

INBREEDING AVOIDANCE AND THE EVOLUTION OF GENDER DIMORPHISM IN *WURMBEA BIGLANDULOSA* (COLCHICACEAE)

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Abstract.—How females establish in populations of cosexuals is central to understanding the evolution of gender dimorphism in angiosperms. Inbreeding avoidance hypotheses propose that females can establish and be maintained if cosexual fitness is reduced because they self-fertilize, and their progeny express inbreeding depression. Here we assess the role of inbreeding avoidance in maintaining sexual system variation in *Wurmbea biglandulosa*. We estimated costs of self-pollination, mating patterns, and inbreeding depression in gender monomorphic (cosexuals only) and dimorphic (males and females) populations. Costs of selfing, estimated from seed set of experimentally self- and cross-pollinated flowers, were severe in both males and cosexuals (inbreeding depression, $\delta = 0.86$). In a field experiment, intact males that could self produced fewer seeds than both emasculated males and females, whereas seed set of intact and emasculated cosexuals did not differ. Thus, pollinator-mediated selfing reduces fitness of males but not cosexuals under natural conditions. Outcrossing rates of males revealed substantial selfing ($t = 0.68$), whereas females and cosexuals were outcrossed (0.92 and 0.97). For males, progeny inbreeding coefficients exceeded parental coefficients (0.220 vs. 0.009), whereas for females and cosexuals these coefficients did not differ and approached zero. Differences in coefficients between males and their progeny indicate that selfed progeny express severe inbreeding depression ($\delta = 0.93$). Combined with inbreeding depression for seed set, cumulative $\delta = 0.99$, indicating that most or all selfed zygotes fail to reach reproductive maturity. We propose that present sexual system variation in *W. biglandulosa* is maintained by high inbreeding depression coupled with differences in selfing rates among monomorphic and dimorphic populations.

Key words.—Gynodioecy, inbreeding depression, mating system, outcrossing rates, selfing, *Wurmbea*.

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Most flowering plants are hermaphroditic and produce both pollen (male function) and ovules (female function) within the same flower. The close proximity of the sexual functions renders plants susceptible to self-pollination and the subsequent costs of selfing owing to inbreeding depression, the reduced fitness of selfed progeny compared with outcrossed progeny. Avoidance of inbreeding depression has long been considered a probable explanation for the widespread occurrence of traits promoting outcrossing in plant populations (Darwin 1876; Charlesworth and Charlesworth 1978, 1987; Lande and Schemske 1985; Schemske and Lande 1985; Barrett 2003). A case in point is the evolutionary transition from monomorphic populations that contain hermaphrodites only to gender dimorphic populations that contain both hermaphrodites and females. Females are obligately outcrossed because they produce ovules only and rely on pollen from other plants to produce seeds (Lloyd 1982; Charlesworth 1999). Thus, females escape any potential costs of selfing, although whether the benefits owing to inbreeding avoidance are sufficient to drive the evolution of gender dimorphism remains controversial (Schultz and Ganders 1996, and references therein).

Theoretical models predict that when nuclear genes control male sterility, females can invade monomorphic populations if they have a greater than twofold fitness advantage relative to hermaphrodites. This advantage could arise from inbreeding avoidance, if the product of the selfing rate and inbreeding depression of hermaphrodites exceeds 0.5, although this requires levels of both parameters to be high (Lloyd 1976; Charlesworth and Charlesworth 1978). Despite these stringent requirements, empirical studies often demonstrate sufficiently high levels of selfing and inbreeding depression to

maintain females in gender dimorphic populations (Kohn and Biardi 1995; Schultz and Ganders 1996; Sakai et al. 1997; Thompson et al. 2004; Medrano et al. 2005; Weller and Sakai 2005; but see Ashman 1992; Eckhart 1992; Koelewijn and van Damme 2005). However, the continued presence of females in dimorphic populations can alter selfing levels, inbreeding depression, and allocation to female function in hermaphrodites, obscuring the conditions that initially favored a newly arisen male-sterile mutant. For example, an overall increase in outcrossing owing to female presence may allow deleterious recessive alleles to accumulate, increasing the magnitude of inbreeding depression compared with that before females established. For these reasons, whether inbreeding avoidance conveys sufficient fitness benefits for male sterility to establish is difficult to assess (Webb 1999).

An alternative approach to address this issue is to study species with the unusual presence of both gender monomorphic and dimorphic populations. Intraspecific variation allows mating patterns of the sex morphs from contrasting sexual systems to be examined without the confounding effects of phylogenetic divergence (Baker 1963; Ganders 1978; Webb 1999). Mating patterns of hermaphrodites from monomorphic populations and of hermaphrodites and females from dimorphic populations can provide insight into the initial spread of females and their maintenance, respectively. Moreover, floral manipulations that alter gender of hermaphrodites from dimorphic and monomorphic populations can elucidate the role of pollination biology in mediating differences in mating patterns. Studies that reveal the basis of shifts in mating patterns are critical because levels of selfing determine the expression of inbreeding depression, which can affect the stability of monomorphic sexual systems and the

potential for male sterility to become established (e.g., Dorken et al. 2002).

