

## Does inbreeding avoidance maintain gender dimorphism in *Wurmbea dioica* (Colchicaceae)?

M. RAMSEY,\* G. VAUGHTON\* & R. PEAKALL†

\*Botany, University of New England, Armidale, NSW, Australia

†School of Botany and Zoology, Australian National University, ACT, Australia

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*Wurmbea*.

### Abstract

The maintenance of females in gender dimorphic populations requires that they have a fitness advantage to compensate for their loss of male reproductive function. We assess whether inbreeding avoidance provides this advantage in two subdioecious *Wurmbea dioica* populations by estimating seed production, outcrossing rates and inbreeding depression. Fruiting males produced less than half as many seeds as females, owing to low outcrossing rates and early acting inbreeding depression. Inbreeding coefficients of fruiting males demonstrated that progeny were more inbred than their parents, implying that few selfed progeny reach maturity, as confirmed by inbreeding depression estimates that exceeded 0.85. In a glasshouse experiment, open-pollinated females exhibited a fitness advantage of 3.7 relative to fruiting males, but when we increased fruiting male outcrossing rate, female advantage was only 1.4. This reduced advantage is insufficient to maintain females if nuclear genes control sex. Thus, inbreeding avoidance could maintain females at high frequencies, although this is contingent upon high frequencies of fruiting males, which can be altered by environmentally determined gender plasticity.

### Introduction

The evolution of separate sexes from hermaphroditic ancestors has occurred repeatedly among angiosperms. The study of the selective mechanisms responsible for this transition is of continuing interest in evolutionary biology (reviewed by Geber *et al.*, 1999). Gynodioecy can be an intermediate stage in the evolution of dioecy and has been a focus of attention because females spread and persist in populations despite their gametic disadvantage relative to hermaphrodites. If nuclear genes control male sterility, then females require a greater than two-fold fitness advantage to persist in populations (Lloyd, 1976; Charlesworth & Charlesworth, 1978; Frank, 1989; Bailey *et al.*, 2003). Two factors that can promote the spread of

females are resource compensation and inbreeding avoidance. Resource compensation occurs if females allocate resources to female reproductive functions that would have been otherwise committed to male functions, resulting directly or indirectly in increased ovule success. Inbreeding avoidance favours females if inbreeding depression following self-fertilization lowers the ovule success of hermaphrodites compared with females that are outcrossed (Charnov, 1982; Lloyd, 1982; Charlesworth, 1999).

To examine the roles of resource compensation and inbreeding avoidance in the initial spread of females in populations the most useful species are those in which females are at the earliest stages of the invasion process. This is because continued female presence in populations alters resource allocation, selfing rates and expression of inbreeding depression, rendering it difficult to determine the factors that initially favoured female invasion (Webb, 1999). However, the study of populations in which females are established can provide insight into how

Correspondence: Mike Ramsey, Botany, University of New England, Armidale, NSW, 2351, Australia.  
Tel.: +61 26773 3006; fax: +61 26673 3283;  
e-mail: mramsey@une.edu.au

females are maintained, and inform on the stability of the sexual system. Subdioecy affords opportunities to address these issues because it is an extreme form of gynodioecy considered to be a late stage in the evolutionary transition to dioecy (Delph & Wolf, 2005). In subdioecious populations, females coexist with two polliniferous phenotypes, males and hermaphrodites (hereafter, fruiting males). Females exhibit canalized sex expression and can be maintained at high frequencies approaching 50%. By contrast, some males may exhibit canalized sex expression, but others express either male or fruiting male phenotypes during different flowering episodes (i.e. gender plasticity: Delph, 1990; Wolfe & Shmida, 1997; Barrett *et al.*, 1999; Olson, 2001). Combined with resource compensation and inbreeding avoidance, gender plasticity can contribute to high female frequencies because in some years fruiting males produce few or no seeds, which increases relative fitness of females over their lifetime. Additionally, gender plasticity could stabilize subdioecy and hinder the evolution to dioecy, providing that seed production by fruiting males contributes to their overall fitness (Delph, 1990; Barrett *et al.*, 1999; reviewed by Delph & Wolf, 2005).

The genus *Wurmbea* (Colchicaceae) consists of about 47 species of small, insect-pollinated geophytes occurring in temperate Africa and Australia. African species are uniformly cosexual, whereas at least 10 of the approximately 30 Australian taxa are gender dimorphic (Macfarlane, 1987; Barrett & Case, 2006). Recent phylogenetic work indicates that gender dimorphism in *Wurmbea* has probably evolved independently on several occasions (A. L. Case, S. W. Graham, T. D. Macfarlane and S. C. H. Barrett, unpublished data). The sexual system variation displayed by the Australian taxa ranges from hermaphroditism through gynodioecy to dioecy and this variability provides an excellent opportunity for understanding factors underlying the evolution and maintenance of separate sexes. Subdioecy occurs in many populations of *Wurmbea dioica* ssp. *dioica* in southeastern Australia. In these populations, about 70% of polliniferous plants exhibit gender plasticity and can flower as either males that produce staminate flowers or fruiting males that produce varying proportions of staminate and perfect flowers. The expression of female function in gender plastic males increases as environmental conditions become more favourable (Barrett, 1992; Barrett *et al.*, 1999; Ramsey & Vaughton, 2001). Based on floral biology and controlled pollinations, Vaughton & Ramsey (2003) proposed that inbreeding avoidance could be important in maintaining females at high frequencies (see also Case & Barrett, 2004). Specific tests of this proposal require that high selfing rates and inbreeding depression be demonstrated in fruiting males. Further, these factors should be considered jointly with gender plasticity to predict whether subdioecy is stable or whether populations are evolving towards dioecy.

