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# Sex-specific fitness variation in gynodioecious *Beta vulgaris* ssp. *maritima*: do empirical observations fit theoretical predictions?

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## Abstract

In gynodioecious species, in which hermaphroditic and female plants co-occur, the maintenance of sexual polymorphism relies on the genetic determination of sex and on the relative fitness of the different phenotypes. Flower production, components of male fitness (pollen quantity and pollen quality) and female fitness (fruit and seed set) were measured in gynodioecious *Beta vulgaris* spp. *maritima*, in which sex is determined by interactions between cytoplasmic male sterility (CMS) genes and nuclear restorers of male fertility. The results suggested that (i) female had a marginal advantage over hermaphrodites in terms of flower production only, (ii) restored CMS hermaphrodites (carrying both CMS genes and nuclear restorers) suffered a slight decrease in fruit production compared to non-CMS hermaphrodites and (iii) restored CMS hermaphrodites were poor pollen producers compared to non-CMS hermaphrodites, probably as a consequence of complex determination of restoration. These observations potentially have important consequences for the conditions of maintenance of sexual polymorphism in *B. vulgaris* and are discussed in the light of existing theory on evolutionary dynamics of gynodioecy.

## Introduction

Flowering plant species exhibit a large range of reproductive strategies. After hermaphroditism, gynodioecy is one of the most common breeding systems (Webb, 1999). In gynodioecious species, two sexual phenotypes coexist in natural populations: individuals can be classified as females or hermaphrodites, depending on their ability to produce pollen. Since females reproduce only through seeds, they should transmit most of their genes only half as frequently as hermaphrodites, which possess both sexual functions. The maintenance of females in gynodioecious species has intrigued evolutionary biologists for decades and numerous theoretical models have been developed (e.g. Charlesworth & Ganders, 1979; Frank, 1989; Gouyon *et al.*, 1991; Couvet *et al.*, 1998; Bailey *et al.*, 2003; Dufay *et al.*, 2007). All existing models share one common condition for the maintenance of gynodioecy: females must compensate for their gametic disadvantage relative to hermaphrodites *via* higher female reproductive fitness (Lewis, 1941; Lloyd, 1974). A number of empirical studies have confirmed that female plants outperform hermaphrodites in one or several aspects of female reproduction (e.g. Assouad *et al.*, 1978; Ashman, 1999; Graff, 1999; Barr, 2004; Olson *et al.*, 2006). This female advantage can result from two nonexclusive mechanisms: (i) females produce more seeds or seeds of better quality than hermaphrodites due to reallocation of resources that are not used to produce male gametes (e.g. Avila-Sakar & Domínguez, 2000) and/or (ii) females benefit from inbreeding avoidance and produce, on average, higher quality seeds than hermaphrodites that partly self-pollinate (e.g. Chang, 2007).

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The magnitude of female advantage (i.e. seed fitness of females relatively to hermaphrodites) needed to maintain females in natural populations critically depends on how sex is genetically determined. In the case of simple nuclear determination, females need to produce on average at least twice as many offspring through seeds as hermaphrodites (as a result of resource reallocation and/or avoidance of inbreeding depression) to compensate for their loss of pollen production (Lewis, 1941; Charlesworth & Charlesworth, 1978). However, in most gynodioecious species, sex determination involves cytonuclear interactions, leading to a more complex mode of inheritance (Saumitou-Laprade *et al.*, 1994; Delph *et al.*, 2007). Sexual phenotype then depends on interactions between cytoplasmic (maternally inherited) male sterility genes (CMS genes) and nuclear (biparentally inherited) male fertility restorers. To develop as a female, an individual must carry a nonrestored CMS gene. To develop as a hermaphrodite, an individual must either carry a CMS gene in combination with the matching restoration allele (restored CMS hermaphrodite), or carry a non-CMS cytoplasm. As in nuclear determination, females must have some fecundity advantage compared to hermaphrodites, but do not need to benefit from a two-fold advantage for gynodioecy to be maintained (e.g. Gouyon *et al.*, 1991; Dufay *et al.*, 2007). This is because the absence of male gamete production does not affect the cytoplasmic fitness of an individual and a moderate increase of seed quantity or quality suffice to confer a selective advantage to CMS genes (Cosmides & Tooby, 1981).

On the other hand, since nuclear genes are biparentally transmitted, the loss of pollen production directly reduces their transmission. Consequently, when CMS genes become frequent in a population, nuclear restorers of male fertility should be selected for. Theoretical models suggest that there must be some forces opposing to the fixation of restorer alleles to maintain cytonuclear polymorphism within populations. This can be achieved through two distinct processes. First, silent restorer alleles (i.e. restorers associated with another cytoplasm than the one they restore) are frequently thought to be associated with a cost acting on either male or female reproductive success. This cost prevents the fixation of restorer alleles and allows the maintenance of cytonuclear polymorphism (Charlesworth & Ganders, 1979; Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufay *et al.*, 2007). Alternatively, metapopulation dynamics, with recurrent extinctions and recolonizations of demes, have been shown to maintain cytonuclear polymorphism without requiring any cost of restoration, by preventing local populations from reaching equilibrium (Couvet *et al.*, 1998).

In addition to the female advantage and the cost of restoration, in the particular case of species containing nonsterilizing cytotypes, hermaphrodites that do carry a male-sterility mutation (i.e. restored CMS hermaphrodites) are expected to have a disadvantage in female fitness compared to those that carry a nonsterilizing cytotype (Dufay *et al.*, 2007). This cost of CMS is theoretically necessary to maintain non-CMS genes that would otherwise be eliminated because they are never associated with female advantage. Altogether, female advantage, cost of restoration and cost of CMS genes theoretically allow frequency-dependent selection to maintain cytonuclear polymorphism, often through large oscillations of sex ratios across time, with CMS genes being selected for when restorer alleles are rare and restorers being selected for when CMS genes are frequent (Dufay *et al.*, 2007).

Theoretical models generally assume that the restoration of male fertility is achieved through one allele (but see Frank, 1989; Bailey & Delph, 2007). However, empirical work suggests that genetic determination may be more complex, as many gynodioecious species also include intermediate sexual phenotypes, either (i) producing flowers with nondehiscent/less numerous anthers or producing lower quantity or quality of pollen (e.g. Koelewijn & van Damme, 1996; Poot, 1997; Dufay *et al.*, 2008) or (ii) carrying a mixture of female and hermaphroditic flowers (gynomonoecious individuals, e.g. Shykoff, 1992; Lopez-Villavicencio *et al.*, 2005; Dufay *et al.*, 2010). This intra-individual variation, which often translates into a quantitative variation of gender, has been proposed to be the result of a polygenic restoration of male fertility (Koelewijn & van Damme, 1996; Ehlers *et al.*, 2005). In case of polygenic determination of restoration, individuals carrying a CMS cytoplasm may rarely be fully restored in natural populations and male fitness may vary quantitatively among restored CMS hermaphrodites (e.g. Dufay *et al.*, 2008). This could slow down the selection of restorers and ultimately modify the conditions of maintenance of cytonuclear polymorphism, thereby favouring the maintenance of females in gynodioecious populations (Bailey & Delph, 2007).

Female advantage, cost of silent restorers, cost of CMS genes and incomplete restoration of male fertility are all important parameters that directly influence the reproductive output of individuals in gynodioecious populations. Knowing the relative male and female fitness of the different genotypes is thus a central issue when attempting to understand the maintenance of sexual polymorphism associated with cytonuclear gynodioecy. From this point of view, gynodioecious *Beta vulgaris* ssp. *maritima* (L.) Arcangeli is a relevant model for investigating sex-specific fitness variations. Indeed, the genetic basis of sex is well known: male sterility is associated with four particular mitochondrial types, called CMS *E*, *G*, *Svulg* and *H*. These sterilizing cytoplasms coexist with male-fertile cytoplasms and all these cytotypes can be identified with molecular markers (Cuguen *et al.*, 1994; Forcioli *et al.*, 1998;

Desplanque *et al.*, 2000; Fénart *et al.*, 2006). By coupling data on genotypes and sexual phenotypes, it is possible to directly identify females, non-CMS hermaphrodites and restored CMS hermaphrodites. These three sexual phenotypes potentially group together individuals with various nuclear restorer genotypes. On the one hand, CMS hermaphrodites are all expected to carry restorer alleles, but with potential inter-individual differences in their exact genotype in case of polygenic restoration. On the other hand, silent restorers – associated with another cytoplasm than the one they restore – may be present in some non-CMS hermaphrodites and may account for some of the variability among individuals for male function. This paper reports the results of an investigation designed to evaluate the reproductive differences between females and the two hermaphroditic types existing within *B. vulgaris* ssp. *maritima* populations. While a silent cost of restoration has already been suggested in this species in the wild (Dufay *et al.*, 2008), the other conditions for the maintenance of cytonuclear gynodioecy have not yet been explored. By investigating sex-related fitness differences in controlled conditions, we tested the following hypotheses:

1. Do female individuals have a reproductive advantage compared to conspecific hermaphroditic plants? Because the study species is self-incompatible, inbreeding avoidance cannot account for potential reproductive advantage of females compared to hermaphrodites. Therefore, if any female advantage is detected, it should be exclusively due to resource reallocation.
2. Is there any evidence for a cost of CMS genes when examining the female reproductive output of restored CMS hermaphrodites?
3. Because previous studies have suggested somewhat complex patterns of restoration determination and an incomplete restoration of male fertility in some restored CMS hermaphrodites in the wild (Dufay *et al.*, 2008), we attempted to evaluate the difference in pollen production between the two hermaphroditic types in controlled experimental conditions, where differences in pollen quality cannot be attributable to age differences or local soil properties. Pollen quantity and quality were measured several times during the flowering season, allowing us to explore variation among anthers and among flowers within individuals and to document all possible differences in male fitness between the two hermaphroditic types. If some CMS hermaphrodites are only partially restored for male fertility, we expect a lower male fertility in these individuals, and possibly among flowers variation in pollen quantity/quality, as found in partial male sterile individuals in some gynodioecious species .