Shifts in pollination biology that occur in association with changed habitat conditions have been implicated in the evolution of gender dimorphism in several taxa (Ganders 1978; Delph 1990; Weller and Sakai 1990; Case and Barrett 2004a,b). Changes in pollinators, their abundance or foraging behavior can increase selfing and alter the expression of inbreeding depression, generating selection to increase outcrossing. In *Hebe*, Delph (1990) found that gender dimorphism was positively correlated with altitude and proposed that changes in pollinators from bees at low altitudes to less efficient flies and beetles at high altitude increased selfing, leading to conditions that favored gender dimorphism. Similarly, Weller and Sakai (1990) proposed that, in *Schiedea*, changes from insect pollination in mesic habitats to wind pollination in drier habitats that increased selfing favored gender dimorphism. Finally, Case and Barrett (2004b) found differences in pollination biology between sympatric monomorphic and dimorphic populations of *Wurmbea dioica* ssp. *alba*. They proposed that increased selfing in drier habitats where dimorphic populations are now found facilitated the evolution of gender dimorphism.

In this study, we investigate the role of inbreeding avoidance in the establishment and maintenance of gender dimorphism in *Wurmbea biglandulosa*. This lilioid herb from southeastern Australia exhibits intraspecific variation in sexual system with either monomorphic or dimorphic populations. In dimorphic populations, female frequency ranges from 4–55%, and hermaphrodites retain substantial female function, indicating that evolution has not progressed far along the pathway from hermaphroditism to dioecy (Vaughton and Ramsey 2002). Several features of *W. biglandulosa* might be expected to influence pollination biology and mating patterns. First, hermaphrodites are self-compatible but require insect vectors to transfer pollen to stigmas (Ramsey and Vaughton 2002). Second, dimorphic populations occur in drier sites than do monomorphic populations, and hermaphrodites in these populations produce smaller flowers (Vaughton and Ramsey 2004). Finally, the major floral visitors are unspecialized nectar-collecting flies and small native bees that might cause increased levels of self-pollination when foraging on smaller hermaphroditic flowers. Because selfing rate mediates the expression of inbreeding depression, shifts in pollination biology could provide insight into evolutionary transitions between gender monomorphism and dimorphism.

Here we examine two monomorphic and dimorphic populations of *W. biglandulosa* and address three specific issues. We begin by using controlled hand self- and cross-pollinations to assess the potential costs of selfing caused by inbreeding depression in hermaphrodites from both sexual systems. Next, we conduct a field experiment and alter gender of hermaphrodites to prevent pollinator-mediated selfing. This assesses whether mating patterns differ and whether costs of selfing actually reduce seed set under natural conditions. Finally, we estimate outcrossing rates and inbreeding coefficients of the sex morphs from the contrasting sexual systems and examine the magnitude of inbreeding depression using a marker-based approach. In dimorphic populations,

high costs of selfing coupled with a high selfing rate in hermaphrodites would indicate that the obligately outcrossed females should be maintained. In monomorphic populations, low selfing rate would indicate sexual system stability regardless of the magnitude of selfing costs, whereas a high selfing rate coupled with high selfing costs would indicate instability and susceptibility to invasion by females.

MATERIALS AND METHODS

Study Species and Sites

Plants of *Wurmbea biglandulosa* ssp. *biglandulosa* have a corm, an annual shoot with three leaves, and an erect cymose inflorescence with one to four flowers. Hermaphrodites in monomorphic and dimorphic populations produce mostly perfect and occasionally staminate flowers. Females produce pistillate flowers only. Ovaries of perfect and pistillate flowers have about 65 ovules contained within three locules, each associated with a separate style. In dimorphic populations, females produce on average twice as many seeds as hermaphrodites (Vaughton and Ramsey 2002, 2004). The sex determination mechanism is unknown, but probably involves both nuclear and cytoplasmic factors (M. Ramsey, unpubl. data). Hereafter, we refer to hermaphrodites in monomorphic and dimorphic populations as cosexuals and males, respectively, reflecting the sexual function by which each sex morph contributes most genes to the next generation (Lloyd 1976, 1980; Lloyd and Bawa 1984).

We studied two monomorphic populations (RR: 30°36.56'S, 151°28.73'E, 1052 m above sea level [asl]; MY: 30°29.01'S, 151°27.54'E, 907 m asl) and two dimorphic populations (GR: 29°49.68'S, 151°07.50'E, 739 m asl; WG: 31°17.49'S, 149°02.57'E, 761 m asl) in northern New South Wales, Australia. Recent molecular work indicates that the two monomorphic populations and one of the dimorphic populations (WG) are sister taxa, but the other dimorphic population (GR) is more distantly related (A. L. Case, S. W. Graham, T. D. Macfarlane, and S. C. H. Barrett, unpubl. ms.). We follow current taxonomic treatment and refer to all populations as *W. biglandulosa* ssp. *biglandulosa* (Macfarlane 1987). Populations contained several thousand plants growing in short grass on rocky clay loam over granite. Over three years, female frequencies averaged 19% at GR and 9% at WG.