Here we examine the role of inbreeding avoidance in maintaining high female frequencies in two subdioecious populations of *W. dioica* ssp. *dioica*. We first estimate the seed advantage of females over 5 years by assessing seed set of naturally pollinated fruiting males and females. Next, we assess natural levels of inbreeding of both sex morphs using allozymes as genetic markers. Finally, we assess the fitness consequences of inbreeding in fruiting males by estimating the magnitude of inbreeding depression using marker based and experimental approaches. We observed high selfing rates and inbreeding depression in fruiting males and discuss how these factors may interact with gender plasticity in maintaining females and determining the stability of subdioecy.

## Materials and methods

### Study species and populations

*Wurmbea dioica* (R. Br.) F. Muell. ssp. *dioica* (Colchicaceae) occurs in grasslands, woodlands and forests in southeastern Australia. Plants have a corm, an annual shoot with three leaves, and flower in spring producing an erect cymose inflorescence with one to 10 flowers. Tepals are white, each with a conspicuous purple nectary near the base. Fruit capsules have up to 50 seeds (Macfarlane, 1987). The sex determination mechanism is unknown.

We conducted this study at two woodland sites (YY, 37°57'S, 145°10'E; WA, 37°42'S, 145°00'E), about 50 km north of Melbourne, Australia. Populations were about 25 km apart and each contained several thousand plants. Frequencies of females, males and fruiting males were similar over 5 years (0.43, 0.50 and 0.07, respectively;  $n$  = about 1000 plants/year). Flowers are open for up to 7 days and are visited mainly by generalist dipteran pollinators that collect nectar. Other floral visitors include native bees and butterflies (Vaughton & Ramsey, 1998). Fruiting males are partially self-fertile, but do not autonomously self-pollinate. Separation of anther dehiscence and stigma receptivity is incomplete, providing opportunities for pollinator-mediated selfing (Vaughton & Ramsey, 2003).

### Open-pollinated seed set

For 5 years at YY, females and fruiting males were marked as they started to flower. The number of ovuliferous flowers on each plant was noted, and fruits of 25 plants were collected 6 weeks later. Numbers of filled seeds, aborted seeds and undeveloped ovules were counted in the first fruit on each plant for 3 years, and for 2 years numbers of seeds per plant were counted. Aborted seeds were smaller and shriveled compared with filled seeds. Per cent seed set was determined as: (seeds)/(seeds + aborted seeds + ovules). To compare per cent seed set, we used a two-factor analysis of variance

(ANOVA), with sex morph and year as fixed and random factors respectively. The morph  $\times$  year interaction was not significant ( $P = 0.472$ ), and was omitted from the final ANOVA. For seeds per plant, we used a two-factor analysis of covariance (ANCOVA) with sex morph (fixed) and year (random) as factors, and the number of ovuliferous flowers as a covariate. Interactions involving the covariate were not significant (year  $\times$  flowers,  $P = 0.788$ ; morph  $\times$  flowers,  $P = 0.331$ ), and they were omitted from the analysis. The morph  $\times$  year interaction was also not significant ( $P = 0.911$ ), and it was omitted from the final ANCOVA.

### Mating system

We collected mature seeds from open-pollinated fruiting male and female plants in each population and assayed progeny for allozyme variation using cellulose acetate electrophoresis. In total, 720 progeny representing 24 plants each for YY and WA (12 males and 12 females per population, 15 seeds per family) were analyzed. For each plant, 20–30 seeds were germinated in Petri dishes, and 15 randomly selected seedlings per family were stored at  $-80^{\circ}\text{C}$  until electrophoresis. Seedlings were ground in an extraction buffer modified from Peakall & Beattie (1991). Three putative loci showed consistently clear and interpretable banding patterns: glucose-phosphate isomerase (*Gpi*) and phosphoglucosyltransferase (*Pgm-1* and *Pgm-2*). *Gpi* and *Pgm* were resolved using 0.1 M Tris-EDTA-maleate  $\text{MgCl}_2$  pH 7.4 buffer and 0.025 M Tris-glycine pH 8.5 buffer respectively. Three to five alleles were observed at each locus.

We estimated multilocus outcrossing rates ( $t_m$ ) for fruiting males and females in each population using the maximum-likelihood program MLTR (Version 2.4, Ritland, 2002). MLTR uses maximum-likelihood procedures to infer maternal plant genotypes, allele frequencies in the pollen pool, the proportion of progeny that are outcrossed and parental inbreeding coefficients ( $f$ ). Separate estimates of  $t_m$  and  $f$  were calculated for fruiting males and females in each population; the estimates did not differ from those obtained when we used entire population data sets to calculate pollen allele frequencies. Expectation-maximization procedure was used for the iterations. We also estimated parental and progeny inbreeding coefficients separately for each sex morph using the software program GENETIC DATA ANALYSIS (GDA, Version 1.1, Lewis & Zaykin, 2001). This allowed us to assess directly differences in  $f$  between the two generations. We estimated parental  $f$  using the inferred parental genotypes from the MLTR output.

For each sex morph, we calculated inbreeding depression ( $\delta = 1 - \text{fitness of selfed progeny}/\text{fitness of outcrossed progeny}$ ) for survival from seed germination to reproductive maturity using the estimates of  $t_m$  and parental  $f$  from the MLTR output as:  $\delta = 1 - [2f(t_m)/(1 - t_m)(1 - f)]$  (Ritland, 1990). This method of estimat-

ing  $\delta$  assumes that  $t_m$  and adult  $f$  do not fluctuate among generations (i.e. inbreeding equilibrium; Ritland, 1990).