All our results are discussed in the light of existing theoretical models.

## Materials and methods

### Study species

Wild beet, *B. vulgaris* ssp. *maritima*, is a diploid species ($2n = 18$) widely distributed along the western European coast and around the Mediterranean basin where it colonizes coastal habitats just at the upper level of high tides (Viard *et al.*, 2004; Fievet *et al.*, 2007). It is a short-lived perennial and wind-pollinated species (Letschert, 1993). *B. vulgaris* is known to be self-incompatible, with up to four gametophytic S loci (Owen, 1942; Larsen, 1977). Each individual bears one to several hundred floral stems carrying a long, dense racemose inflorescence at their apex. In addition to the main inflorescence, each floral stem also commonly develops several secondary flowering axes. An individual plant can bear few to several thousand flower clusters. Only some of the flowers open simultaneously along the floral stems within an individual plant, and flower size tends to decrease in the course of the flowering season. Fruits are the product of the clustered, joint development of several flowers that mature into a single, hard, woody seed ball. Each cluster of flowers contains one to eight flowers and, because the flowers are uniovulate, each of these aggregated fruits (hereafter simply referred to as 'fruits') contains one to eight seeds. Fruits have no particular dispersal mechanism: dispersal is thought to be mainly local (Arnaud *et al.*, 2009; De Cauwer *et al.*, 2010b), although hydrochory may lead to occasional long distance dispersal events (Fievet *et al.*, 2007).

In contrast to some other gynodioecious species, a large part of cytoplasmic diversity in *B. vulgaris* is associated with nonsterilizing factors. Only four of 20 mitochondrial haplotypes described in wild populations are clearly associated with male sterility: CMS *E*, CMS *G*, CMS *H* and CMS *Svulg* (Cuguen *et al.*, 1994; Forcioli *et al.*, 1998; Desplanque *et al.*, 2000; Fénart *et al.*, 2006). Historical relationships between CMS and non-CMS haplotypes suggest that the different CMS haplotypes, although carrying distinct functional factors of male sterility, belong to a single lineage (Darracq *et al.*, 2011).

### Plant material

Plants surveyed in this study were derived from seeds that were collected during summer 2007 from two study sites located in Brittany (western France) and named *MOR* (N 48°34 168, E −2°34 831) and *PAL* (N 48°40 497, E −2°52 911). Individuals growing in these two sites have been exhaustively sampled, phenotyped and genotyped for another study (De Cauwer, unpublished data). Based on these investigations, the sexual phenotypes and cytotypes of all plants located in these two study sites (including the mother plants used in the present study) were known. Overall sex-ratio (i.e. proportion of

females) was 1.6% in *MOR* (with 8.4% of individuals carrying a CMS cytoplasm) and 12.3% in *PAL* (with 25.7% of individuals carrying a CMS cytoplasm).

We chose to focus on one particular CMS cytotype: CMS *E*, which is also the most common male-sterile cytotype in natural populations in the study area (see Dufay *et al*., 2009), as well as in the study sites selected for this study (among individuals carrying a CMS cytoplasm, 100% were carrying CMS *E* in *MOR* and 92.9% were carrying CMS *E* in *PAL*). Seeds used in this study came from 29 mother plants from *MOR* (9 females, 8 restored CMS hermaphrodites and 12 non-CMS hermaphrodites) and 28 mother plants from *PAL* (3 females, 7 restored CMS hermaphrodites and 18 non-CMS hermaphrodites). For each mother plant, ten fruits were sown in October 2007 with the aim to obtain at least five offspring. All seedlings from these 57 progenies were grown in pots of 19 cm in diameter, in a greenhouse, until seedlings reached a six-leaves stage. At the end of December 2007, five to ten offspring per mother plant were randomly selected and were vernalized at 6 °C for 9 weeks to induce flowering. These 292 individuals (mean number of offspring per progeny ± SD = 5.12 ± 1.56) were equally distributed between the two study sites: 144 plants were originating from *MOR* (mean number of offspring per progeny ± SD = 4.97 ± 1.38) and 148 plants were originating from *PAL* (mean number of offspring per progeny ± SD = 5.29 ± 1.74). The same plants were used in the second year of the study, the same vernalization protocol being applied in December 2008.

At the end of March, pots were set out in an experimental garden. The spatial arrangement of plants was random and different in spring 2008 and spring 2009. The average local sex ratio (i.e. proportion of females within a radius of 1 m around focal plants) was 0.115 ± 0.065 in 2008 and 0.127 ± 0.079 in 2009, and did not differ among the three sexual phenotypes ($P > 0.05$ for both study years). Random arrangement of plants, resulting in homogeneous pollen availability, reduces the potential effect of local sex ratios on female fitness traits, as can be found in natural conditions (De Cauwer *et al*., 2010a).

## Plant characterization

The 57 progenies were surveyed for two consecutive flowering seasons (2008 and 2009), allowing us to verify the consistency of results across years. At the onset of flowering, sexual phenotype was determined (i.e. female or hermaphrodite) by examining several flowers on different parts of each surveyed individual. Plants with brown or white reduced anthers were considered to be females and plants with yellow anthers with obvious pollen production were considered to be hermaphrodites. Some individuals showed intermediate phenotypes (light-coloured yellow anthers with little pollen production) and were also classified as hermaphrodites. The sexual phenotype was further confirmed by pollen counts (see below). For each mother plant, the cytotype (CMS *E* or non-CMS) was known. Among individuals that were expressing a hermaphroditic phenotype, it was thus possible to discriminate restored CMS hermaphrodites and non-CMS hermaphrodites. Among all the individuals that flowered at least once (2008 and/or 2009), we obtained 37 females, 103 restored CMS hermaphrodites and 134 non-CMS hermaphrodites ($N_{TOT}$ = 274 flowering individuals).

### *General descriptors of flowering*

Because flowering duration and flowering synchrony potentially have important effects on individual fitness, flowering phenology was investigated in the three sexual phenotypes (females, restored CMS hermaphrodites and non-CMS hermaphrodites). The flowering survey started on 13 May 2008 and lasted 62 days the first year of the study. In the second study year, flowering started on 18 May 2009 and lasted 63 days. For each individual, the dates of the onset and end of flowering were noted and the duration of flowering was calculated.

Because individuals can produce up to several thousand flowers distributed among several floral stems, measurements of fitness were limited to the main inflorescence of the largest floral stem for each surveyed plant. Every eight days, starting from the onset of flowering, the number of flowers and the number of flower clusters (potential fruits) that opened along the main floral stem were counted. For most individuals, three consecutive sections were obtained. Monitoring was standardized for all individuals, allowing us to compare male and female traits among plants (see below).

Additionally, the number of floral stems was counted for all individuals at the end of the flowering season. The total number of flowers as well as the total number of flower clusters (potential fruits) produced during the whole flowering season were also counted on a subsample of 81 plants in 2009 (11 females, 40 restored CMS hermaphrodites and 30 non-CMS hermaphrodites).

### *Descriptors of female fitness*

Mid-August, several weeks after the end of flowering, ripe fruits were collected along the main floral stem. For all plants for which the main stem flowered during three temporal sections, we calculated fruit set as the number of fruits relative to the number of flower clusters (potential fruits) that were produced during these three sections. Given the structure of fruits in *B. vulgaris* (i.e. woody seedballs), it was not possible to directly count the number of seeds per fruit to assess seed set. Measuring the germination rates for the collected fruits – by counting the number of seedlings that emerged from fruits – was the best way to estimate the number of viable seeds per fruit. To compare germination of seeds

produced by the three different sexual phenotypes, 3–40 fruits from the main floral stem were sown (depending on individual fruit production). For mother plants that produced more than 40 fruits on the main floral stem, fruits surveyed for germination were chosen randomly. For mother plants producing fewer than 40 fruits, all fruits were harvested from the main floral stem for the estimation of seed set. Overall, we sowed 1483 fruits in 2008 (203 produced by females, 400 produced by restored CMS hermaphrodites and 880 produced by non-CMS hermaphrodites) and 4360 fruits in 2009 (681 produced by females, 1941 produced by restored CMS hermaphrodites and 1738 produced by non-CMS hermaphrodites). After monitoring for 2 months and removing all seedlings that germinated, all fruits were stored in dry conditions at room temperature for 4 weeks, to break dormancy. Final germination rates were determined after a second 2 months survey. We then estimated seed set as the ratio between the total number of seedlings and the estimated number of available ovules for each plant. The number of available ovules was estimated by multiplying the number of sown fruits by the mean number of flowers per flower cluster (each flower being uniovulate). This estimator of seed set not only describes seed production, but also germination ability.

Finally, a combined estimator of female fitness was calculated by multiplying the total number of flower clusters produced along the main floral stem, the fruit set and the seed set.