Effects of Selfing on Seed Production

We assessed potential costs of selfing using hand-pollination experiments. Males, females, and cosexuals were excavated; re-established in pots containing 1:1:1 sand, loam, and peat; and grown in a glasshouse for one year. From each population, 20 males and 20 cosexuals were randomly allocated to each of the following pollination treatments: (1) self-pollen from the same plant, (2) outcross pollen from a different plant from the same population, and (3) self-pollen on one of the three stigmas per flower and outcross pollen on a different stigma (self + cross). Treatment 3 tests whether selfing reduces seed set when both self- and cross-pollen are deposited on the same flower, as probably occurs frequently under natural conditions. In all treatments, two stigmas per flower were pollinated by direct contact with recently de-

hiscid anthers. Pollinating two rather than three stigmas did not reduce seed set ($P > 0.10$). We also cross-pollinated 20 females, enabling comparisons with selfed, crossed, and self + cross males. On all plants, only the first flower was pollinated.

We also tested for costs of self-pollination in the field during peak flowering by emasculating males and cosexuals, rendering them incapable of selfing. In each population, plants on which the first flower was about to open were randomly assigned to either an emasculated or a nonemasculated control (intact) treatment (both $n = 25$). In both treatments, unopened flowers were removed, leaving one experimental flower per plant. We removed the anthers on flowers of the emasculated plants with fine forceps, and left flowers on control plants intact. In dimorphic populations, we also removed all but the first flower on 25 females for comparison with the emasculated and intact males. Our experimental design provides a conservative estimate of the effects of selfing because plants had only one flower, and geitonogamous self-pollination was not possible. Although geitonogamy can have important effects in plants with large flowering displays (de Jong et al. 1993), *W. biglandulosa* plants have on average only two flowers, and most self-pollination probably occurs as a result of insect-mediated pollen movement within flowers.

In the pollination and emasculation experiments, we harvested the mature fruits from males, females, and cosexuals and counted the numbers of unfertilized ovules, aborted seeds, and viable seeds. Aborted seeds were shriveled and were smaller than viable seeds but at least twice as large as ovules. Nevertheless, abortions were probably underestimated because we would not have detected zygotes that were aborted very early in development. We calculated percent seed abortion as the number of abortions divided by the number of seeds + aborted seeds (i.e., fertilized ovules).

We compared the number of seeds and percentage of aborted seeds per fruit of males and cosexuals with partially nested analyses of variance (ANOVA). Pollination or emasculation treatment and sexual system were considered fixed factors. Populations nested within sexual system were considered random factors. In these analyses, sexual system is tested over population, resulting in low power for determining the significance of sexual system. However, this was of little consequence to our overall interpretation of the analyses. We were primarily interested in the treatment \times sexual system interaction, which, if significant, indicates that selfing affected males differently than cosexuals. Nonsignificant treatment \times population interactions ($P > 0.20$) were pooled with the error term. For the pollination experiment, we used non-orthogonal planned contrasts with Bonferroni adjustment to compare seed set and seed abortion between the three selfing and crossing treatments. For dimorphic populations, we compared seed set and seed abortion of males and females with two-way ANOVAs, with treatment and population as fixed and random factors, respectively. As above, nonsignificant treatment \times population interactions ($P > 0.20$) were pooled with the error term. We used orthogonal planned contrasts to compare females with the polliniferous plants in both experiments. To meet assumptions of ANOVA, seed number (+1) was transformed using natural logarithms, and percent

abortion was square-root arcsine transformed. Analyses were performed using JMP (ver. 5.01a, SAS Institute 2002). Means \pm SE are presented.

Mating System

We collected seeds from open-pollinated plants in each population and assayed progeny for allozyme variation using cellulose acetate electrophoresis. In total, 800 progeny representing 15 plants from each monomorphic population, 30 plants from WG (15 males and 15 females), and 20 plants from GR (10 males and 10 females) were analyzed. For each plant, 12–19 seeds were germinated in petri dishes, and 10 randomly selected seedlings per plant were assayed. We ground seedlings in an extraction buffer modified from Peakall and Beattie (1991). Five putative loci showed clear and interpretable banding patterns: glucose-phosphate isomerase (*Gpi*), phosphoglucumutase (*Pgm-1* and *Pgm-2*), mannose-phosphate isomerase (*Mpi*), and glutamate oxaloacetate transaminase (*GOT*). All enzymes were resolved on 0.025 M Tris-glycine pH 8.5 buffer. Two to five alleles were observed at each locus.

Multilocus outcrossing rates (t_m) were estimated for each population using the mating system program MLTR (ver. 1.0, Ritland 1990a). This program uses maximum-likelihood procedures to infer the genotypes of maternal plants, allele frequencies in the pollen pool, and the proportion of progeny that are outcrossed. Separate t_m estimates were calculated for males and females, using the entire population dataset to calculate pollen allele frequencies. Expectation-maximization procedure was used for the iterations. We also estimated parental and progeny inbreeding coefficients (f) separately for each gender using the program Genetic Data Analysis (GDA, ver. 1.1, Lewis and Zaykin 2001). To estimate parental f , we used the inferred parental genotypes from the MLTR output. When parental and progeny inbreeding coefficients differed significantly, we calculated inbreeding depression ($\delta = 1 - \text{fitness of selfed progeny}/\text{fitness of outcrossed progeny}$) for survival from seed germination to reproductive maturity from the estimates of t_m and parental f using Ritland's (1990b) equilibrium estimator:

$$\delta = 1 - [2f(t_m)/(1 - t_m)(1 - f)]. \quad (1)$$

Standard errors of the outcrossing rates and inbreeding coefficients were based on 1000 bootstrap samples, using families as the unit of resampling. For tests of statistical significance, we examined the distribution of 1000 bootstrap values following Eckert and Barrett (1994). Using this method, a one-tailed test of a given parameter is considered to be either significantly less than or greater than the test value if 100% ($1 - \alpha$) of bootstrap values are either less than or greater than the test value, respectively. To reduce Type I errors when multiple comparisons were performed, we used sequential Bonferroni adjustments for α (Rice 1989). In dimorphic populations, we considered males to have significantly lower outcrossing rates than females if 100% ($1 - \alpha$) of the differences in bootstrap samples were less than zero. Differences between average outcrossing rates of males and cosexuals, and females and cosexuals, were tested similarly.