Standard errors of  $t_m$ ,  $f$  and  $\delta$  were based on 1000 bootstrap samples generated from the MLTR program. For tests of statistical significance, we examined the distribution of 1000 bootstrap values following Eckert & Barrett (1994). A one-tailed test of a given parameter is considered to be either significantly less than or greater than reference values such as zero or one if 100  $(1 - \alpha)$  per cent of bootstrap values are either less than or greater than the reference value, respectively. In each population, we tested whether estimates of  $t_m$  and  $\delta$  differed from one and parental  $f$  and progeny  $f$  differed from zero. We then compared  $t_m$  of the sex morphs and  $f$  of parents and their progeny by testing whether 100  $(1 - \alpha)$  per cent of the differences in bootstrap samples were less than zero. To reduce Type I errors when multiple comparisons were performed, we used Bonferroni adjustments to maintain  $\alpha = 0.05$ .

Inbreeding depression at the seed production and germination stages results in a lower selfing rate in seedlings than in zygotes. We estimated the zygote selfing rate at fertilization for fruiting males by adjusting the seedling selfing rate ( $s_m = 1 - t_m$ ) for inbreeding depression as:  $r = s_m / (1 - \delta_s + s_m \delta_s)$  (Maki, 1993; Lande *et al.*, 1994). We estimated  $\delta_s = 0.613$  from seed set and germination for selfed and crossed males from YY and used the average male  $t_m$  from the two populations (Tables 1 and 2).

### Relative performance and inbreeding depression

#### Pollination procedures

From YY, we excavated 34 fruiting males and 15 females on which the first flowers were just opening. Plants were potted using soil from the study site, sand and peat (2 : 1 : 1), and placed in an unheated, pollinator-free glasshouse. Fruiting males were either cross- or self-pollinated ( $n = 15$  and 19, respectively). We emasculated flowers on crossed fruiting males before anthers opened and crossed them using pollen from several male plants. We selfed fruiting males using pollen from the same plant. Females were crossed similarly to fruiting males ( $n = 15$ ). Other fruiting males and females (both  $n = 15$ ) were marked in the field as open-pollinated controls. Shortly after flowering, these plants were excavated, established in the glasshouse and treated as the hand-pollinated plants. We watered and fertilized all plants regularly.

#### Seed set and progeny performance

We harvested fruits from each plant in the five pollination treatments and counted the numbers of seeds and aborted seeds. We compared numbers of seeds and aborted seeds among treatments with ANCOVAs, with the number of flowers per plant as a covariate. The pollination  $\times$  covariate interactions were not significant

| Population | $t_m$                | $f_{\text{parental}}$            | $f_{\text{progeny}}$            | $\delta^\dagger$                |
|------------|----------------------|----------------------------------|---------------------------------|---------------------------------|
| YY         |                      |                                  |                                 |                                 |
| Males      | 0.390*** $\pm$ 0.108 | -0.068 <sup>NS</sup> $\pm$ 0.194 | 0.514*** $\pm$ 0.054            | 0.954 <sup>NS</sup> $\pm$ 0.141 |
| Females    | 0.748*** $\pm$ 0.079 | -0.118 <sup>NS</sup> $\pm$ 0.114 | 0.144** $\pm$ 0.037             | 1.000 <sup>NS</sup> $\pm$ 0.024 |
| WA         |                      |                                  |                                 |                                 |
| Males      | 0.336*** $\pm$ 0.113 | -0.108 <sup>NS</sup> $\pm$ 0.073 | 0.441*** $\pm$ 0.072            | 1.000 <sup>NS</sup> $\pm$ 0.039 |
| Females    | 0.855*** $\pm$ 0.064 | -0.118*** $\pm$ 0.012            | 0.074 <sup>NS</sup> $\pm$ 0.056 | 1.000 <sup>NS</sup> $\pm$ 0.002 |

Standard errors were estimated from 1000 bootstrap values. In each population, 180 seedlings from 12 families of each gender were used. Superscripts denote estimates that are significantly different from either one ( $t_m$ ,  $\delta$ ) or zero ( $f$ ).

$^\dagger$ Calculated as:  $1 - [2(t_m)f_{\text{parental}} / (1 - t_m)(1 - f_{\text{parental}})]$ ;  $t_m$  and  $f$  from MLTR output.

<sup>NS</sup> $P > 0.05$ ; \*\* $P = 0.006$ ; \*\*\* $P < 0.001$ .

| Trait                   | Males           |                 |                 | Females         |                 |
|-------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                         | Self            | Open            | Cross           | Open            | Cross           |
| Number of seeds         | 20.9 $\pm$ 3.4  | 27.2 $\pm$ 2.1  | 42.6 $\pm$ 4.8  | 55.3 $\pm$ 7.6  | 60.1 $\pm$ 7.4  |
| Number of aborted seeds | 15.9 $\pm$ 2.8  | 16.3 $\pm$ 2.5  | 7.4 $\pm$ 0.7   | 8.2 $\pm$ 1.1   | 7.7 $\pm$ 1.4   |
| Seed mass (mg)          | 1.01 $\pm$ 0.06 | 0.97 $\pm$ 0.04 | 1.35 $\pm$ 0.07 | 1.21 $\pm$ 0.05 | 1.28 $\pm$ 0.06 |
| Seed germination (%)    | 74.7 $\pm$ 4.7  | 80.9 $\pm$ 4.7  | 94.8 $\pm$ 1.8  | 96.4 $\pm$ 1.4  | 94.7 $\pm$ 1.3  |
| Leaf length (mm)        | 51.4 $\pm$ 3.0  | 51.4 $\pm$ 2.0  | 65.9 $\pm$ 3.3  | 62.4 $\pm$ 1.7  | 71.5 $\pm$ 3.1  |
| Corm mass (mg)          | 13.1 $\pm$ 2.2  | 14.2 $\pm$ 2.1  | 21.4 $\pm$ 1.5  | 19.0 $\pm$ 1.7  | 20.1 $\pm$ 2.3  |
| Total plant mass (mg)   | 15.4 $\pm$ 2.6  | 16.2 $\pm$ 2.5  | 25.2 $\pm$ 1.7  | 21.2 $\pm$ 2.0  | 22.2 $\pm$ 2.8  |
| Survival (%)            | 55.7 $\pm$ 6.4  | 72.5 $\pm$ 5.2  | 88.5 $\pm$ 3.6  | 82.3 $\pm$ 4.5  | 83.3 $\pm$ 4.7  |