*Descriptors of male fitness*

Pollen production was determined for all flowering individuals. For each plant and for each of the three temporal sections, two nearly opened buds located on the main floral stem were collected and dissected to obtain two anthers per bud. Anthers were stored separately in 95% ethanol until pollen counts were performed. Details on the counting procedure and the utilization of the particle counter [CASY® model TT (Innovatis, Bielefeld, Germany)] are described in Dufay *et al.* (2008). The number of detected particles was determined for 400 size classes ranging from 0.125 to 50 $\mu$m. Typical peaks of particles with sizes ranging from 10 to 24 $\mu$m were observed in samples collected from hermaphrodites. These peaks did not occur in blank samples. As a result, we only considered this 10–24 $\mu$m zone for pollen counts. Prior observations showed that nonviable pollen grains in *B. vulgaris* ssp. *maritima* are smaller than viable pollen grains (Dufay *et al.*, 2008), with two sub-peaks that are clearly recognizable. As in Dufay *et al.* (2008), we categorized particles within these two size classes, and considered three variables for subsequent analyses: (i) total pollen quantity, (ii) proportion of large (viable) pollen grains and (iii) quantity of large pollen grains (see Table 1 for the number of individuals). The total number of pollen grains and the number of large pollen grains were obtained from the values provided by the particle counter after correcting for the dilution ratio. Four different counts (one per anther) were obtained for each individual within each temporal section, yielding a total number of 744 samples for the 2008 flowering season and 2616 for the 2009 flowering season. Within each of the three temporal sections, the average and the coefficient of variation (ratio of the standard deviation to the mean) of the four counts were calculated prior to statistical analyses. We measured these variables on several flowers in order to obtain estimates representative of male fitness over the whole flowering season. The coefficients of variation were recorded in order to assess whether restored CMS hermaphrodites showed stronger within-individual variation in male fertility, as this could be expected in case of partial male sterility.

Finally, as in Dufay *et al.* (2008), we verified whether the proportion of large pollen grains was a reliable

**Table 1** Summary of the variables and the number of individuals used to characterize phenology, female fitness and male fitness in *B. vulgaris* for the two study years (2008 and 2009).

| Variable | Number of non-CMS hermaphrodites | Number of restored CMS hermaphrodites | Number of females |
|---|---|---|---|
| **General descriptors of flowering** | | | |
| Flowering onset (2008/2009)* | 49/126 | 26/100 | 9/36 |
| Flowering duration (2008/2009)* | 13/126 | 10/100 | 4/36 |
| Number of flowers along the main floral stem (2008/2009)* | 27/67 | 18/66 | 7/23 |
| Number of floral stems (2008/2009)* | 49/126 | 26/100 | 9/36 |
| **Female fitness descriptors** | | | |
| Fruit set (2008/2009)* | 35/80 | 22/73 | 7/25 |
| Seed set (2008/2009)* | 30/61 | 14/67 | 6/22 |
| Estimation of overall female fitness (2008/2009)* | 30/61 | 14/67 | 6/22 |
| **Male fitness descriptors** | | | |
| Mean number of pollen grains (2008/2009)† | 34/104 | 21/80 | 7/34 |
| CV of the number of pollen grains (2008/2009) † | 34/104 | 21/80 | 7/34 |
| Mean proportion of large pollen grains (2008/2009) † | 34/104 | 21/80 | 7/34 |
| CV of the proportion of large pollen grains (2008/2009) † | 34/104 | 21/80 | 7/34 |
| Mean number of large pollen grains (2008/2009) † | 34/104 | 21/80 | 7/34 |
| CV of the number of large pollen grains (2008/2009) † | 34/104 | 21/80 | 7/34 |

CV: coefficient of variation.
*Variables consisting of single data points per plant.
†Variables for which three within-plant replicates were available along the main floral stem.

estimator of the proportion of viable pollen grains, using Alexander staining (Alexander, 1969) for anthers collected during the first temporal section on all individuals characterized for pollen production. Within 3 h of collection, pollen was removed from the anthers 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 of phenol, 5 mL 1% acid fuschin in $H_2O$, 0.5 mL 1% orange G in $H_2O$, 2 mL glacial acetic acid, 25 mL glycerol and 50 mL water), which stains viable pollen purple, was added to each pollen sample. These samples were then examined under a light microscope at 100 × magnification. Two hundred pollen grains per sample, when available, were scored as either purple or green. The proportion of viable pollen grains (i.e. ratio of purple-stained pollen grains to the total number of pollen grains) was then compared to the proportion of large pollen grains obtained from the particle counter.

### Data analyses

Data generated in 2008 and 2009 were analysed separately. The purpose of the study was to compare sexual phenotypes while controlling for possible population and mother plant effects. For all variables consisting of a single data point per plant (i.e. general descriptors of flowering and descriptors of female fitness, see Table 1), these comparisons were performed using general linear models, while when within-plant replicates were available (i.e. descriptors of male fitness, see Table 1), we used repeated-measurement general linear models. Because of the particular determination of sex in gynodioecious *B. vulgaris*, it was not possible to build complete statistical models that could simultaneously test the effect of population (*MOR* and *PAL*), identity of the mother plant (57 mother plants) and the sexual phenotype of the individual (female, restored CMS hermaphrodite and non-CMS hermaphrodite). This is because male sterility is maternally inherited, and therefore the sex of an individual is not independent of the identity of the mother plant: non-CMS mother plants produce only non-CMS hermaphrodites, while CMS mother plants produce both females and restored CMS hermaphrodites. As a consequence, instead of using a single test, the effect of sexual phenotype was assessed by working on subsamples of the data sets including only two sexual phenotypes. For each study year, three different data sets were thus generated: (i) a data set including non-CMS hermaphrodites and restored CMS hermaphrodites, (ii) a data set including non-CMS hermaphrodites and females and (iii) a data set including females and restored CMS individuals. Two different statistical models were then used: one for comparisons involving individuals carrying different cytotypes (non-CMS hermaphrodites vs. restored CMS hermaphrodites and non-CMS hermaphrodites vs. females) and another one for comparisons of individuals carrying the same cytotype (females vs. restored CMS individuals). In the first case, we tested for an effect of population of origin, mother plant cytotype and mother plant identity (nested within the interaction between population and mother plant cytotype). As we used truncated data sets including only two sexual phenotypes, testing for mother plant cytotype (CMS *E* vs. non-CMS) also directly tested for an effect of the individual sexual phenotype. In the second case (when comparing individuals carrying the same cytotype, i.e. females and restored CMS hermaphrodites), it was possible to test directly for an effect of population, mother plant identity (nested within population) and sexual phenotype of the individual. In these models, mother plant cytotype (or plant sexual phenotype, depending on the model) and mother plant population were treated as fixed factors, while mother plant identity was treated as a random factor. For each variable, three different comparisons were thus conducted (non-CMS hermaphrodites vs. restored CMS hermaphrodites, non-CMS hermaphrodites vs. females and females vs. restored CMS hermaphrodites). A standard Bonferroni procedure was used to reduce Type I errors: the significant criterion was reduced according to the number of statistical tests ($\alpha/k$, where $k$ is three, i.e. the number of comparisons conducted for each variable).

In the particular case of variables describing male fitness (i.e. total pollen quantity, quantity of large pollen grains and ratio of large pollen grains, as well as the associated coefficients of variation, see Table 1), comparisons only involved non-CMS hermaphrodites and restored CMS hermaphrodites.

All proportions (i.e. fruit set, seed set and proportion of viable pollen grains) were arcsine-root square transformed to improve the normality of the residuals. All analyses were performed using PROC GLM in SAS (version 9.1, SAS Institute Inc., Cary, NC, USA).

## Results

Among the total number of available plants ($n$ = 292), nearly all flowered during at least one flowering season ($n$ = 274). The proportion of flowering individuals was quite low in 2008 compared to 2009 (0.57 in 2008 and 0.96 in 2009). For all flowering individuals, we determined the sexual phenotype (female or hermaphrodite) in the experimental garden. Measurements of pollen production (see below) were then used to confirm the assigned sexual phenotypes. Indeed, as in Dufay *et al.* (2008), we observed a threshold in particle quantity within CMS individuals, with some plants producing very little pollen and other individuals that were at least partially restored for male fertility and that produced more than 7000 pollen grains per anther (Figure S1). Visual sexual phenotyping in the experimental garden and pollen counts yielded the same results for 98% of

individuals. In the few cases where these results were contradictory, we used the sexual phenotype determined by pollen counts. Overall, the 274 flowering individuals included 37 females, 103 restored CMS hermaphrodites and 134 non-CMS hermaphrodites.

### General descriptors of flowering

On average, individuals flowered for 35.54 days in 2008 and for 35.84 days in 2009. The three sexual phenotypes were statistically indistinguishable with regard to the date of flowering onset and total flowering duration (Table 2 and Fig. 1).

The mean number of flowers produced per day along the main floral stem (±SD) was 5.39 ± 3.15 in 2008 and 5.26 ± 3.30 in 2009. Females produced significantly more flowers than the two hermaphroditic types in 2008 and more flowers than non-CMS hermaphrodites in 2009 (although these differences were not significant after Bonferroni correction for multiple tests, see Table 2 and Fig. 1). In both study years, the two hermaphroditic types could not be differentiated with regard to flower production. Given that *B. vulgaris* flowers are uniovulate, flower production is directly correlated with ovule production. Our results then suggest that females may have some slight advantage over hermaphrodites (regardless of their cytotype) in terms of ovule production. The number of flowers produced along the main floral stem was positively correlated with the total number of flowers, estimated on a subsample of 81 individuals surveyed in 2009 ($R_{PEARSON} = 0.239$, $P = 0.0246$). Besides, as the three sexual phenotypes were statistically similar in terms of number of floral stems in both flowering seasons, this suggests no major differences in plant architecture among sex categories (Table 2 and Fig. 1). In the same way, the number of floral stems was correlated with the total number of flowers, estimated on this subsample of individuals ($R_{PEARSON} = 0.3401$, $P = 0.0020$; $n = 81$). Comparing sexual phenotypes using only a restricted part of each individual (i.e. the main floral stem of each individual) is thus relevant.