Inbreeding depression at seed development results in a

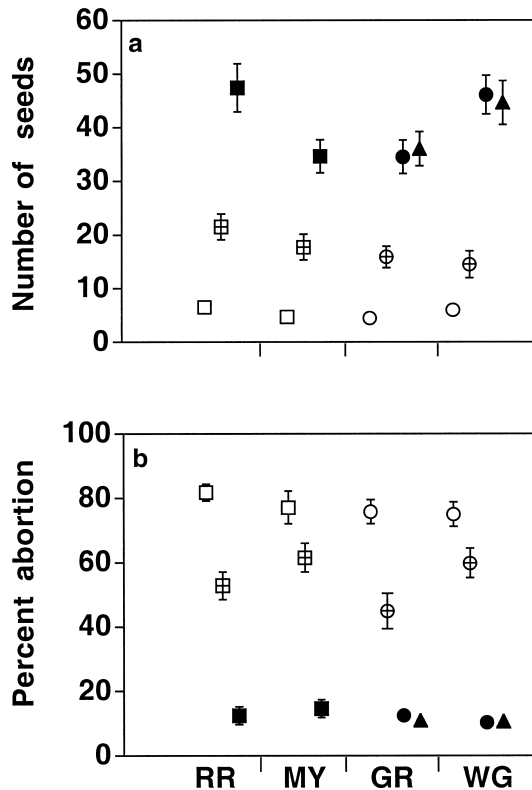


FIG. 1. Mean (\pm SE) number of seeds (a) and percent seed abortion (b) of *Wurmbea biglandulosa* cosexuals (squares, monomorphic populations RR and MY) and males (circles, dimorphic populations GR and WG) that were self-pollinated (open symbols), self- + cross-pollinated (+ symbols) or cross-pollinated (filled symbols). Also given are seed set and abortion of crossed females (filled triangles) from dimorphic populations.

lower selfing rate in seedlings than in zygotes. We estimated the zygote selfing rate at fertilization for males by adjusting the seedling selfing rate ($s_m = 1 - t_m$) for inbreeding depression as:

$$r = s_m / (1 - \delta_s + s_m \delta_s) \quad (2)$$

(Maki 1993; Lande et al. 1994). Using seed set from selfed and crossed males, we estimated that $\delta_s = 0.86$ (Fig. 1).

RESULTS

Effects of Selfing on Seed Production

Experimental self-pollination, either alone or with crossing, reduced seed set (Fig. 1, Table 1). Males and cosexuals were affected similarly, as indicated by the nonsignificant pollination \times sexual system interactions for seed set and abortion (Table 1). Seed set following crossing was 2.4-fold and 7.5-fold greater than following mixed selfing + crossing and selfing, respectively, and was 3.2-fold greater following mixed pollinations than following selfing. Abortions showed the opposite trend. Following selfing, abortions were 1.4-fold and 6.3-fold greater than following mixed pollinations and crossing, respectively, and were 4.5-fold greater following mixed pollinations than following crossing (Fig. 1, Table 1). Aborted seeds from selfed fruits varied greatly in size, as expected when different lethal recessive genes are expressed at different stages during seed development. Overall, costs of selfing were similarly severe for males and cosexuals. Inbreeding depression (δ_s), estimated as $\delta_s = 1 - \text{self-seed set/cross-seed set}$, ranged from 0.85 to 0.87 for all populations.

In dimorphic populations, pollination treatment also significantly affected seed set and abortion (seeds: $F_{3,3} = 66.64$, $P = 0.003$; abortions: $F_{3,3} = 63.97$, $P = 0.003$; Fig. 1). Seed set and seed abortion of crossed females and males did not differ significantly, indicating that the two sex morphs had similar reproductive potential on a per flower basis (planned contrasts: seeds and abortions, both $F_{1,3} < 0.02$, $P > 0.908$). Crossed females produced 7.7-fold more seeds than did selfed or self + cross pollinated males, respectively, but had significantly fewer aborted seeds (planned contrasts: seeds and abortions, all $F_{1,3} > 45.05$, $P < 0.007$). Neither population nor the pollination \times population interactions were significant (population: both $F_{1,152} < 1.35$, $P > 0.246$; interaction: both $F_{3,152} < 2.41$, $P \geq 0.070$).

In the emasculation experiment, anther removal that prevented selfing affected males but not cosexuals, as indicated by significant emasculation \times sexual system interactions for seed set and abortion (Fig. 2, Table 2). Emasculated males produced 66% more seeds and 15% fewer aborted seeds than

TABLE 1. Results of partially nested ANOVAs examining the effects of pollination (self, cross, or self + cross), sexual system (monomorphic or dimorphic), and population nested within sexual system on number of seeds and percentage of aborted seeds. Planned contrasts between treatments were conducted and Bonferroni correction was used ($\alpha = 0.016$). Pollination treatment and sexual system were considered fixed factors, and population a random factor. The pollination \times population interaction for seed set was not significant ($P = 0.41$) and was pooled with the error. Analyses refer to data in Figure 1.