**Table 1** Multi-locus outcrossing rates ( $t_m$ ) of fruiting males and females, inbreeding coefficients of parents and their progeny ( $f$ ), and inbreeding depression ( $\delta$ ) in two *Wurmbea dioica* populations.

**Table 2** Mean ( $\pm$ SE) traits for fruiting male and female maternal families in *Wurmbea dioica* following five different pollination treatments. Between nine and 15 families per treatment were used. Analyses are given in Table 3.

(seeds,  $P = 0.713$ ; abortions,  $P = 0.508$ ), and they were omitted from final analyses. The covariate was positively related to both traits (seeds and abortions, both  $P < 0.001$ ).

We weighed individually either 15 seeds or all of the seeds produced from each of 15 families in the five pollination treatments ( $n = 1111$  seeds). Of the 19 selfed fruiting males, five produced between 11 and 14 seeds and four produced too few seeds for the subsequent experiments. We calculated mean seed mass per family and compared treatments using a one-factor ANOVA, with families as replicates.

We assessed seed germination by placing 11–15 seeds from each family in separate Petri dishes on moistened filter paper ( $n = 1111$  seeds). Three dishes per treatment were assigned to each of five shelves in a germination cabinet set at 12-h light (16°C) and 12 h darkness (8°C). Dishes were rearranged within shelves weekly to reduce microenvironmental variation. Seed germination was scored every week for 4 months. Ungerminated seeds had brown embryos and were not viable. We initially compared per cent seed germination using a two-factor random block ANOVA with pollination treatment and cabinet shelves and as fixed and random block factors, respectively. Shelves and the shelves  $\times$  treatment interaction were not significant (shelves,  $P = 0.266$ ; interaction,  $P = 0.337$ ), and they were omitted from the final analysis. We then compared germination among treatments using a one-factor ANOVA.

We planted eight seedlings from 11 selfed and 10 open-pollinated fruiting male families, and 12 families each of crossed fruiting males and crossed and open females ( $n = 456$  seedlings). Seedlings were planted into 12, 64-cell seedling trays (cell volume, 52 cm<sup>3</sup>) containing sand, loam and peat (1 : 1 : 1). Most trays contained one family of each of the five pollination treatments. Trays were placed in a growth cabinet at 12-h light (20°C) and 12-h darkness (10°C) for 4 weeks; all seedlings survived transplanting. Trays were then transferred to a glass-house and their positions were rearranged every 10–14 days to minimize micro-environmental variation. Plants were watered regularly and fertilized every month with half-strength liquid fertilizer.

Before seedlings entered summer dormancy, they all produced a single leaf that we measured ( $n = 456$  seedlings). During dormancy, we reduced the frequency of watering to every 14 days. After 4 months, the previous watering and fertilizing regimes were resumed, and resprouting was monitored. At the end of the second growing season prior to the dormancy period, we counted the number of surviving individuals in each family. We then excavated plants and weighed corms and leaves separately after at drying 65°C.

We calculated per cent survival and means for leaf length, and corm and plant mass for each family, and compared treatments using random block ANOVAs with trays as blocks and families as replicates. In all analyses, trays were not significant (all  $P > 0.70$ ), and they were

omitted from further analyses. We then compared treatments using one-factor ANOVAS. We were interested in five nonorthogonal, planned contrasts: (i) selfed vs. crossed fruiting males, to estimate inbreeding depression; (ii) open fruiting males vs. open females, to examine differences in fitness of the sex morphs following natural pollination; (iii) crossed fruiting males vs. crossed females, to examine differences in fitness of the sex morphs when outcrossing rate was equal; (iv) open vs. cross-pollinated fruiting males, to examine effects of inbreeding following natural pollination on fruiting male fitness; and (v) open vs. crossed females, to examine effects of biparental inbreeding following natural pollination on female fitness. We used sequential Bonferroni adjustments to maintain an overall significance level of  $\alpha = 0.05$ .

Here and elsewhere, we transformed counts, measurements and masses using natural logarithms, and percentages using arc-sine square-root. Means ( $\pm$ SE) are given. Analyses were computed using JMP (version 5.01a, SAS Institute Inc., NC, USA).

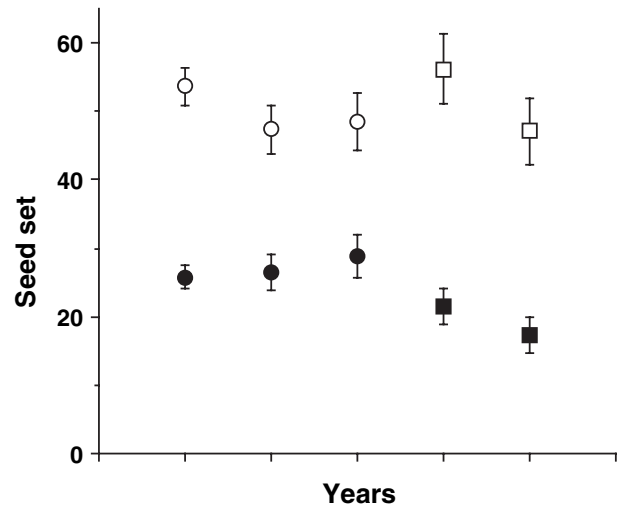
#### Relative performance and inbreeding depression