Finally, while we observed a significant correlation between the two study years for the date of flowering onset as well as for the number of floral stems ($R_{PEARSON} = 0.171$ and 0.154, respectively, $P < 0.05$ in both cases), no significant correlations were found for the flowering duration and the number of flowers produced along the main floral stem.

### Female fitness descriptors

In *B. vulgaris*, each fruit is derived from the joint development of all flowers belonging to the same flower cluster. Overall, 52.49% of the 3010 flower clusters observed in 2008 produced a fruit. In 2009, fruit set was also limited, with 51.01% of the 10 957 flower clusters observed producing a fruit. When investigating the effect of sexual phenotype on fruit set, non-CMS hermaphrodites had a significant advantage over restored CMS hermaphrodites in 2008 (significant after Bonferroni correction, see Table 3 and Fig. 1), while other comparisons were nonsignificant. This possibly suggests a cost of CMS genes for restored CMS hermaphrodites, although none of the differences among sexual phenotypes were significant in 2009 (Table 3 and Fig. 1). Besides, we observed no correlation between the two study years for fruit set ($P > 0.05$).

Seed set, calculated as the ratio between the number of emerging seedlings and the estimated number of available ovules, was quite low for both study years (mean ± SD: 49.63% ± 19.83 in 2008 and 40.31% ± 23.40 in 2009). The vast majority of fruits germinated before dry-storage (79.84% in 2008 and 85.81% 2009), suggesting low seed dormancy. Seed germination rates did not significantly differ among females, restored CMS hermaphrodites and non-CMS hermaphrodites (Table 3 and Fig. 1). In addition, seed set was significantly correlated between study years ($R_{PEARSON} = 0.821$, $P = 0.044$).

The global investment in female function along the main floral stem was estimated by multiplying the total number of flower clusters, the fruit set and the seed set. Although the value estimated in female plants was always higher than those estimated in hermaphroditic plants, no statistically significant differences were detected among the three sexual phenotypes (first year of the study : mean ± SD; 58.52 ± 44.17 for female individuals, 43.61 ± 20.84 for restored CMS hermaphrodites and 43.95 ± 19.59 for non-CMS hermaphrodites; second year of the study : mean ± SD; 51.87 ± 22.95 for female individuals, 46.63 ± 26.11 for restored CMS hermaphrodites and 44.01 ± 32.05 for non-CMS hermaphrodites; all at $P > 0.05$). The ratio of the average value observed in females to the average value observed in hermaphrodites (regardless of their cytotype) was 1.34 in 2008 and 1.14 in 2009, providing an estimate of the level of female advantage in *B. vulgaris*.

### Male fitness descriptors

Pollen counts on the particle counter confirmed the occurrence of two clearly distinct peaks, with some individuals producing mainly small pollen grains, some individuals producing mainly large pollen grains and some individuals producing both types, has previously observed by Dufay *et al.* (2008). However, the positions of these peaks were slightly different: the peak for large pollen grains was detected for sizes ranging from 13.5 to 24 $\mu$m in our study and for sizes ranging from 16 to 24 $\mu$m in Dufay *et al.* (2008). The proportions of large pollen grains (estimated with the particle counter) and of viable pollen grains (estimated with the staining method) were statistically correlated

**Table 2** Results of general linear models carried on the general descriptors of flowering: the flowering onset, the flowering duration, the number of flowers along the main floral stem and the number of floral stems.

| | | 2008 | | | | 2009 | | | |
|---|---|---|---|---|---|---|---|---|---|
| Variable | Source of variation | d.f. | MS | *F* | *P* | d.f. | MS | *F* | *P* |
| **(a) Comparison between non-CMS hermaphrodites and CMS hermaphrodites** | | | | | | | | | |
| *Flowering onset* | Population | 1 | 19.51 | 0.09 | 0.7620 | 1 | 357.28 | 3.85 | 0.0530 |
| | Cytotype | 1 | 6.76 | 0.03 | 0.8583 | 1 | 42.06 | 0.45 | 0.5019 |
| | Mother plant (population × cytotype) | 33 | 212.10 | 1.04 | 0.4446 | 54 | 98.87 | 1.33 | 0.0898 |
| | Error | 39 | 203.02 | | | 169 | 74.55 | | |
| *Flowering duration* | Population | 1 | 42.07 | 0.52 | 0.4828 | 1 | 0.38 | 0.01 | 0.9416 |
| | Cytotype | 1 | 4.16 | 0.05 | 0.8240 | 1 | 0.42 | 0.01 | 0.9389 |
| | Mother plant (population × cytotype) | 15 | 83.17 | 1.41 | 0.3728 | 54 | 71.49 | 1.06 | 0.3871 |
| | Error | 5 | 58.80 | | | 169 | 67.68 | | |
| *Number of flowers along the main floral stem* | Population | 1 | 72.25 | 0.06 | 0.8043 | 1 | 4161.01 | 1.71 | 0.1952 |
| | Cytotype | 1 | 1617.71 | 1.40 | 0.2451 | 1 | 4030.03 | 1.66 | 0.2023 |
| | Mother plant (population × cytotype) | 24 | 1245.66 | 1.63 | 0.1457 | 49 | 2505.10 | 1.15 | 0.2867 |
| | Error | 18 | 764.58 | | | 81 | 2181.07 | | |
| *Number of floral stems* | Population | 1 | 8.00 | 2.94 | 0.0938 | 1 | 9.65 | 1.40 | 0.2405 |
| | Cytotype | 1 | 2.05 | 0.76 | 0.3866 | 1 | 0.03 | 0.00 | 0.9514 |
| | Mother plant (population × cytotype) | 33 | 97.37 | 1.64 | 0.0689 | 54 | 378.21 | 1.05 | 0.3937 |
| | Error | 39 | 1.80 | | | 169 | 6.66 | | |
| **(b) Comparison between non-CMS hermaphrodites and females** | | | | | | | | | |
| *Flowering onset* | Population | 1 | 82.84 | 0.37 | 0.5449 | 1 | 260.47 | 2.72 | 0.1033 |
| | Cytotype | 1 | 133.40 | 0.60 | 0.4410 | 1 | 15.62 | 0.17 | 0.6835 |
| | Mother plant (population × cytotype) | 26 | 223.86 | 1.03 | 0.4662 | 45 | 102.49 | 1.26 | 0.1658 |
| | Error | 29 | 217.25 | | | 114 | 81.45 | | |
| *Flowering duration* | Population | 1 | 28.78 | 0.31 | 0.5885 | 1 | 0.31 | 0.00 | 0.9450 |
| | Cytotype | 1 | 36.15 | 0.40 | 0.5406 | 1 | 29.12 | 0.46 | 0.4995 |
| | Mother plant (population × cytotype) | 8 | 92.33 | 1.13 | 0.5109 | 45 | 66.24 | 1.11 | 0.3255 |
| | Error | 6 | 81.67 | | | 114 | 59.74 | | |
| *Number of flowers along the main floral stem* | Population | 1 | 47.03 | 0.02 | 0.8883 | 1 | 1526.48 | 0.71 | 0.4014 |
| | Cytotype | 1 | 10112.00 | 4.89 | 0.0385* | 1 | 9521.23 | 4.46 | 0.0384* |
| | Mother plant (population × cytotype) | 18 | 2744.08 | 6.27 | **0.0008***** | 37 | 2142.48 | 1.01 | 0.4785 |
| | Error | 13 | 437.96 | | | 50 | 2117.17 | | |
| *Number of floral stems* | Population | 1 | 0.33 | 0.15 | 0.7002 | 1 | 0.28 | 0.04 | 0.8419 |
| | Cytotype | 1 | 2.58 | 1.30 | 0.2612 | 1 | 0.22 | 0.03 | 0.8588 |
| | Mother plant (population × cytotype) | 26 | 65.63 | 2.14 | 0.0245* | 45 | 325.91 | 1.06 | 0.3959 |
| | Error | 29 | 1.18 | | | 114 | 6.84 | | |
| **(c) Comparison between CMS hermaphrodites and females** | | | | | | | | | |
| *Flowering onset* | Population | 1 | 7.60 | 0.04 | 0.8464 | 1 | 5.65 | 0.07 | 0.7966 |
| | Sexual phenotype | 1 | 283.02 | 0.82 | 0.3799 | 1 | 103.37 | 1.50 | 0.2230 |
| | Mother plant (population) | 17 | 145.98 | 0.42 | 0.9549 | 25 | 85.59 | 1.24 | 0.2197 |
| | Error | 15 | 345.71 | | | 108 | 68.81 | | |
| *Flowering duration* | Population | 1 | 108.25 | 4.42 | 0.1703 | 1 | 34.61 | 0.65 | 0.4275 |
| | Sexual phenotype | 1 | 120.05 | 4.90 | 0.1573 | 1 | 15.57 | 0.28 | 0.5963 |
| | Mother plant (population) | 6 | 59.21 | 2.42 | 0.3213 | 25 | 53.36 | 0.97 | 0.5159 |
| | Error | 5 | 24.50 | | | 108 | 55.17 | | |
| *Number of flowers along the main floral stem* | Population | 1 | 2978.44 | 1.01 | 0.3250 | 1 | 4673.58 | 2.11 | 0.1572 |
| | Sexual phenotype | 1 | 22602.00 | 5.64 | 0.0493* | 1 | 120.59 | 0.06 | 0.8020 |
| | Mother plant (population) | 15 | 2629.93 | 0.66 | 0.7672 | 24 | 2243.28 | 1.18 | 0.2946 |
| | Error | 7 | 4009.10 | | | 62 | 1900.89 | | |
| *Number of floral stems* | Population | 1 | 3.70 | 1.36 | 0.2534 | 1 | 6.65 | 0.74 | 0.3964 |
| | Sexual phenotype | 1 | 1.89 | 0.59 | 0.4527 | 1 | 0.01 | 0.00 | 0.9653 |
| | Mother plant (population) | 17 | 43.54 | 0.80 | 0.6703 | 25 | 236.46 | 1.79 | 0.0215* |
| | Error | 15 | 3.19 | | | 108 | 5.28 | | |

Results are presented for the 2 years of survey. $^{*}P < 0.05$, $^{***}P < 0.001$, with *P*-values remaining significant after Bonferroni correction ($P < 0.05$ or less) shown in bold.