Source of variation	Number of seeds				Percent aborted seeds			
	df	MS	F	P	df	MS	F	P
Pollination	2	75.59	196.10	<0.001	2	40,185.0	126.99	<0.001
self vs. self + cross	1	36.96	95.87	<0.001	1	8011.3	25.32	0.007
cross vs. self + cross	1	38.64	100.25	<0.001	1	35,420.8	111.94	<0.001
self vs. cross	1	151.18	392.19	<0.001	1	77,122.8	243.72	<0.001
Sexual system	1	0.85	0.67	0.499	1	371.2	3.02	0.225
Population (sexual system)	2	1.26	3.28	0.039	2	123.0	0.39	0.701
Pollination \times sexual system	2	0.39	1.02	0.363	2	19.5	0.06	0.941
Pollination \times population (sexual system)	—	—	—	—	4	316.4	2.11	0.081
Error	232	0.39	—	—	228	150.3	—	—

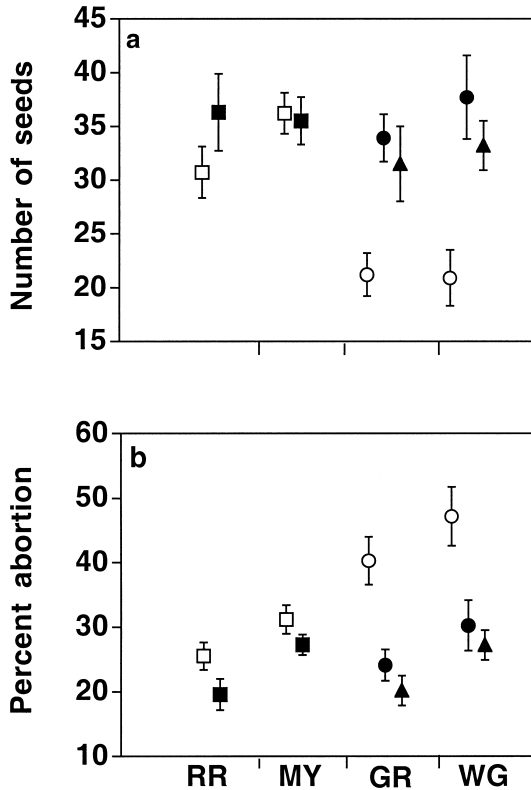


FIG. 2. Mean (\pm SE) number of seeds (a) and percent seed abortion (b) of *Wurmbea biglandulosa* open-pollinated cosexuals (squares, monomorphic populations RR and MY) and males (circles, dimorphic populations GR and WG) that were either left intact (open symbols) or emasculated (filled symbols). Also given are seed set and abortion of open-pollinated females (filled triangles) from dimorphic populations.

did intact males. Seed set and abortion of emasculated males and emasculated and intact cosexuals was similar.

In dimorphic populations, the emasculatation \times population interactions for seed set and abortion were not significant (both $P > 0.650$) and were pooled with the error term. The emasculatation effect for seed set and abortion was significant (seeds: $F_{2,146} = 15.04, P < 0.0001$; abortions: $F_{2,146} = 18.07, P < 0.0001$; Fig. 2). Seed set and abortion did not differ between females and emasculated males (planned contrasts: both $F_{1,146} < 2.51, P > 0.115$), but females produced 45% more seeds and 20% fewer aborted seeds than did intact males (both $F_{1,146} > 15.11, P < 0.001$). Populations did not differ

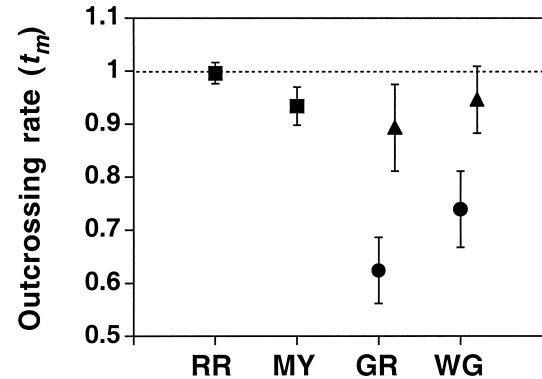


FIG. 3. Mean (\pm SE) outcrossing rates (t_m) of *Wurmbea biglandulosa* cosexuals (squares, monomorphic populations RR and MY) and males and females (circles and triangles, respectively, dimorphic populations GR and WG). Cosexual and female outcrossing rates did not differ significantly from one. In both dimorphic populations, male outcrossing rates were significantly less than those of females (GR, $P = 0.027$; WG, $P = 0.033$).

for seed set ($F_{1,146} = 0.11, P = 0.740$), but differed for seed abortion ($F_{1,146} = 8.98, P = 0.003$). For all treatments, abortions were about 7% greater at WG than at GR (Fig. 2).