We estimated the relative performance (RP) of selfed to crossed fruiting males as:  $RP = w_s/w_c$ , where  $w_s$  and  $w_c$  are the performances of selfed and crossed progeny, respectively. RP was calculated for seed production, seed germination, seedling survival and corm mass, the latter included as an indicator of differences in growth rate that may affect future survival and fecundity. Pollination treatments were undertaken on different plants, and RPs were calculated as the ratios of trait means without regard to family, providing population rather than family estimates. Cumulative RP was estimated as the product of the RPs of the above traits. Inbreeding depression was summarized as:  $\delta = 1 - \text{cumulative RP}$  (Charlesworth & Charlesworth, 1987). To correspond with the planned contrasts described in the previous section, we also calculated individual and cumulative RPs for open fruiting males to open females, crossed fruiting males to crossed females, open to crossed fruiting males and open to crossed females. We calculated standard errors for RP estimates using 1000 bootstrap samples randomly resampled from the original data (Chernick, 1999). We tested whether the estimates, or differences between estimates, differed from one or zero, respectively, using methods described above for the mating system analyses.

## Results

### Open-pollinated seed set

Fruiting males produced fewer seeds than did females in all years (Fig. 1). For fruiting males, both per cent seed set and number of seeds per plant were only about half that of females (per cent:  $F_{1,146} = 75.28$ ,  $P < 0.0001$ ; number:  $F_{1,96} = 73.27$ ,  $P < 0.0001$ ). Seed production did



**Fig. 1** Mean ( $\pm$ SE) seed set of open-pollinated fruiting males (closed symbols) and females (open symbols). Per cent seed set of the first flower on plants (circles) and the number of seeds per plant (squares) were examined for 3 and 2 years, respectively (all years,  $n = 25$ ). Females produced more seeds than did fruiting males in all years ( $P < 0.001$ ).

not differ between years (per cent:  $F_{2,146} = 0.45$ ,  $P = 0.637$ ; number:  $F_{1,96} = 1.28$ ,  $P = 0.261$ ). The number of seeds per plant was positively related to the number of ovuliferous flowers in fruiting males and females ( $F_{1,96} = 11.06$ ,  $P = 0.001$ ).

### Mating system

Outcrossing rates ( $t_m$ ) of fruiting males were less than those of females in both populations ( $P < 0.001$ ; Table 1). Females experienced biparental inbreeding as evidenced by outcrossing rates that were less than one ( $P < 0.001$ ). Although outcrossing rates of fruiting males and females differed, parental inbreeding coefficients ( $f$ ) were similar ( $P > 0.270$ ) and did not differ from, or were less than, zero (Table 1). All progeny were inbred ( $f > 0$ ), except for the progeny of females at WA ( $f = 0.07$ ). Progeny were more inbred than their parents (all  $P < 0.005$ ), although the differences in  $f$  between fruiting males and their progeny were substantially greater than those between females and their progeny. All marker-based estimates of inbreeding depression exceeded 0.95, and none differed from one (Table 1). Fruiting males experienced considerable selfing and early-acting inbreeding depression as evidenced by the difference between the adjusted zygote selfing rate (0.80) and the average seedling selfing rate (0.63).

### Relative performance and inbreeding depression

Pollination treatment had pronounced effects on seed production and progeny fitness (Tables 2 and 3). All traits

**Table 3** Results of ANOVAS comparing five pollination treatments conducted on fruiting males and females in *Wurmbea dioica*.

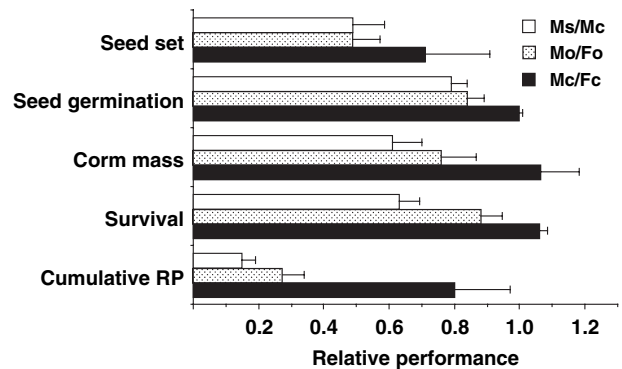
| Trait                   | ANOVAS    |      |       |       |         | Planned comparisons |               |           |                   |           |
|-------------------------|-----------|------|-------|-------|---------|---------------------|---------------|-----------|-------------------|-----------|
|                         |           | d.f. | MS    | F     | P       | Ms vs. Mc           | Mo vs. Fo     | Mc vs. Fc | Mo vs. Mc         | Fo vs. Fc |
| Number of seeds         | Treatment | 4    | 4.708 | 17.29 | <0.0001 | <b>&lt;0.0001</b>   | <b>0.0003</b> | 0.0423    | 0.0307            | 0.3731    |
|                         | Error     | 73   | 0.272 |       |         |                     |               |           |                   |           |
| Number of aborted seeds | Treatment | 4    | 1.636 | 6.81  | <0.0001 | <b>0.0013</b>       | <b>0.0008</b> | 0.4206    | <b>0.0005</b>     | 0.2968    |
|                         | Error     | 73   | 0.240 |       |         |                     |               |           |                   |           |
| Seed mass (mg)          | Treatment | 4    | 0.328 | 7.94  | <0.0001 | <b>&lt;0.0001</b>   | <b>0.0017</b> | 0.2726    | <b>&lt;0.0001</b> | 0.2475    |
|                         | Error     | 70   | 0.041 |       |         |                     |               |           |                   |           |
| Seed germination (%)    | Treatment | 4    | 1409  | 11.08 | <0.0001 | <b>&lt;0.0001</b>   | <b>0.0001</b> | 0.4253    | <b>0.0013</b>     | 0.1983    |
|                         | Error     | 70   | 127   |       |         |                     |               |           |                   |           |
| Leaf length (mm)        | Treatment | 4    | 0.248 | 10.16 | <0.0001 | <b>0.0001</b>       | <b>0.0022</b> | 0.1019    | <b>0.0007</b>     | 0.0253    |
|                         | Error     | 52   | 0.024 |       |         |                     |               |           |                   |           |
| Corm mass (mg)          | Treatment | 4    | 0.776 | 4.49  | 0.003   | <b>0.0006</b>       | 0.0236        | 0.2726    | <b>0.0071</b>     | 0.4223    |
|                         | Error     | 52   | 0.173 |       |         |                     |               |           |                   |           |
| Total plant mass (mg)   | Treatment | 4    | 0.776 | 3.85  | 0.008   | <b>0.0009</b>       | 0.0331        | 0.1513    | <b>0.0056</b>     | 0.4921    |
|                         | Error     | 52   | 0.201 |       |         |                     |               |           |                   |           |
| Survival (%)            | Treatment | 4    | 1198  | 5.23  | 0.001   | <b>0.0001</b>       | 0.0472        | 0.2802    | 0.0186            | 0.3911    |
|                         | Error     | 52   | 229   |       |         |                     |               |           |                   |           |