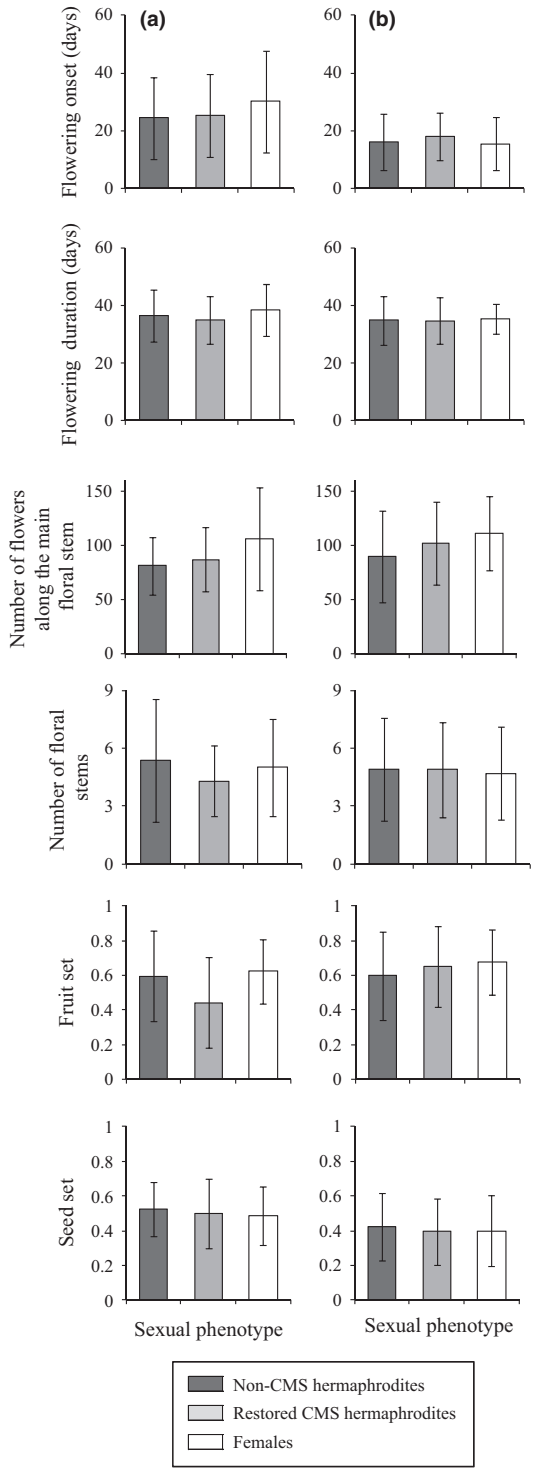


**Fig. 1** Effect of the sexual phenotype (non-CMS hermaphrodites, restored CMS hermaphrodites and females) on the general descriptors of flowering (flowering onset, flowering duration, number of flowers along the main floral stem and total number of floral stems) and on the descriptors of female fitness (fruit set and seed set) during the two study years: 2008 (a) and 2009 (b).

($R_{PEARSON} = 0.660$ in 2008 and $R_{PEARSON} = 0.816$ in 2009, $P < 10^{-4}$ in both cases).

The two hermaphroditic types were statistically indistinguishable with regard to the quantity of pollen (average for the four anthers collected within each temporal section) in both study years (Table 4 and Fig. 2). However, non-CMS hermaphrodites showed significantly lower coefficients of variation (among anthers, for each date) for the quantity of pollen when compared to restored CMS hermaphrodites in both flowering seasons (Table 4 and Fig. 2). Our results also show that the quantity of pollen in 2009 varied over time, with a decrease from the beginning to the end of the flowering season.

Sexual phenotype was found to have a significant effect on the proportion of large pollen grains (viable pollen grains, i.e. particle size ranging between 13.5 and 24 $\mu$m), with restored CMS hermaphrodites producing a lower proportion of large pollen grains than non-CMS ones (Table 4 and Fig. 2). Contrary to what was observed for the total number of pollen grains, no difference in the coefficient of variation (among anthers, for each date) of the proportion of large pollen grains was detected between the two types of hermaphrodites. Time had no effect on the quality of pollen or on intra-individual variation of pollen quality in either study year (Table 4 and Fig. 2). Altogether, our results suggest that pollen quantity is independent of sexual phenotype and decreases during the flowering season, whereas pollen quality is constant during the flowering season but varies with sexual phenotype. Additionally, the proportion of large pollen grains per anther varied greatly among plants, particularly in individuals carrying a CMS gene (Fig. 3).

When combining both variables, an estimator of the number of large (viable) pollen grains per anther was obtained. As shown in Table 4, there was a significant effect of sexual phenotype on this variable as well as on its coefficient of variation. Non-CMS hermaphrodites produced a significantly higher number of large pollen grains per anther and were characterized by lower levels of intra-individual variance in both study years (Fig. 2). We found that the number of large pollen grains significantly decreased with time only in 2009 (Table 4). The coefficient of variation was also positively affected by time, with an increase of intra-individual variation during the 2009 flowering season.

Individual pollen production in the two consecutive study years were significantly correlated ($R_{PEARSON} = 0.779$, $P < 10^{-4}$ for the total number of pollen grains per anther in the first temporal section; $R_{PEARSON} = 0.709$, $P < 10^{-4}$ for the proportion of viable pollen grains in the first temporal section, $R_{PEARSON} = 0.808$, $P < 10^{-4}$ for total number of viable pollen grains per anther in the first temporal section). Similar levels of correlation were obtained for subsequent temporal sections (data not shown).

**Table 3** Results of general linear models carried on the descriptors of female fitness: fruit set and seed set, measured along the main floral stem. Fruit set and seed set were arcsine-square root transformed before statistical tests.

| | | 2008 | | | | 2009 | | | |
|---|---|---|---|---|---|---|---|---|---|
| Variable | Source of variation | d.f. | MS | *F* | *P* | d.f. | MS | *F* | *P* |
| **(a) Comparison between non-CMS hermaphrodites and CMS hermaphrodites** | | | | | | | | | |
| *Fruit set* | Population | 1 | 0.007 | 0.06 | 0.8043 | 1 | 0.065 | 0.61 | 0.4386 |
| | Cytotype | 1 | 0.838 | 7.70 | **0.0082**** | 1 | 0.301 | 2.81 | 0.0979 |
| | Mother plant (population × cytotype) | 30 | 0.112 | 1.17 | 0.3536 | 52 | 0.116 | 1.52 | 0.0380* |
| | Error | 24 | 0.096 | | | 98 | 0.076 | | |
| *Seed set* | Population | 1 | 0.047 | 1.82 | 0.1855 | 1 | 0.043 | 0.76 | 0.3874 |
| | Cytotype | 1 | 0.001 | 0.05 | 0.8297 | 1 | 0.022 | 0.38 | 0.5397 |
| | Mother plant (population × cytotype) | 25 | 0.022 | 0.45 | 0.9651 | 49 | 0.063 | 1.64 | 0.0257* |
| | Error | 16 | 0.048 | | | 76 | 0.038 | | |
| **(b) Comparison between non-CMS hermaphrodites and females** | | | | | | | | | |
| *Fruit set* | Population | 1 | 0.004 | 0.05 | 0.8275 | 1 | 0.038 | 0.41 | 0.5256 |
| | Cytotype | 1 | 0.000 | 0.01 | 0.9419 | 1 | 0.069 | 0.75 | 0.3905 |
| | Mother plant (population × cytotype) | 23 | 0.084 | 0.76 | 0.7302 | 41 | 0.100 | 1.28 | 0.1885 |
| | Error | 16 | 0.110 | | | 61 | 0.078 | | |
| *Seed set* | Population | 1 | 0.091 | 3.78 | 0.0620 | 1 | 0.016 | 0.35 | 0.5593 |
| | Cytotype | 1 | 0.000 | 0.00 | 0.9484 | 1 | 0.000 | 0.00 | 0.9796 |
| | Mother plant (population × cytotype) | 22 | 0.024 | 1.12 | 0.4409 | 37 | 0.049 | 1.35 | 0.1699 |
| | Error | 11 | 0.022 | | | 43 | 0.036 | | |
| **(c) Comparison between CMS hermaphrodites and females** | | | | | | | | | |
| *Fruit set* | Population | 1 | 0.001 | 0.01 | 0.9353 | 1 | 0.062 | 0.73 | 0.3986 |
| | Sexual phenotype | 1 | 0.323 | 3.14 | 0.1067 | 1 | 0.000 | 0.00 | 0.9728 |
| | Mother plant (population) | 16 | 0.091 | 0.89 | 0.5990 | 24 | 0.086 | 1.32 | 0.1836 |
| | Error | 10 | 0.103 | | | 71 | 0.065 | | |
| *Seed set* | Population | 1 | 0.030 | 0.58 | 0.4661 | 1 | 0.025 | 0.32 | 0.5772 |
| | Sexual phenotype | 1 | 0.010 | 0.09 | 0.7725 | 1 | 0.012 | 0.48 | 0.4908 |
| | Mother plant (population) | 12 | 0.021 | 0.20 | 0.9898 | 24 | 0.081 | 3.14 | **0.0002***** |
| | Error | 5 | 0.106 | | | 62 | 0.026 | | |

Results are presented for the two years of survey (2008 and 2009). $^{*}P < 0.05$, $^{**}P < 0.01$; $^{***}P < 0.001$, with *P*-values remaining significant after Bonferroni correction ($P < 0.05$ or less) shown in bold.

