such a restricted data set.

Inbreeding Depression and Consequences for the Reproductive System

We found evidence for severe inbreeding depression in gynodioecious and hermaphroditic lineages in controlled conditions for both pre- and postdispersal traits. Selfing resulted in a decrease of both seed mass and seed germination when compared within each maternal progeny. While seed mass affected seed germination, it was not the only factor that explained the difference in germination between selfed and outcrossed seeds, suggesting that other mechanisms than seed provision are involved in the effect of the breeding treatment. Inbreeding depression was also found when measuring offspring vegetative growth in both optimal and more restrictive growth conditions. In both environments, outcrossed progeny reached larger vegetative size and dry mass than selfed progeny. Because one of the environmental conditions tested in this study was closer to natural conditions in terms of temperature and water supply, this suggests that such inbreeding depression should also be found in natural populations. Furthermore, although an effect of the environmental conditions was consistently shown on vegetative growth, we found no interaction between the environment and the breeding treatment, indicating that outcrossed progeny did not show a better resistance to stressful conditions compared with selfed progeny.

Overall, the value of inbreeding depression, based on the inbred/outbred differences in cumulative fitness for early stages of life cycle, was found to be quite severe (0.3), similar to results found in other gynodioecious species (Mutikainen and Delph 1998; Delph 2004; Chang 2007). Furthermore, the measurement of offspring dry mass prevented the observation of selfed and outcrossed progeny in the later steps of their life cycle. Hence, inbreeding depression in *S. nutans* may be stronger than estimated by this study, particularly if vegetative growth at later stages and flowering probability are also affected, as shown in other *Silene* species (Mutikainen and Delph 1998; Emery and McCauley 2002; Glaetli and Goudet 2006).

In self-compatible gynodioecious species, inbreeding depression is an important parameter to consider because female fitness of female plants should be increased compared with hermaphrodites, providing cytoplasmic male sterility genes with a selective advantage. To evaluate the role of inbreeding depression for sex polymorphism in *S. nutans*, future studies will have to measure the actual selfing rate in natural populations. Although the large floral display in *S. nutans* (up to more than 200 flowers; M. Dufay, personal observations) is expected to increase the probability of geitonogamy, the natural rate of self-pollination could be limited by the ability of maternal choice between self- and cross-pollen, as shown in the same species by Hauser and Siegismund (2000).

*Gynodioecious Plants in *Silene nutans**

Gynodioecious plants were found to be frequent in *S. nutans* and to form a heterogeneous category according to

the proportion of female flowers. These results are similar to results found for other gynodioecious-gynomonoecious *Silene* species (Shykoff 1992; Maurice 1999; Guitian and Medrano 2000). Overall, the few studies that have attempted to compare gynomoecious individuals with other sex morphs have found that they were intermediate between females and hermaphrodites either for the number of flowers (Poot 1997; Lafuma and Maurice 2006), seed set, or fruit set (Agren and Willson 1991; Lafuma and Maurice 2006) or for offspring quality (Delph and Mutikainen 2003). In *S. nutans*, we found female and perfect flowers of gynomoecious plants to be statistically intermediate for flower mass and petal size, compared with flowers of females and hermaphrodites. More interestingly perhaps, we found the gynomoecious category to be heterogeneous not only for their relative proportion of female and perfect flowers but also for their floral traits: gynomoecious plants that carried a high proportion of perfect flowers had perfect flowers that resemble more those of hermaphrodites, with larger petals and heavier flowers. To our knowledge, such correlation has not been investigated in other gynodioecious-gynomonoecious species. Because it stresses a high variance within the gynomoecious category, this could indeed partially explain why gynomoecious plants are often described as statistical intermediates for their floral traits or for plant fitness.

In this study, we also found a difference between gynomoecious plants and hermaphrodites for pollen production. Even without considering male fitness at the level of the individual, which was consistently found to be in favor of hermaphrodites that produce pollen from all flowers, we found pollen quality to be higher in hermaphrodites compared with gynomoecious plants. In *S. italica*, Lafuma and Maurice (2006) found similar results, although this difference was not found in all plant families. These results raise the question of both the conditions of maintenance of gynomoecious plants in populations and of the determination of their gender morph.