Mating System

Outcrossing rates (t_m) of males were significantly less than one, indicating moderate levels of self-fertilization and a mixed mating system (both dimorphic populations $P < 0.001$; Fig. 3). Males were also less outcrossed than females ($P < 0.034$). By contrast, outcrossing rates of females and cosexuals did not differ from one, confirming that progenies of these plants were cross-fertilized (all $P > 0.06$; Fig. 3). When populations were considered together, males were less outcrossed than females (pooled males vs. pooled females: 0.68 ± 0.05 vs. $0.92 \pm 0.06, P < 0.001$) and cosexuals (0.68 ± 0.05 vs. $0.97 \pm 0.03, P < 0.001$), but outcrossing rates of females and cosexuals did not differ ($P = 0.17$). In dimorphic populations, the adjusted zygote selfing rate of males was substantially higher than the seedling selfing rate ($s_m = 1 - t_m$); on average, 78% of zygotes compared with 32% of seedlings were self-fertilized.

For males, progeny inbreeding coefficients significantly exceeded zero (both populations $f \geq +0.22, P < 0.001$), whereas parental coefficients did not differ from zero ($P > 0.35$; Fig. 4). The inferred inbreeding depression to account for these differences averaged 0.93 ± 0.26 and exceeded 0.50

TABLE 2. Results of partially nested ANOVAs examining the effects of anther removal (emasculated or intact), sexual system (monomorphic or dimorphic), and population nested within sexual system on number of seeds and percentage of aborted seeds. Emasculatation treatment and sexual system were considered fixed factors and population a random factor. The emasculatation \times population interactions were not significant ($P > 0.62$) and were pooled with the error. Analyses refer to data in Figure 2.

Source of variation	df	Number of seeds			Percent aborted seeds		
		MS	F	P	MS	F	P
Emasculatation	1	5.21	19.21	<0.001	2047.9	20.17	<0.001
Sexual system	1	4.99	21.83	0.043	2143.2	3.16	0.218
Population (sexual system)	2	0.23	0.84	0.432	678.4	6.68	0.002
Emasculatation \times sexual system	1	3.92	14.48	<0.001	425.1	4.19	0.042
Error	194	0.27			101.5		

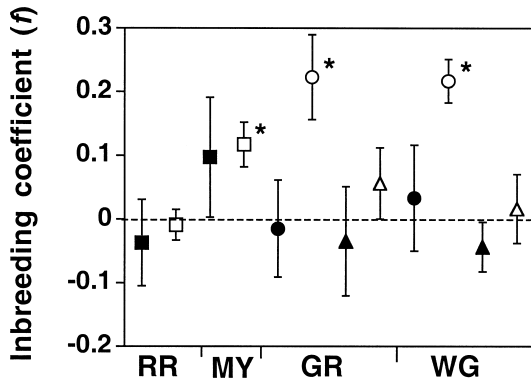


FIG. 4. Mean (\pm SE) inbreeding coefficients for parents (filled symbols) and progeny (open symbols) for two monomorphic (RR, MY) and two dimorphic (GR, WG) populations of *Wurmbea biglandulosa*. Values for cosexuals (squares) from monomorphic populations and for males (circles) and females (triangles) from dimorphic populations are given. Asterisks indicate values significantly different from zero following Bonferroni correction ($\alpha = 0.008$).

($P < 0.041$) but did not differ from one ($P > 0.10$). This severe inbreeding depression indicates that selfed progeny are culled by selection during the life cycle and are unlikely to contribute to the next generation. Consistent with the high female outcrossing rates, progeny f and parental f did not differ from zero (range, -0.044 to $+0.056$; all $P > 0.11$; Fig. 4). In the monomorphic RR population, progeny f and parental f also did not differ from zero ($P > 0.31$). In the MY population, both inbreeding coefficients were about $+0.10$, and although the estimates did not differ from each other ($P = 0.42$), progeny f but not parental f exceeded zero ($P < 0.001$; Fig. 4), implying a slight excess of homozygotes.

DISCUSSION

In southern Australia, the genus *Wurmbea* exhibits remarkable diversity in sexual systems ranging from hermaphroditism through gynodioecy and subdioecy to full dioecy (Macfarlane 1987; Barrett 1992; Vaughton and Ramsey 2002). *Wurmbea biglandulosa* has both monomorphic and dimorphic populations, providing opportunities to examine the role of inbreeding avoidance in the evolution of gender dimorphism (see also Dorken et al. 2002; Case and Barrett 2004a,b). We found striking differences in pollinator-mediated self-pollination between males and cosexuals, even though both sex morphs were equally self-fertile. Males experience moderate self-fertilization, but owing to inbreeding depression no selfed progeny survive, resulting in reduced fecundity relative to females and cosexuals that are fully outcrossed. Higher selfing by males could be a consequence of smaller flowers compared with cosexuals or changes in pollinator assemblages between habitats. Our findings highlight the importance of interactions between pollination biology, selfing rates, and expression of inbreeding depression in the invasion and maintenance of male-sterile mutants. Below, we discuss the pollination biology and mating patterns of dimorphic and monomorphic populations and explore how differences in pollinator-mediated selfing may contribute to sexual system variation in *W. biglandulosa*.