## Discussion

In gynodioecious *B. vulgaris*, three conditions are theoretically necessary for frequency-dependent selection to maintain the sexual polymorphism: females must outperform hermaphrodites for at least one trait related with female fitness, restored CMS hermaphrodites must have a disadvantage in female fitness compared with non-CMS hermaphrodites and restorer alleles must be associated with a cost. While this cost of restoration has already been suggested in *B. vulgaris* ssp. *maritima* (Dufay *et al.*, 2008), the present study was designed to explore the possible existence of a female advantage and a cost of CMS genes for several components of female reproductive output. Besides, because differences in pollen production between CMS and non-CMS hermaphrodites has already been suggested in the wild (Dufay *et al.*, 2008), and because these differences can also impact the dynamics of gynodioecy, we aimed to thoroughly investigate the differences in male fitness through pollen production between the two hermaphroditic types in controlled conditions.

### A female advantage in *B. vulgaris*?

Female individuals are known to be common in natural populations of *B. vulgaris* ssp. *maritima*. In a previous study investigating the occurrence of gynodioecy among 33 natural populations in Brittany (western France), Dufay *et al.* (2009) found that females were present in 91% of the studied sites, with frequencies ranging from 2% to 43%. In the present study, three traits associated with female reproductive output were measured along the main floral stem: fruit set (ratio between the number of flower clusters and the number of fruits), seed set (ratio between the number of emerging seedlings and the estimated number of available ovules) and number of flowers. The two first traits are directly related to female fitness, whereas flower counts also describe potential differences in terms of male function. Our results suggest that differences in fruit set and seed set probably do not account for the maintenance of females in natural populations of *B. vulgaris*, because females did not perform better than hermaphrodites, at least in the conditions examined in this study. Nonetheless, when

**Table 4** Results of repeated-measures analyses carried on the descriptors of male fitness for hermaphroditic plants: (i) the number of pollen grains, (ii) the proportion of large pollen grains (estimating pollen viability) and (iii) the number of large pollen grains.

| | | | 2008 | | | | 2009 | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Variable | | Source of variation | d.f. | MS | *F* | *P* | d.f. | MS | *F* | *P* |
| *Mean number of pollen grains* | Between | Population | 1 | 1.19E+09 | 4.72 | 0.0400* | 1 | 1.52E+09 | 4.39 | 0.0381* |
| | | Cytotype | 1 | 6.80E+08 | 2.69 | 0.1138 | 1 | 3.38E+07 | 0.10 | 0.7552 |
| | | Mother plant (population × cytotype) | 28 | 3.32E+08 | 1.32 | 0.2490 | 53 | 3.49E+08 | 1.01 | 0.4735 |
| | | Error | 24 | 2.53E+08 | | | 128 | 3.46E+08 | | |
| | Within | Time | 2 | 5.79E+07 | 1.20 | 0.3104 | 2 | 1.71E+09 | 21.65 | <.0001*** |
| | | Time × population | 2 | 9.12E+06 | 0.19 | 0.8285 | 2 | 6.21E+07 | 0.79 | 0.4562 |
| | | Time × cytotype | 2 | 2.01E+07 | 0.42 | 0.6623 | 2 | 1.00E+07 | 0.13 | 0.8807 |
| | | Time × mother plant (population × cytotype) | 56 | 3.18E+07 | 0.66 | 0.9337 | 106 | 5.57E+07 | 0.71 | 0.9798 |
| *CV of the number of pollen grains* | Between | Population | 1 | 0.0002 | 0.01 | 0.9211 | 1 | 0.0160 | 0.52 | 0.4708 |
| | | Cytotype | 1 | 0.1544 | 9.60 | 0.0049** | 1 | 0.6200 | 20.27 | <.0001*** |
| | | Mother plant (population × cytotype) | 28 | 0.0283 | 1.76 | 0.0814 | 53 | 0.0273 | 0.89 | 0.6752 |
| | | Error | 24 | 0.0161 | | | 128 | 0.0306 | | |
| | Within | Time | 2 | 0.0542 | 3.69 | 0.0324* | 2 | 0.0540 | 2.72 | 0.0675 |
| | | Time × population | 2 | 0.0083 | 0.57 | 0.5714 | 2 | 0.0099 | 0.50 | 0.6077 |
| | | Time × cytotype | 2 | 0.0181 | 1.24 | 0.2998 | 2 | 0.0229 | 1.15 | 0.3170 |
| | | Time × mother plant (population × cytotype) | 56 | 0.0126 | 0.86 | 0.7078 | 106 | 0.0212 | 1.07 | 0.3336 |
| *Mean proportion of large pollen grains* | Between | Population | 1 | 0.0897 | 1.05 | 0.3167 | 1 | 0.4928 | 8.18 | 0.0049** |
| | | Cytotype | 1 | 1.3281 | 15.48 | 0.0006*** | 1 | 4.0082 | 66.57 | <.0001*** |
| | | Mother plant (population × cytotype) | 28 | 0.0953 | 1.11 | 0.3999 | 53 | 0.1630 | 2.71 | <.0001*** |
| | | Error | 24 | 0.0858 | | | 128 | 0.0602 | | |
| | Within | Time | 2 | 0.0237 | 1.30 | 0.2809 | 2 | 0.0349 | 2.84 | 0.0602 |
| | | Time × population | 2 | 0.0117 | 0.65 | 0.5287 | 2 | 0.0626 | 5.09 | 0.0068** |
| | | Time × cytotype | 2 | 0.0182 | 1.00 | 0.3736 | 2 | 0.0080 | 0.65 | 0.5216 |
| | | Time × mother plant (population × cytotype) | 56 | 0.0118 | 0.65 | 0.9392 | 106 | 0.0189 | 1.53 | 0.0034** |
| *CV of the proportion of large pollen grains* | Between | Population | 1 | 0.0161 | 0.27 | 0.6069 | 1 | 0.3115 | 9.06 | 0.0032** |
| | | Cytotype | 1 | 0.0387 | 0.66 | 0.4260 | 1 | 0.0077 | 0.22 | 0.6373 |
| | | Mother plant (population × cytotype) | 28 | 0.0878 | 1.49 | 0.1639 | 53 | 0.0518 | 1.51 | 0.0323* |
| | | Error | 24 | 0.0591 | | | 128 | 0.0344 | | |
| | Within | Time | 2 | 0.0242 | 0.51 | 0.6024 | 2 | 0.0448 | 1.58 | 0.2088 |
| | | Time × population | 2 | 0.0637 | 1.35 | 0.2691 | 2 | 0.0541 | 1.91 | 0.1509 |
| | | Time × cytotype | 2 | 0.1511 | 3.20 | 0.0496* | 2 | 0.0572 | 2.01 | 0.1358 |
| | | Time × mother plant (population × cytotype) | 56 | 0.0470 | 1.00 | 0.5091 | 106 | 0.0332 | 1.17 | 0.1605 |
| *Mean number of large pollen grains* | Between | Population | 1 | 1.28E+09 | 4.61 | 0.0421* | 1 | 3.00E+09 | 9.63 | 0.0024** |
| | | Cytotype | 1 | 1.80E+09 | 6.49 | 0.0177* | 1 | 2.71E+09 | 8.69 | 0.0038** |
| | | Mother plant (population × cytotype) | 28 | 3.32E+08 | 1.20 | 0.3295 | 53 | 4.37E+08 | 1.40 | 0.0640 |
| | | Error | 24 | 2.77E+08 | | | 128 | 3.12E+08 | | |
| | Within | Time | 2 | 7.14E+07 | 1.54 | 0.2251 | 2 | 1.27E+09 | 21.46 | <.0001*** |
| | | Time × population | 2 | 1.05E+07 | 0.23 | 0.7991 | 2 | 1.35E+08 | 2.28 | 0.1041 |
| | | Time × cytotype | 2 | 2.26E+07 | 0.49 | 0.6181 | 2 | 2.42E+06 | 0.04 | 0.9598 |
| | | Time × mother plant (population × cytotype) | 56 | 3.06E+07 | 0.66 | 0.9335 | 106 | 6.20E+07 | 1.05 | 0.3688 |
| *CV of the number of large pollen grains* | Between | Population | 1 | 0.0072 | 0.28 | 0.6046 | 1 | 0.0160 | 0.45 | 0.5021 |
| | | Cytotype | 1 | 0.3716 | 14.30 | 0.0009*** | 1 | 1.0717 | 30.27 | <.0001*** |
| | | Mother plant (Population × cytotype) | 28 | 0.0525 | 2.02 | 0.0419* | 53 | 0.0468 | 1.32 | 0.1046 |
| | | Error | 24 | 0.0260 | | | 128 | 0.0354 | | |
| | Within | Time | 2 | 0.0763 | 2.62 | 0.0830 | 2 | 0.0772 | 3.29 | 0.0388* |
| | | Time × population | 2 | 0.0354 | 1.22 | 0.3045 | 2 | 0.0341 | 1.45 | 0.2355 |
| | | Time × cytotype | 2 | 0.0411 | 1.42 | 0.2529 | 2 | 0.0396 | 1.69 | 0.1868 |
| | | Time × mother plant (population × cytotype) | 56 | 0.0169 | 0.58 | 0.9739 | 106 | 0.0294 | 1.25 | 0.0784 |

For each of these descriptors, the mean value and coefficient of variation (CV) were calculated over the four measured anthers. Proportion of nonviable pollen grains was arcsine-square root transformed before statistical tests. Results are presented for the two years of study (2008 and 2009). $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$.

