

## GENDER DIFFERENCES IN REPRODUCTIVE AND PHYSIOLOGICAL TRAITS IN A GYNODIOECIOUS SPECIES, *GERANIUM MACULATUM* (GERANIACEAE)

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Plant species with separate genders often exhibit gender differences in traits related to reproductive allocation. In gynodioecious species, females often produce more seeds than do hermaphrodites, leading to a higher reproductive cost. The mechanisms that allow females to meet the high costs of reproduction are currently under debate. In this study, we test the hypothesis that there are genetically based gender differences in physiological traits that enable females to finance these costs through higher photosynthetic carbon gain in the gynodioecious perennial *Geranium maculatum*. Females and hermaphrodites were compared in a greenhouse study that minimized environmental and selfing rate differences between the genders. We found that females produced smaller flowers but more of them and more fruits than did hermaphrodites. However, genders did not differ in their seed number, seed mass, fruit set, and reproductive allocation. In addition, genders did not differ in photosynthetic rate ( $A$ ), leaf N, and water use efficiency inferred from leaf carbon isotope ratio ( $\delta^{13}\text{C}$ ). Overall, *G. maculatum* shows no genetically based gender differences for most of the reproductive traits or any of the physiological traits measured. Our results suggest that for *G. maculatum*, the gender fitness differences previously identified in natural populations may be caused by gender differences in microhabitat and/or selfing rate.

**Keywords:** carbon isotope ratio, female fitness compensation, gynodioecy, hermaphrodite, photosynthesis, leaf N, water use efficiency.

### Introduction

Gynodioecy is a breeding system that contains both female and hermaphroditic individuals in a population and is often considered to be an intermediate evolutionary stage between hermaphroditism and dioecy (Charlesworth and Charlesworth 1978). Theoretical studies suggest that because females cannot gain fitness through pollen, they must increase their investment in seed quantity and/or quality in order to coexist with hermaphrodites (Darwin 1877; Lewis 1941; Lloyd 1975; Charlesworth and Charlesworth 1978; Charlesworth and Ganders 1979). This increase in female fitness has been observed in most gynodioecious species, with an extreme case of females producing 600% more seeds than hermaphrodites did (Manicacci et al. 1998). Such increases in seed investment are likely to lead to a higher total reproductive cost for females than hermaphrodites because fruit production is generally more expensive than pollen production in terms of energy requirement (Dawson and Geber 1999; but see Eckhart and Chapin 1997; Eckhart and Seger 1999). The mechanism that allows females to meet such high-budget costs has been a major question in the evolution of gynodioecy.

At least three non-mutually exclusive hypotheses may explain how females are able to produce more seeds than hermaphrodites can. First, females and hermaphrodites of gynodioecious plants can be patchily distributed (Graff 1999;

Olson et al. 2006). In some dioecious plants, genders are also spatially segregated and have been shown to associate with different environmental qualities (Dawson and Bliss 1989; Dawson and Ehleringer 1993). If the female- and hermaphrodite-biased patches in gynodioecious plants also differ in quality, the difference in average seed production between these genders found in natural populations may simply reflect the differences in their microhabitat qualities. Second, because females can only outcross, while hermaphrodites often produce a portion of their seeds through self-fertilization, the higher seed production in females might reflect the higher quality of the outcrossed progeny (Darwin 1877; Schultz and Ganders 1996; Glaetli and Goudet 2006; Chang 2007) because of differences in the selfing rates of the two genders in natural populations. Finally, it has also been suggested that females, which generally produce smaller petals and no pollen (reviewed in Eckhart 1999; Shykoff et al. 2003), are able to use resources saved from these structures to increase seed production (Darwin 1877; Eckhart 1992; Ashman 1994).