Several hypotheses have been proposed for the determination of the gynomoecious morph, including the effect of environmental factors (e.g., gynomoecious-gynodioecious *S. italica*: Maurice 1999; gynomoecious *Silene noctiflora*: Folke and Delph 1997), and partial restoration of male fertility (Koloewijn and Van Damme 1996). These two hypotheses are nonexclusive: if multiple nuclear genes are involved in the restoration of male fertility, sex is a quantitative trait, the expression of which may be affected by environmental factors (Koelewijn and Van Damme 1996). In this study, half of gynomoecious plants carried few female flowers (from 3% to 19%), which generally opened at the beginning of the flowering period; these plants resembled hermaphrodites in terms of both pollen quality and flower size. In the other half, the proportion of female flowers quantitatively varied from 30% to 90%, with this proportion being inversely correlated with flower size and pollen quality. These results suggest that restoration of male fertility could be quantitative and involve multiple nuclear loci. In some other gynodioecious species, pollen viability and/or anther development and dehiscence vary quantitatively among individual plants while being apparently constant within each plant (*Plantago coronopus*: Koelewijn and Van Damme 1996; *Thymus vulgaris*: Thompson et al. 2002; *Beta vulgaris*: Dufay et al. 2008). Ehlers et al.

(2005) suggested that such interindividual variation was the likely result of a polygenic restoration of male fertility. In *S. nutans*, such quantitative determination of restoration could affect both the development of anthers within some of the flowers (perfect vs. female) and the quality of pollen produced within perfect flowers in individuals that do not carry all restorer alleles at the different loci.

To date, only Bailey and Delph (2007) investigated the consequences of polygenic restoration of male fertility. However, because their study considered restoration as a threshold trait, it could not apply to species in which male fitness quantitatively varies among individual plants. Theoretical studies are thus needed to investigate whether gynomonoeious plants should be found at equilibrium in natural populations. Indeed, gynomonoeious plants could be a simple by-product of a quantitative determination of sex; during the phase of selection of restorer alleles, one expects to find genotypes carrying only a fraction of the restorer alleles. Gynomonoeious individuals should thus be found only during transitory phases, which are then replaced by fully restored hermaphrodites as long as restorer alleles increase in frequency. Alternatively, to explain the occurrence (and sometimes the large frequencies) of gynomonoeious plants in gynodioecious plant species, Desfeux (1996) postulated that gynomonoeicy could be a bet-hedging strategy. Under this hypothesis, gynomonoeious individuals would gain some advantage in female fitness (saving resources by producing less pollen than hermaphrodites) while being protected against strong pollen limitation. This is at least partially consistent with the findings of Davis and Delph (2005). Carrying out on gynomonoeious *S. noctiflora*, this study showed that perfect flowers were capable of autonomous selfing, providing reproductive assurance when pollination is low, whereas female flowers produced only outcrossed seeds that avoided the cost of inbreeding depression but depended on pollinator availability. Even if such bet-hedging advantage has not been found in gynomonoeious plants within gynodioecious species, one can imagine similar processes to occur.

The dynamics of sex ratios within populations as well as the occurrence of gynomonoeious plants at equilibrium

should strongly depend on male and female fitness components of these intermediates compared with females and hermaphrodites. In *S. nutans*, we found that gynomonoeious plants should experience a reduction in male fitness compared with hermaphrodites by producing less attractive flowers and lower pollen quantity and quality. On the other hand, no advantage in female fitness was found for gynomonoeious plants compared with hermaphrodites in terms of either seed quality or the magnitude of inbreeding depression. As mentioned, the consequences of inbreeding depression strongly depend on the value of selfing rates in natural populations. This holds to explain the maintenance of both females (thereby avoiding the severe inbreeding depression found in this study and consequently benefiting from fitness advantages compared with other sex morphs) and gynomonoeious plants as they compete with hermaphrodites. Whether hermaphroditic and gynomonoeious individuals experience the same rate of self-pollination should therefore be crucial to maintain the polymorphism. At this point, no clear predictions can be made; while one could have guessed that selfing through geitonogamy is less likely in gynomonoeious plants (that carry less perfect flowers), Collin and Shykoff (2003) found no such difference in *Dianthus sylvestris*. Much additional information is therefore needed to understand both the determination and the conditions of maintenance of intermediate sex morphs in both *S. nutans* and other gynodioecious species.

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