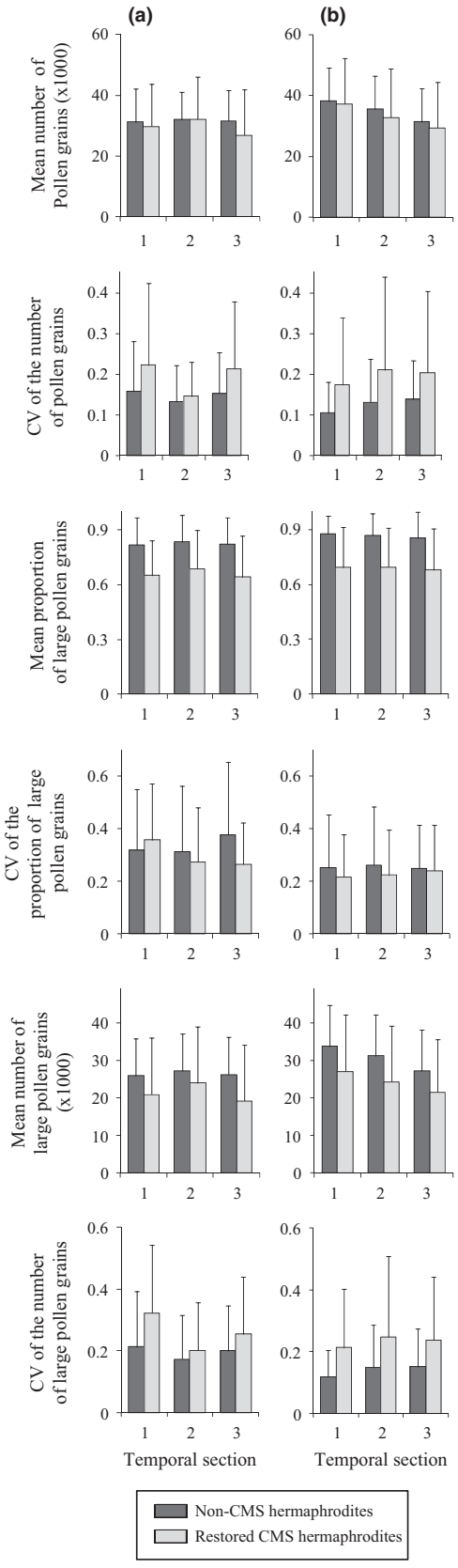


**Fig. 2** Variation in the number of pollen grains, the proportion of large pollen grains and the number of large pollen grains [mean and coefficient of variation (CV) over the four measured anthers] for the three temporal sections along the main floral stem, the two sexual phenotypes (non-CMS hermaphrodites and restored CMS hermaphrodites) and the two study years: 2008 (a) and 2009 (b).

considering the number of flowers produced along the main floral stem, females were found to outperform both hermaphroditic types in 2008 and non-CMS hermaphrodites in 2009 (although these differences were not significant after Bonferroni correction). As flowers of *B. vulgaris* are uniovulate, an increase of the number of flowers directly translates into an increase of the number of available ovules, which may represent an advantage for females. The same trend, although not significant, was observed for the estimator of overall investment in female function. This estimator was used to quantify the level of female advantage in *B. vulgaris* (ratio of the average value observed in females to the average value observed in hermaphrodites) providing an estimate of 1.34 in 2008 and 1.14 in 2009. Compared to other species with cytonuclear gynodioecy, in which female advantage is often significant for several different reproductive traits and usually of larger magnitude (reviewed in Shykoff *et al.*, 2003; Dufay & Billard, in press), the evidence for female advantage may seem somewhat equivocal in *B. vulgaris.* Although quite uncommon in gynodioecious species, there are a number of other species in which no clear female advantage has been documented (e.g. Alonso & Herrera, 2001; Miyake *et al.*, 2009).

Several possible explanations could account for levels of female advantage as low as what was observed here in *B. vulgaris*. First, observations in controlled conditions may not necessarily reflect behaviour in the wild, because fitness measures can differ between laboratory and natural conditions (e.g. Dudash, 1990). Because females have been shown to perform better than hermaphrodites in harsher environments for several gynodioecious species (reviewed in Ashman, 2006), further studies of female reproductive output in *B. vulgaris* should be conducted in varying natural conditions. Additionally, female advantage may occur in traits that were not studied here. Although female fitness was investigated in several traits, the quality of offspring produced by the different sexual phenotypes was not directly examined (although our estimator of seed set also included germination ability). Further studies measuring offspring survival and seedling size are thus needed to compare the female fitness in the three different sexual phenotypes. Because adult survival is a key fitness parameter in perennial species, long-term surveys of individuals should also be performed to define the lifetime reproductive output of the different sexual phenotypes. In *B. vulgaris*, because female fitness seems to vary between consecutive years for a given individual

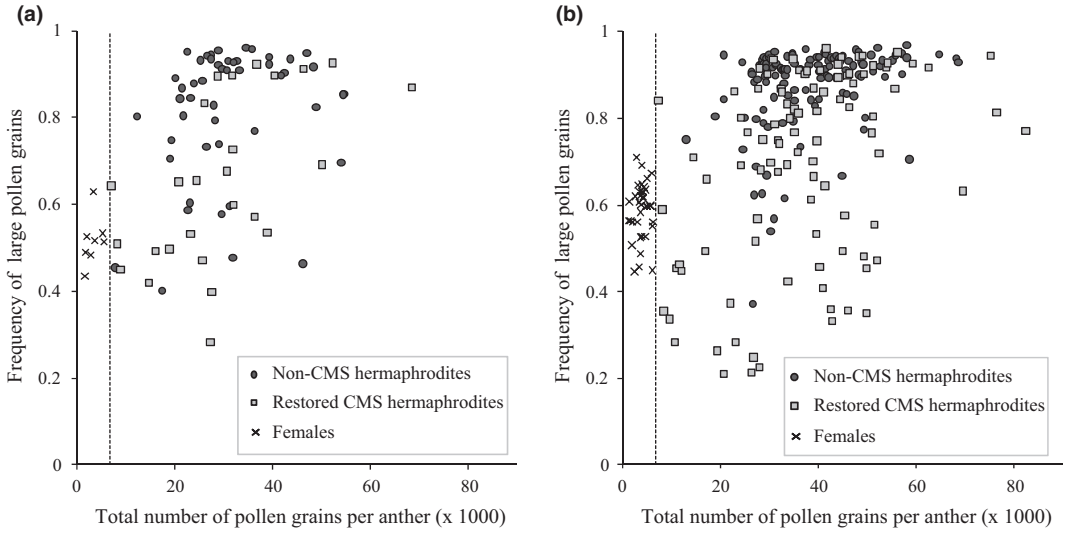


**Fig. 3** Pollen quantity (mean value over the four anthers collected in the first temporal section) and pollen viability (mean frequency of large pollen grains over the four anthers collected in the first temporal section) for all studied individuals in 2008 (a) and 2009 (b). The dashed lines separate females and hermaphrodites.

(with no significant correlation for flower production and fruit set between study years), such lifetime reproductive output measures are potentially necessary to correctly infer fitness differences between the three sexual phenotypes.

Another hypothesis is that female advantage is effectively very low in our study species. Indeed, theoretical models predict that restricted levels of female advantage are sufficient to maintain females in the case of cytonuclear gynodioecy (slightly > 1, see Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufay *et al.*, 2007). A slight selective advantage should be difficult to detect statistically, in particular within a dataset comprizing relatively few females compared to hermaphroditic individuals. To assess this possibility in our study, we performed a power analysis following Siegel & Castellan (1988), based on the sample sizes used in our experiment, the mean measures of global estimates of female fitness for the different sexual phenotypes, and the maximal standard deviation observed for these estimates. Power analysis then suggested that, given the very limited difference in female fitness among sexual phenotypes, and the relatively limited number of surveyed female individuals, we had about 10% of chance to detect a significant female advantage at a 5% level of significance (data not shown). Such low magnitude of female advantage in *B. vulgaris*, which would contrast with many other gynodioecious species, could have several nonexclusive explanations. First, this may derive from the fact that hermaphrodites in our study species are self-incompatible. Whereas females are obligate outcrossers, hermaphrodites in most gynodioecious species are self-compatible (Charlesworth, 1981). The avoidance of selfing and inbreeding depression has been shown to favour females over hermaphrodites in some self-compatible plant species (e.g. Chang, 2007), which could ultimately promote selection for male sterility. As *B. vulgaris* is known to be self-incompatible in the wild (Larsen, 1977; but see Arnaud *et al.*, 2010 for cultivated accessions), inbreeding avoidance cannot confer an advantage to females. This may limit the magnitude of female advantage in our study species, as in some other self-incompatible gynodioecious species (Dufay & Billard, in press). Accordingly, the only process that could yield a female advantage in self-incompatible species is the reallocation of resources saved from male function towards female function. Finally, the low female advantage observed in *B. vulgaris* could also be explained by the occurrence of a CMS cost (see below). If the CMS mutation itself yields a reduction in female function, it should concern all plants carrying such a mutation (i.e. both females and restored hermaphrodites). According to theory, this allows the maintenance of gynodioecy, as soon as the female advantage over-compensates this cost in female individuals (Dufay *et al.*, 2007). In these conditions, even if females reallocate many resources towards female function, the resulting female advantage could be partially hidden by the CMS cost. Overall, self-incompatibility and the occurrence of a CMS cost could both contribute to limit the female advantage, potentially explaining the restricted fitness differences between females and hermaphrodites in *B. vulgaris*.