There is another plausible hypothesis regarding physiological differences between genders that has received much less attention and has rarely been tested in gynodioecious species. This hypothesis predicts that the higher seed production observed in natural populations could result from higher physiological capacity that allows females to increase their investment in reproduction compared with hermaphrodites (Poot et al. 1996, 1997; Eckhart and Chapin 1997; Case and Barrett 2001; Caruso et al. 2003; Schultz 2003; reviewed in Case and Ashman 2005). The few empirical studies that investigate physiological differences between genders in gynodioecious species have

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produced inconsistent results. For example, for *Lobelia siphilitica* grown in a controlled greenhouse environment, females had higher photosynthetic rates than did hermaphrodites, which may contribute to financing their higher seed production (Caruso et al. 2003). In contrast, in a natural population of *Sidalcea hirtipes*, females had lower photosynthetic rates, lower photosynthetic water use efficiency (measured from gas exchange), and higher predawn water potentials than hermaphrodites (Schultz 2003). Finally, for *Plantago lanceolata* grown in a controlled greenhouse environment, there were no gender differences in photosynthetic rates, leaf nutrient content, or chlorophyll content (Poot et al. 1996). Contrasting results in physiological differences between genders have also been found for dioecious species, where females often have significantly higher reproductive investment (e.g., Dawson et al. 1990; Dawson and Ehleringer 1993; Marshall et al. 1993).

Many of the previous studies that investigated gender differences may not have directly tested the physiological differences because they were performed in natural populations where females and hermaphrodites may experience different conditions for other factors, such as microhabitat conditions and selfing rates (e.g., Molina-Freaner and Jain 1992; Klinkhamer et al. 1994; Schultz 2003). In order to isolate the effect of genetically based physiological differences between genders, comparisons that control for the environmental conditions and the selfing rate are needed. To do this, individuals must be grown in similar environments, a condition difficult to achieve when using plants grown in the field because genders may be patchily distributed in natural populations (Graff 1999; Olson et al. 2006). Additionally, differences in the selfing rates in natural populations should be eliminated by providing only non-self-pollen to avoid the effect of inbreeding depression because selfing hermaphrodites may incur more inbreeding depression and, as a result, produce fewer seeds than females.

The goal of our study was to determine whether genders differed in reproductive and physiological traits in a gynodioecious species, *Geranium maculatum*. Previous studies in natural populations have shown that females of this species produce 14%–63% more seeds (Ågren and Willson 1991; Chang 2006) and 6%–18% heavier seeds (Chang 2006) than do hermaphrodites. To isolate the physiological differences, we designed our experiment to reduce the contribution of the environmental variation and to eliminate the differences in the selfing rate on our measurements; hence, we focused our study on genetically based gender differences. We tested two hypotheses: (1) that females produce more seeds or better-quality seeds in order to compensate for the loss of fitness through pollen and (2) that females have a physiological mechanism to increase resource availability for seed production. In addition, we incorporated two light levels to encompass the range of light environments in which this species occurs in the natural populations.

## Material and Methods

### Study Species

*Geranium maculatum* is a herbaceous perennial species with a distribution ranging from the southeastern United States to Canada (USDA, NRCS 2007). Populations grow predomi-

nantly under a deciduous hardwood tree canopy, though they are sometimes found in habitats such as the forest edge or gaps that receive more light (S.-M. Chang, personal communication). Natural populations generally contain five to several thousand flowering individuals. Individuals start flowering in early spring (late March–early April) before canopy closure and continue flowering for several weeks, producing, on average, six flowers per inflorescence in natural populations around Athens, Georgia (Chang 2006). Pollination is performed by generalist pollinators, including bumblebees, honeybees, halictid bees, and butterflies. Although hermaphrodites are self-compatible, the temporal separation of male and female functions, that is, protandry, limits self-pollination within a flower. Ca. 4 wk after fertilization, mature seeds are released by the elastic dehiscence of the schizocarp. This mechanism disperses seeds to, on average, 3 m from the maternal plant (Stamp and Lucas 1983). Seedlings spend their first year as vegetative ramets and may flower in their second year in the greenhouse, although the average time to flowering in natural populations has not been determined.

*Geranium maculatum* is gynodioecious, with genetically female or hermaphroditic individuals coexisting within a population. The type of genetic determination for gender has not been described for this species, but studies are under way to determine whether genders are controlled by nuclear genes or by interactions between nuclear and cytoplasmic (most likely mitochondrial) genes. Female individuals are easily identified by their small aborted anthers and smaller petal sizes (Chang 2006). In natural populations, females produce more seeds and better-quality seeds that are more likely to germinate and survive until flowering (Chang 2006). Populations range in female frequency from 0% to 50% (Chang 2006).