Costs of Self-Pollination

Costs of selfing are central to models of inbreeding avoidance for the evolution of gender dimorphism and occur when self-fertilization reduces seed set and progeny fitness compared with cross-fertilization (e.g., Lloyd 1976; Charlesworth and Charlesworth 1978). The severity of these costs depends on how much self-fertilization occurs and the magnitude of the subsequent inbreeding depression expressed by selfed progeny. Our experimental pollinations demonstrated that, although the potential seed fecundity of the three sex morphs after crossing was high, selfing significantly reduced seed set and increased seed abortion in both males and cosexuals. This indicates that early-acting inbreeding depression was similarly severe in both of these sex morphs. Further, costs of selfing depend on the relative amounts of self- versus cross-pollination, as demonstrated by the intermediate seed set when we pollinated males and cosexuals with self-pollen + cross-pollen. Collectively, these results show that selfing in both males and cosexuals usurps ovules, rendering them unavailable for cross-fertilization. Because flowers do not self autonomously, the amount of self-pollination and the subsequent fitness of males and cosexuals will depend on interactions between insect pollinators and floral biology.

We experimentally assessed the costs of pollinator-mediated self-pollination under natural conditions by removing anthers of males and cosexuals to prevent selfing (emasculature). Emasculated males and cosexuals produced similar numbers of seeds, as did open-pollinated females and plants that were experimentally cross-pollinated. By contrast, seed set of intact plants depended on sexual system. Intact males produced about 35% fewer seeds than did emasculated males, whereas seed set of intact and emasculated cosexuals did not differ. Early-acting inbreeding depression is similarly severe in males and cosexuals, and these differences between sexual systems are consistent with differing amounts of pollinator-mediated self-pollination.

Differences in pollinator-mediated selfing between dimorphic and monomorphic *W. biglandulosa* populations may be a consequence of two factors. First, pollinator assemblages may be altered in the drier dimorphic populations. Although flies are major floral visitors in populations of both sexual systems, native bees visit flowers more frequently in monomorphic populations (M. Ramsey, unpubl. data). In other *Wurmbea* species, bees are more likely to cross-pollinate flowers than flies that forage unsystematically, visiting the same flower repeatedly and several flowers per plant (Vaughton and Ramsey 1998; Case and Barrett 2004b).

Second, the ability of pollinators to cross-pollinate flowers on males may be reduced in dimorphic populations because flowers are smaller (Vaughton and Ramsey 2004). Flowers on males are 10% smaller than those on cosexuals and are likely to have smaller floral nectarines and fewer floral rewards. Consequently, some pollinators such as bees that are more likely to deposit cross-pollen may avoid these smaller flowers. Furthermore, distances between nectarines, anthers, and stigmas on smaller flowers are reduced, and unspecialized floral visitors are likely to deposit self- rather than cross-pollen onto stigmas (Vaughton and Ramsey 1998; Ramsey and Vaughton 2001; Case and Barrett 2004b). Regardless of

the cause of the shifts in pollination biology between sexual systems, pollinator-mediated selfing is likely to maintain gender dimorphism in *W. biglandulosa*. However, whether these shifts were the proximate factor favoring male sterility, or whether plant traits that cause such shifts evolved in response to female presence, remains to be determined.

Mating Patterns

Our study is one of only a few to use genetic markers to compare plant mating between conspecific dimorphic and monomorphic populations (see also Dorken et al. 2002). Supporting the findings from the emasculation experiment, we found substantial differences in outcrossing rates among the sex morphs, presumably because of variation in the amount of pollinator-mediated self-pollination. Male outcrossing rates were moderate (pooled populations: $t_m = 0.68$), indicating that males are self- and cross-pollinated (i.e., mixed mating). By contrast, females and cosexuals were highly outcrossed (pooled populations: female $t_m = 0.92$; cosexual $t_m = 0.97$).

Male outcrossing rates are lower than those indicated when mortality of selfed progeny prior to electrophoresis of seeds is taken into account (Maki 1993; Weller and Sakai 2005). The adjusted male selfing rate at the time of zygote formation was 0.78 ($t = 0.22$), indicating that males experience appreciable selfing under natural conditions. This adjusted selfing rate is comparable with estimates of selfing in fruiting males of subdioecious *W. dioica* ssp. *dioica* using Charlesworth's (1988) nongenetic method and allozyme electrophoresis (0.76 and 0.80: Vaughton and Ramsey 2003; M. Ramsey, unpubl. data). In both *Wurmbea* species, differences between seedling and adjusted outcrossing rates indicate that early-acting inbreeding depression is severe and similar to plants with predominantly outcrossing mating systems (Husband and Schemske 1996).

Our estimates of inbreeding depression for males using Ritland's (1990b) marker-based approach averaged 0.93, indicating that selfed progeny are only 7% as fit as outcrossed progeny. This estimate includes life-cycle stages from seed germination to reproductive maturity. If we incorporate our experimental estimate of inbreeding depression for seed production ($\delta = 0.86$), then cumulative inbreeding depression is 0.99. This estimate is indistinguishable to that using the adjusted selfing rate in the marker-based approach ($\delta = 1.0$). Moreover, our analyses of inbreeding coefficients showed an excess of homozygotes among the progeny of males, indicative of selfing, but the coefficients of the parents did not differ from zero, supporting the view that inbreeding depression is severe. Thus, despite substantial selfing, very few, if any, of the selfed progeny of males survive to reproduce. This reduces opportunities for purging recessive lethal alleles and could maintain the high genetic load in the dimorphic populations (Lande et al. 1994).