Means ( $\pm$ SE) are given in Table 2.

Number of seeds and abortions were analysed with ANCOVAs with the number of flowers per plant as covariates (both  $P \leq 0.001$ ). Other traits were analysed with one-way ANOVAs. Planned, nonorthogonal comparisons were: male self vs. male cross; male open vs. female open; male cross vs. female cross; male open vs. male cross; and female open vs. female cross. For these comparisons,  $P$ -values presented in bold were significant following sequential Bonferroni adjustments.

for crossed fruiting males and open and crossed females were greater than those of open and selfed fruiting males. For the planned contrasts, crossed fruiting males outperformed selfed fruiting males for all traits. Open females outperformed open fruiting males for all traits, although corm mass, total plant mass and survival were not significant following Bonferroni adjustment. Crossed females produced more seeds than did crossed fruiting males, but this was not significant following Bonferroni adjustment. No other traits differed. Crossed fruiting males outperformed open fruiting males for all traits, although seed set and seedling survival were not significant following Bonferroni adjustment. Finally, crossed and open females did not differ for any of the traits (Tables 2 and 3).

Inbreeding depression following self-pollination was severe (Fig. 2). Relative performances (RP) of selfed to crossed fruiting males for the four life-cycle stages ranged from 0.49 to 0.79. Cumulative RP was less than one ( $P < 0.001$ ), and inbreeding depression was  $0.85 \pm 0.04$ , resulting in a 6.7-fold fitness advantage for crossed fruiting males. Following natural pollination of fruiting males and females, females had a 3.7-fold fitness advantage (Fig. 2). Relative performance for seed set was only 0.49 and about 0.80 for the other traits. Cumulative RP was less than one ( $P < 0.001$ ). When we experimentally equalized outcrossing rates of fruiting males and females, female fitness advantage was reduced substantially (Fig. 2). Relative performance for seed set was only 0.71, but about 1.0 for the other traits. RP for seed set was low because females produced more ovules,



**Fig. 2** Relative performances of self- to cross-pollinated fruiting males (Ms/Mc), open-pollinated fruiting males to open-pollinated females (Mo/Fo) and cross-pollinated fruiting males to cross-pollinated females (Mc/Fc). Cumulative relative performances are the product of the relative performances of the individual traits. Data are ratios of treatment means, with standard errors estimated from 1000 bootstrap values randomly resampled from the original data.

and hence more seeds. Cumulative RP with and without seed set were  $0.80 \pm 0.17$  and  $1.13 \pm 0.14$ , respectively, and although both estimates did not differ from one ( $P > 0.125$ ), they differed from each other ( $P < 0.015$ ).

Inbreeding following natural pollination substantially reduced fruiting male fitness. Relative performances of open to crossed fruiting males for the four life-cycle stages ranged from 0.64 to 0.85 and cumulative RP was less than one ( $0.23 \pm 0.07$ ,  $P < 0.001$ ). By contrast,

biparental inbreeding had minimal effect on female fitness. Relative performances of open- to cross-pollinated females ranged from 0.92 to 1.02. Cumulative RP was  $0.88 \pm 0.21$  and did not differ from one ( $P = 0.264$ ).

## Discussion

Our data highlight the potential importance of inbreeding avoidance in maintaining females at high frequencies in populations in *Wurmbea dioica*. Under natural conditions, more than half of the seeds produced by fruiting males were self-fertilized. By contrast, females were predominantly outcrossed, resulting in superior performance relative to fruiting males and a 3.7-fold fitness advantage. The reduced fitness of fruiting males was caused by the expression of severe inbreeding depression following selfing. By experimentally increasing outcrossing rate, we increased fitness of fruiting males by 6.7-fold, which reduced the female fitness advantage to 1.4-fold. Below we discuss how high selfing rates and inbreeding depression maintain females in populations and consider how these factors interact with gender plasticity to determine the stability of subdioecy in *W. dioica*.