In case of a low female advantage, one should consider whether other factors may help the maintenance of gynodioecy. Indeed, nonequilibrium processes can have important effects on the spatial distribution and the maintenance of sex-determining genes, both through recurrent introduction of new CMS cytotypes via

mutation (e.g. Belhassen *et al.*, 1993) and founder events (Couvet *et al.*, 1998). Since previous studies performed on *B. vulgaris* on large geographical scales have shown that females are always associated with the same four sterilizing cytoplasms (Dufay *et al.*, 2009), these CMS genes probably did not arise recently. As a consequence, the maintenance of females in *B. vulgaris* populations is unlikely to rely only on recurrent introductions of new CMSs *via* new mutations. However, metapopulation dynamics, with extinctions and recolonizations of populations, could result in local mismatches between CMS genes and restorer alleles. *B. vulgaris* is typically found along the coastline, where populations are potentially subject to high tides and storm disturbances, and several empirical studies have indeed suggested that founder events may be common within natural *B. vulgaris* population (Fievet *et al.*, 2007; De Cauwer *et al.*, 2010b). Whether females could be maintained in recurrently disturbed populations without benefiting from a fitness advantage (or benefiting from a very low advantage) remains an open question, and needs to be investigated theoretically.

### A cost of CMS and/or restorer alleles?

In gynodioecious species where nonsterilizing cytotypes coexist with CMS genes, restored CMS hermaphrodites are theoretically expected to show a decrease in female reproductive output compared to non-CMS hermaphrodites. This condition allows non-CMS cytotypes to be maintained in the population even if they are never associated with a female advantage (Dufay *et al.*, 2007). This difference in female fitness can have at least two proximal (not easily distinguishable) causes: a cost of expressed restorer alleles acting on female fitness and/or a cost of CMS genes that is not compensated by female advantage in restored CMS hermaphrodites. Because very few gynodioecious species have been found to contain both CMS and non-CMS cytotypes, this condition can only be verified in a restricted number of cases. To date, *Raphanus sativus* is the only documented case in which non-CMS hermaphrodites outperform restored CMS hermaphrodites in female fitness (Miyake *et al.*, 2009).

In the current study, non-CMS hermaphrodites did not outperform restored CMS hermaphrodites for any of the measured traits associated with female fitness, except for fruit set in the 2008 flowering season. However, as it was not detected in the second year of the study, this trend remains inconclusive. As for female advantage, the low performance of CMS hermaphrodites could also have been overlooked because the fitness parameters were measured in nonnatural conditions or because differences occur through other components of female fitness. However, because the female advantage is apparently low in *B. vulgaris*, only a small difference between the two types of hermaphrodites is theoretically necessary to maintain the polymorphism within a population (Dufay *et al.*, 2007), and may be, again, particularly difficult to detect experimentally.

### Restoration of male fertility and the dynamics of cytonuclear gynodioecy

Although theoretical models generally consider very simple genetic determination for restoration of male fertility (but see Frank, 1989; Bailey & Delph, 2007), empirical studies of the genetics of restoration have repeatedly rejected this assumption (e.g. Charlesworth & Laporte, 1998; Koelewijn, 2003; Ehlers *et al.*, 2005). A complex determination of restoration means that some restored CMS hermaphrodites may be only partially restored. Partial restoration could decrease pollen quantity and/or quality and affect the degree of selection for restorer alleles in natural populations.

The present study characterized pollen quantity and quality in a standardized environment, using plants of the same age and controlling for the potential effect of flowering time on male function. Our results definitively confirmed the previous observations made by Dufay *et al.* (2008): (i) pollen quality varied importantly among restored CMS hermaphrodites and (ii) restored CMS hermaphrodites were inferior to non-CMS hermaphrodites in terms of pollen quality. As the quantity of large pollen grains has been shown to affect the number of sired seedlings in natural populations of *B. vulgaris* (I. De Cauwer, unpublished data), our results suggest that the observed differences in pollen production at the flower level must result in differences in male reproductive output at the plant level. The characterization of pollen production during the whole flowering season along the main floral stem showed that, along with a global decrease in pollen quantity over time (probably associated with a decrease in flower size), the difference of pollen quality between the two types of hermaphrodites was constant across time. This suggests that CMS hermaphrodites suffer a reduction in their male fitness that cumulates over the several weeks of flower production. Such difference in pollen quality between the two types of hermaphrodites directly resulted from a high inter-individual variance among restored CMS hermaphrodites, with some of these individuals producing pollen of very low quality while others produced pollen equivalent to non-CMS hermaphrodites. Because of our experimental design, such differences in pollen quality cannot be attributable to age differences or local soil properties. Besides, we found that sexual phenotype not only affected the mean quality (number of large pollen grains), but also the intra-individual variance in the number of large pollen grains. The number of large (viable) pollen grains produced by restored CMS hermaphrodites during a given temporal section was, on average, not only lower than what was observed for non-CMS hermaphrodites, but also more variable. All these

results can be interpreted as a consequence of quantitative restoration: an insufficient number of restorer alleles may not entirely cancel the effects of CMS genes, yielding a lower pollen quality on average, with a variable efficiency among flowers. In other words, restorer alleles may then not only offset male sterility by counteracting the action of CMS genes, but also determine the quality of pollen produced by restored CMS hermaphrodites and its variability. This can be compared to other gynodioecious species that contain intermediate sexual phenotypes, with individuals carrying flowers with nondehiscent and/or less numerous anthers, producing lower quantity and/or quality of pollen (e.g. Koelewijn & van Damme, 1996; Poot, 1997) or carrying a mixture of female and perfect flowers (e.g. Shykoff, 1992; Lopez-Villavicencio *et al.*, 2005).

It is not clear how continuous variation in viable pollen production would change the prediction of existing models. Dufay *et al.* (2007) showed that it is not possible to maintain male-fertile and CMS cytotypes if non-CMS hermaphrodites are systematically better pollen producers than restored CMS hermaphrodites (i.e. in the case of an expressed cost of restoration). However, in wild populations, incompletely restored CMS hermaphrodites may constitute a reservoir of restorers in the population until the appearance of fully restored hermaphrodites, which would efficiently produce pollen and change the population sex ratio. Such complex determination may then slow down the positive selection of restorer factors and help maintain females in populations, as suggested by Bailey & Delph (2007).

## Conclusion

Altogether, our results suggest that (i) females have a low advantage over hermaphrodites, (ii) restored CMS hermaphrodites (carrying both CMS genes and nuclear restorers) may suffer a slight decrease in female function compared to non-CMS hermaphrodites and (iii) restored CMS hermaphrodites are poor pollen producers compared to non-CMS hermaphrodites, probably as a consequence of complex determination of restoration. While this last point is typically not taken into account in theoretical models, the restricted female advantage and the trend for a cost of CMS genes found in this study, along with the existence of a cost of restoration (see Dufay *et al.*, 2008) could theoretically allow the maintenance of cytonuclear polymorphism in gynodioecious populations (Dufay *et al.*, 2007). However, models generally consider infinite panmictic populations and do not account for the potential effects of sex structure (but see McCauley & Taylor, 1997). In *B. vulgaris*, as well as in various other gynodioecious species, sexual phenotypes are classically strongly structured in space (e.g. Tarayre & Thompson, 1997; Olson & McCauley, 2002; Asikainen & Mutikainen, 2003; Alonso, 2005; Nilsson & Agren, 2006; Cuevas *et al.*, 2008; De Cauwer *et al.*, 2010b), which could modify importantly the expected results of selection. For example, female reproductive output has been shown to decrease in female-biased environments in several gynodioecious species, including *B. vulgaris*, due to pollen limitation (Widen & Widen, 1990; Graff, 1999; Alonso, 2005; Zhang *et al.*, 2008; De Cauwer *et al.*, 2010a). This could locally offset female advantage in demes where pollen donors are rare, in particular in species where female advantage is restricted, such as our study species. Population structure at sex-determining genes could also modify the expected results of selection on restorer alleles in different ways. On the one hand, spatial genetic structure should greatly limit the probability of appearance of fully restored CMS hermaphrodites in local demes, particularly in species growing in disturbed environments, such as *B. vulgaris*, because of the increased risk of stochastic loss of restoration alleles. On the other hand, population structure may also favour restored CMS hermaphrodites over non-CMS ones, by clustering restored CMS hermaphrodites with females, as observed in a particular population of *B. vulgaris*, where restored CMS hermaphrodites, although being poor pollen producers, sired significantly more seedlings than non-CMS hermaphrodites (De Cauwer *et al.*, 2010b). Because population structure can modify the effects selection in different ways, theoretical models taking into account the effects of both population structure and complex genetic determination are clearly needed to further explore the conditions of maintenance of sex polymorphism.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Figure S1** Quantity of pollen (average over the four measured anthers) produced by the first flowers that opened along the main floral stem, for all studied *B. vulgaris* ssp. *maritima* individuals. For individuals that flowered both in 2008 and 2009, the mean value over the both study years was calculated. Individuals are ranked according to the quantity of pollen per anther. Plants that were initially typed as females are represented in white, as hermaphrodites in grey. The dashed box contains all plants that were considered as females in statistical analyses (mean pollen quantity produced per anther in the first flowering week <7000 pollen grains).

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