Marker-based estimates of inbreeding depression avoid the problems of experimental measures, such as environmentally dependent inbreeding depression (e.g., Ramsey and Vaughton 1998), but are based on two main assumptions (Ritland 1990b). Violation of the first assumption, inbreeding equilibrium, overestimates inbreeding depression, whereas vio-

lation of the second assumption, temporal stability of selfing rate, underestimates inbreeding depression. We cannot address these assumptions directly. However, the similarity of male selfing rates in both dimorphic populations implies that the rates are stable, and our inbreeding depression estimates are reliable. Further, the differences in progeny and parental inbreeding coefficients imply that selection culls selfed progeny, which is consistent with severe inbreeding depression.

Implications for Mating System Evolution

For females to establish in cosexual populations they require a more than twofold fecundity advantage in the case of nuclear controlled male sterility, or in the case of nucleocytoplasmic control, a smaller advantage, but other complex mechanisms such as costs of nuclear restorers of male fertility (Gouyon and Couvet 1987). The genetic control of sex in *W. biglandulosa* is unknown, but may be more complex than simple nuclear control. Open-pollinated males produce male progeny only, whereas females produce, on average, 70% female progeny, although some produce all females. This implies that both nuclear and cytoplasmic factors probably determine sex (M. Ramsey, unpubl. data). Nevertheless, the use of models that assume nuclear control should elucidate the roles of inbreeding avoidance and resource reallocation that compensates for the loss of pollen production in the evolution of gender dimorphism (Charlesworth 1999).

Models that assume nuclear control predict that females will establish when:

$$k + 2s\delta > 1, \quad (3)$$

where k is relative increase in female fecundity (F) owing to reallocation of resources ($k = [\text{female}_F - \text{male}_F]/\text{male}_F$), s is the male selfing rate, and δ is the inbreeding depression expressed by males (Charlesworth and Charlesworth 1978). When $k = 0$, the product of the selfing rate and inbreeding depression ($s\delta$) must exceed 0.5 for females to establish. In our monomorphic populations, cosexuals are outcrossed ($s = 0$) and avoid costs of selfing. Thus, substantial values of k would be necessary to provide a fitness advantage sufficiently large to permit females to establish. To estimate k , we compare cosexuals with females from dimorphic populations as a surrogate for a newly arisen male-sterile mutant. Cosexuals and females produce the same number of total flowers and ovules per flower, but females produce more ovuliferous flowers (2.3 vs. 1.9; Vaughton and Ramsey 2004). Using ovuliferous flower production, $k = 0.21$. However, because $s\delta = 0$, the left side of inequality (3) is only 0.11, which is substantially less than the threshold for females to establish. The lack of females in monomorphic populations, despite comparable values of k to dimorphic populations ($k = 0.35$), provides evidence that inbreeding avoidance is important for females to establish. In the absence of inbreeding avoidance, a minimum fecundity advantage provided by k alone would have to exceed substantially our estimate of 0.21 for females to establish. Such extreme resource reallocation in association with a newly arisen male-sterility mutation is unlikely.

For dimorphic populations, Lloyd (1976) proposed that

when nuclear genes control male sterility, the expected female frequency (p_f) would be:

$$p_f = 1 - [2c/2(1 - c)], \quad (4)$$

where c is the relative seed fitness of males (i.e., male seed set/female seed set). Selfing and inbreeding depression can be included by defining c as: (male seed set) t_m /female seed set, where t_m is the seed outcrossing rate, which assumes that all selfed progeny are culled by selection. This is justified for dimorphic populations of *W. biglandulosa* because inbreeding depression is so severe. We parameterized equation (4) by first using outcrossing rates for intact males, and then using male and female seed sets per flower from the emasculation experiment multiplied by the number of ovuliferous flowers per plant (males, 1.7; females, 2.3). Using intact males that exhibit mixed mating, $p_f = 0.51$ and 0.43 for GR and WG, respectively. These expected frequencies exceed the observed frequencies (GR, 0.19; WG, 0.09) and may indicate nonequilibrium female frequencies under nucleocytoplasmic control of male sterility. However, using emasculated males that are outcrossed, $p_f = 0$ for both populations. Because only outcrossing rate differed between intact and emasculated males, the differences in female frequency as determined by Lloyd's model clearly indicate that inbreeding avoidance alone could maintain females in dimorphic populations. Furthermore, this modeling implies that increases in male outcrossing rate in present day dimorphic populations could reduce the female fecundity advantage, resulting in sexual system reversal from dimorphism to monomorphism.

In conclusion, we propose that gender dimorphism in *W. biglandulosa* is maintained by selection to avoid inbreeding under pollination conditions that promote pollinator-mediated selfing. In dimorphic populations, moderate selfing rates coupled with severe inbreeding depression reduces fitness of males relative to females to the extent that male sterility is maintained. By contrast, although inbreeding depression is as severe in cosexuals as in males, hermaphroditism is stable in monomorphic populations because selfing rates are negligible and costs of inbreeding are avoided. These conditions render the establishment of male-sterile mutants unlikely because of advantages related to inbreeding avoidance alone. Collectively, our data strongly support the view that interactions between pollination biology, mating system, and inbreeding depression can result in selection to avoid inbreeding, which promotes sexual system variation in species such as *W. biglandulosa*.

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