### Seed set and outcrossing rates

As with many gynodioecious and subdioecious species (reviewed by Shykoff *et al.*, 2003) females of *W. dioica* produced significantly more seeds than fruiting males. Over the 5 years of our study, seed set of open-pollinated fruiting males was consistently lower than that of open-pollinated females, providing females with a two- to three-fold seed advantage. Similar female advantages have been reported by previous single-year studies of *W. dioica* (Barrett *et al.*, 1999; Ramsey & Vaughton, 2001). Seed set of fruiting males is unlikely to be limited by either insufficient pollination or insufficient resources. Pollinators deposit excess pollen onto stigmas, and seed set can be increased substantially when flowers are experimentally cross-pollinated (Vaughton & Ramsey, 2003).

One factor that contributes to the low seed set of *W. dioica* fruiting males is the production of fewer ovules. Fruiting males produce 11% fewer ovuliferous flowers and 24% fewer ovules per flower than do females, a possible consequence of resource compensation or the spread of genetic modifiers in males that reduce female function and facilitate increases in male function (Ramsey & Vaughton, 2001). The disadvantage of producing fewer ovules can be assessed independently of pollen quality by experimentally increasing and equalizing outcrossing rates and then comparing seed production of the sex morphs. Cross-pollinated fruiting males converted a similar proportion of ovules to seeds as did females, but they still produced fewer seeds because they produced fewer ovules. However, cross-pollination

reduced the relative female seed advantage to only 1.4, which is less than the two-fold advantage required to maintain females at low frequencies in populations, if sex is controlled by nuclear factors (Charlesworth & Charlesworth, 1978). These findings indicate that the female ovule advantage is insufficient to maintain females at high frequencies in *W. dioica*, and other factors such as inbreeding avoidance and gender plasticity must also be involved.

Seedling outcrossing rates of fruiting males were significantly less than those of females in both populations ( $t_m \approx 0.36$  vs. 0.80). The adjusted selfing rate ( $s = 1 - t$ ) for fruiting males, which accounts for early-acting inbreeding depression at stages preceding seed germination (Maki, 1993), implied even greater selfing than the seedling outcrossing rates ( $s = 0.80$  vs. 0.64). The differences in seedling outcrossing rates between the sex morphs are comparable to those reported for other self-compatible dimorphic species (reviewed by Collin & Shykoff, 2003). In our study, the fitness consequence of mixed mating by the fruiting males can be assessed in the light of the findings of the pollination experiment. Seed set and abortion of open-pollinated fruiting males were intermediate to that following either selfing or crossing, as would be expected if plants were pollinated with both cross and self pollen, and selfed zygotes were aborted owing to early-acting inbreeding depression. Collectively, our findings for *W. dioica* mirror those for dimorphic populations of *W. biglandulosa* (Ramsey *et al.*, 2006). In both species, high selfing rates leading to the expression of early-acting inbreeding depression are major factors contributing to the seed advantage enjoyed by females under natural conditions.

### Inbreeding depression in fruiting males

Cumulative inbreeding depression ( $\delta$ ) was substantial, indicating that selfed progeny of fruiting males bear a severe fitness cost throughout their life cycle. High estimates of inbreeding depression were found using both Ritland's (2002) marker-based approach ( $\delta \geq 0.95$ ), and an approach that compares the fitness of experimentally self- vs. cross-pollinated plants ( $\delta = 0.85$ ). Owing to this inbreeding depression, fruiting males that were crossed exhibited a 6.7-fold fitness advantage compared with those that were selfed. Inbreeding depression also reduced fitness of open-pollinated fruiting males that exhibited mixed mating. Both crossed males and open females exhibited almost four-fold fitness differences relative to open fruiting males. Our inbreeding depression estimates are similar to those reported for *W. biglandulosa* and several other dimorphic species (Sakai *et al.*, 1989; Kohn & Biardi, 1995; Schultz & Ganders, 1996; Sakai *et al.*, 1997; Ramsey *et al.*, 2006). In such species, high levels of inbreeding depression in combination with moderate to high selfing are likely to be important in maintaining females in populations. Not

all dimorphic species, however, exhibit these traits (see Table 4 in Mutikainen & Delph, 1998). Other factors such as nucleo-cytoplasmic control of sex, maternal effects on seed quality, pleiotropic effects of male restorer genes and/or resource compensation are likely to be important in maintaining females in such species (Ashman, 1992; Eckhart, 1992; Delph & Mutikainen, 2003).

High marker-based estimates of inbreeding depression arise when parental inbreeding coefficients are near zero in populations experiencing substantial selfing, implying that selfed progeny are culled by selection (e.g. Eckert & Barrett, 1994). In *W. dioica*, inbreeding coefficients of the progeny of fruiting males were greater than zero and exceeded those of their parents, indicating that excess homozygotes are produced owing to high selfing, but few selfed progeny survive to reproduce. Although electrophoretic data may overestimate inbreeding depression if detrimental genes are linked to marker genes (Charlesworth, 1991), our estimate of inbreeding depression for 2 years of the life cycle using experimentally self- and cross-pollinated plants was almost as high ( $\delta = 0.85$ ). Differences between the two estimates occur because the experimental approach examines early life-cycle stages, whereas the marker-based approach accounts for most of the life cycle. Also, our experimental estimates are conservative because inbreeding depression was examined in the glasshouse rather than in the field where conditions are more severe (e.g. Dudash, 1990; Ramsey & Vaughton, 1998).

Self-pollinated fruiting males expressed inbreeding depression at a variety of life-cycle stages. Inbreeding depression was greatest for seed production ( $\delta = 0.51$ ), but was also high for seed germination ( $\delta = 0.21$ ), corm mass ( $\delta = 0.39$ ) and survival ( $\delta = 0.37$ ). Overall, these findings are consistent with those for predominantly outcrossing cosexual species in which substantial inbreeding depression is expressed early in development, owing to the involvement of recessive deleterious alleles (Husband & Schemske, 1996). In *W. dioica*, high genetic load could be maintained because the predominantly outcrossed females produce most of the seeds in a population. Opportunities for selection to purge deleterious alleles could be afforded by fruiting males that self, although purging would be limited if no selfed progeny survive to reproduce (i.e. selective interference, Lande *et al.*, 1994). Gender plasticity would further reduce opportunities for purging because fruiting males cannot self when they flower as males.