### Experimental Individuals and Growing Conditions

Rhizomes used in this study were initially collected from natural populations in the State Botanical Garden of Georgia, Athens, with a sex ratio of ca. 50%. These rhizomes were subsequently grown in greenhouse conditions at the University of Georgia, Athens, for more than two growing seasons to homogenize environmental effects. Dormant rhizomes of both genders were randomly selected from plants that had flowered the previous spring. Each rhizome was cut to ca. 8 cm in length. Because it was not possible to obtain rhizomes of exactly the same size, we measured the initial rhizome size (length, width, largest diameter, and mass) as a potential source of variation for traits examined in this study. Thirty-two rhizomes were individually planted in 15-cm-diameter pots filled with fresh pine bark medium (2500 fine-grade pine bark : 1024 coarse-grade vermiculite : 8.6 dolomitic limestone : 2 superphosphate : 1 calcium nitrate : 1 potassium nitrate : 1 gypsum : 1 micromax micronutrients). Throughout the experiment, plants were fertilized once a week with 300 ppm N (Peters Peatlite Special, 20 : 10 : 20) and watered as necessary.

Two levels of light intensity were created in the greenhouse in a split plot design. Plants were randomly assigned to one of two light levels: shade or light (35% and 100% of full sun, respectively) and one of two replications. These light levels were chosen because they are within the range of light

levels observed in the natural populations (S.-M. Chang, personal communication). Shade treatments were constructed as  $1 \times 1 \times 1$ -m PVC pipe frames with shade cloths attached to the tops and sides. Two greenhouse benches were used as two replications in this experiment. Each replication contained one shade and one light treatment, each randomly placed on half of the bench. Within each shade and light treatment, four randomly selected individuals per gender were placed in random locations, for a total of 32 plants in the study. In this design, gender is the subplot, and light level is the whole plot.

### *Reproductive and Biomass Traits*

We measured both pre- and postpollination traits in order to evaluate whether genders differ in their reproductive traits and to determine the potential to set seeds when the growing conditions and selfing rates were controlled. For each individual, the number of flowers per inflorescence and the number of inflorescences were recorded before hand pollination was carried out. Additionally, in order to examine differences in petal size, we removed and scanned petals from two to three flowers per individual. The length and width of the petals were measured using imaging software ImageJ (Abramoff et al. 2004). The product of the petal length and width was used to estimate the petal area ( $\text{mm}^2$ ), using an equation obtained from a preliminary study that used 80 flowers in a regression analysis:  $\text{area} = 0.0039 \times (\text{petal length} \times \text{width}) + 7.1691$  ( $r^2 = 0.967$ ; M. L. Van Etten, unpublished data). To examine differences in the capacity of seed production, we hand pollinated each flower using pollen collected from non-experimental plants of the same population. Sufficient pollen (one to two anthers) was used to fully saturate the stigmatic surface, allowing the potential for full fruit and seed set. The number of pollinated flowers that developed into fruits containing at least one seed was recorded for each individual. All fruits containing at least one seed were collected just before dehiscence, and the number of seeds within each of these fruits (seed set) was counted. Average seed mass was determined for each individual by dividing the total seed mass by the total number of seeds it produced.

Several biomass measurements were also taken from all individuals, for two reasons. First, we wanted to determine whether genders differ in their investment in aboveground and belowground biomass; a greater investment in aboveground biomass could allow females to produce more seeds. Aboveground biomass included inflorescences and leaves that were harvested, dried, and weighed after all individuals had completed fruit development. Belowground biomass included rhizomes and the attached roots that were cleaned of soil, dried, and weighed. Second, we also wanted to determine whether genders differ in their reproductive allocation. To do so, we first constructed two composite biomass measurements: the reproductive biomass (the sum of the dry mass of fruits, seeds, and inflorescences) and vegetative biomass (the sum of the mass of the rhizomes and dried leaves). An index of reproductive allocation for each individual was then calculated by dividing its reproductive biomass by its vegetative biomass.