### Biparental inbreeding in females

In gender-dimorphic taxa, females require pollen from the polliniferous morphs to produce seeds and are thus obligately outcrossed, although biparental inbreeding caused by matings between relatives is possible (Kohn & Biardi, 1995; Weller & Sakai, 2005). Biparental inbreeding was detected in females from both *W. dioica*

populations, as indicated by multi-locus outcrossing estimates that were less than one ( $t_m \approx 0.80$ ). A major factor causing biparental inbreeding is spatial substructuring of genotypes within populations. In *W. dioica*, such matings probably result from limited dispersal of pollen by generalist pollinators and limited movement of seeds, which lack dispersal mechanisms. In some dimorphic species, biparental inbreeding reduces the fertility advantage of females (Schultz & Ganders, 1996; Thompson & Tarayre, 2000). In *W. dioica*, average inbreeding coefficients of female parents were less than those of their progeny ( $-0.118$  vs.  $+0.109$ ), leading to high estimates of inbreeding depression that did not differ from one. This implies that biparentally inbred progeny are culled by selection. However, relative performances for individual traits calculated from open- and cross-pollinated females were similar and cumulative relative performance did not differ from one (0.88). Thus, although selection culls biparentally inbred progeny, the level of biparental inbreeding that we observed might have only minimal effects on the overall fitness of open-pollinated females.

### Maintenance of females

Equilibrium female frequencies in dimorphic populations are influenced by the genetic control of male sterility, and the relative seed production and progeny fitness of fruiting males and females (Charlesworth, 1999). If nuclear genes govern sex expression, then the predicted equilibrium frequency of females ( $p$ ) can be estimated as:  $p = (k + 2s\delta - 1)/2(k + s\delta)$ , where  $k$  is the relative increase in female fecundity ( $F$ ) owing to resource reallocation [i.e.  $(\text{female}_F - \text{male}_F)/\text{male}_F$ ],  $s$  is the zygote selfing rate of fruiting males, and  $\delta$  is inbreeding depression (Charlesworth & Charlesworth, 1978). For *W. dioica*, we can estimate  $k$  using either the number of ovules per flower multiplied by the number of ovuliferous flowers per plant (Ramsey & Vaughton, 2001) or the cross-pollinated seed set from this study (Table 2). We estimated female fecundity of males (i.e.  $\text{male}_F$ ) using both fruiting males and nonfruiting males, weighted by their frequencies relative to both polliniferous morphs. This provides a better estimate of female fecundity of males, which is overestimated if fruiting males only are used. For both ovule and seed approaches,  $k = 10.5$ . Substituting this  $k$ , the zygote selfing rate (0.80) and inbreeding depression (0.98) into the equation above, the predicted female frequency is 0.49, which is marginally greater than the observed frequency of 0.43.

This small discrepancy can be explained if female frequency has not yet attained equilibrium. Alternatively, gender plasticity could select for lower female frequency if it increases average male seed fecundity, causing  $k$  and relative female fitness to decrease. This assumes that gender plasticity increases female fitness without decreasing male fitness as probably occurs when

males express female function only under favourable conditions (Delph & Wolf, 2005). In *W. dioica*, gender plasticity can affect average female fecundity of males in two ways. First, the frequency of fruiting males may be >7% in some years. Frequencies of up to 35% have been observed in the field and glasshouse under favourable growing conditions, although we do not know how regularly such frequencies occur. Second, fruiting males increase ovuliferous flower production under favourable conditions, which should increase seed production (Barrett, 1992; Barrett *et al.*, 1999; Ramsey & Vaughton, 2001).

By influencing the average female fecundity of males and the magnitude of  $k$ , gender plasticity can alter the importance of selfing rate and inbreeding depression in maintaining females. When average female fecundity of males is low (i.e.  $k$  is large), inbreeding avoidance has little influence on female frequency. For example, assuming  $k = 10.5$  as above and all seeds are outcrossed ( $s\delta = 0$ ), then the expected female frequency is 0.45, which is marginally <0.49 using our values of  $s = 0.80$  and  $\delta = 0.98$ . By contrast, when average female fecundity of males is high (i.e.  $k$  is small), inbreeding avoidance becomes important. If fruiting male frequency is 35%, then  $k$  is reduced substantially (1.02). Using  $k = 1.02$  and  $s\delta = 0$ , the expected female frequency is <0.01, which is substantially <0.44 when  $k = 1.02$ ,  $s = 0.80$  and  $\delta = 0.98$ . Such modelling indicates that despite high selfing rates and high inbreeding depression, inbreeding avoidance may have only minimal bearing on the maintenance of females in these *W. dioica* populations when the frequency of fruiting males remains low.

Several authors have argued that the presence of gender plasticity in dimorphic species may hinder the evolution of dioecy because males express female function only when conditions are favourable, which reduces potential trade-offs with male function (reviewed by Delph & Wolf, 2005). Based on our present findings, we suspect that subdioecy is unstable and gender plasticity will not hinder evolution of dioecy in *W. dioica*. This is because although most males are plastic in their allocation to female function, under natural conditions few males flower as fruiting males and produce seeds. However, because gender plasticity depends on the environment, changes that improve growth conditions could increase the frequency that males express female function. If this occurs, then the stability of subdioecy will depend upon the selfing rate and the expression of inbreeding depression.

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