The aboveground and belowground biomass was analyzed with an ANOVA, and the reproductive traits were analyzed

with either an ANOVA or an ANCOVA. To satisfy the assumptions of ANOVA and ANCOVA, data were normalized using appropriate transformations, and outliers were removed if they were greater than 2 SD from the mean. Total flower number per plant, flowers per inflorescence, fruit number, and reproductive allocation were log transformed, inflorescence number was square root transformed, and seed number was natural log transformed. Fruit set (number of fruits per number of flowers) could not be normalized, and this was tested for gender differences using a Wilcoxon rank sum test separately for each light level. The remaining traits were not transformed. Two outliers were removed for their respective analysis; one hermaphrodite had exceptionally high reproductive allocation (0.1), and another hermaphrodite had very high seed mass (1.8 mg). The outliers were removed in order to satisfy the normality assumption of ANOVA and ANCOVA. However, results of the analyses remained qualitatively the same when outliers were retained in the analyses.

The ANOVA and ANCOVA models for reproductive traits had gender and light level and their interaction as independent variables. Several plant size measurements were considered as possible covariates for these analyses. First, because we did not find gender difference for the biomass traits (see details in “Results” and in table 1), we considered the variation in final vegetative biomass to be a potential source of variation for reproductive traits that is not related to gender. In addition, the length, width, diameter, and mass of the initial rhizomes may also account for a portion of the variation found in the reproductive traits. To determine whether to include these plant size measurements as covariates in the ANOVA for a particular trait, we first tested their necessity individually, following steps outlined by Littell et al. (1996). These tests involved including each size trait individually in a regression analysis for each reproductive trait and then determining the slope for their relationship. A regression slope that significantly differs from 0 suggests that the size trait can explain a significant amount of variation in the reproductive trait, and it was included in the final analysis of that reproductive trait. Among the five size traits tested, only final vegetative biomass consistently covaried with the reproductive trait data. After including the covariate, the analysis was further tested to determine whether there was an interaction between the covariate and the main effects: gender and light treatment. If there was no interaction, then a common slopes model was used. In one case (inflorescence number), vegetative biomass showed a significant interaction with gender, suggesting that the relationship (or the slopes) between inflorescence number and gender varied depending on the value of the covariate. In this case, trait means were compared at the twenty-fifth, fiftieth, and seventy-fifth percentiles of the covariate values (Littell et al. 1996).

In summary, four traits—total flower number, number of inflorescences, total fruit number, and seed set—were analyzed using ANCOVA with gender, light level, and their interaction as main fixed effects, final vegetative biomass as the covariate, and the block as a random effect (Littell et al. 1996). Results for these analyses were qualitatively the same when the covariate was not included. The remaining reproductive and biomass traits (see table 1) were analyzed using ANOVA with the same independent variables as the analysis

**Table 1**  
**F Values from the Analysis of Reproductive and Biomass Traits**

	df 1, df 2	Gender	Treatment	Gender × treatment
Reproductive traits:				
Petal size	1, 9	21.99***	.55	.30
Total flower number <sup>a</sup>	1, 16	5.25*	.03	.35
Inflorescence number <sup>a</sup>	1, 24	1.05	2.77	.57
Flowers per inflorescence	1, 17	3.73	.74	.71
Total fruit number <sup>a</sup>	1, 16	2.98 <sup>+</sup>	.65	.03
Fruit set (fruit no./flower no.)	1, 7;	4.54 shade;		
	1, 10	.09 light		
Total seed number	1, 17	2.78	.35	.78
Seed set (seeds/fruit) <sup>a</sup>	1, 16	2.36	.06	3.64
Average seed mass	1, 16	.39	2.28	.02
Biomass traits:				
Belowground biomass	1, 25	.06	2.64	1.29
Aboveground biomass	1, 25	.04	4.59*	2.86
Reproductive allocation	1, 24	.10	.00	1.28

Note. Numbers in the degrees of freedom column are the numerators (df = 1) and denominators (df = 2) for the corresponding *F* values. Only petal size and flower number were significantly different between hermaphrodites and females (gender). Aboveground biomass was significantly different between the light and shade treatments (treatment). Values were obtained using either an ANOVA or an ANCOVA.

<sup>a</sup> ANCOVA result with vegetative biomass as covariate.

<sup>+</sup> *P* = 0.055.

\* *P* < 0.05.

\*\*\* *P* < 0.001.

above but without the covariate. All of these analyses were done using PROC MIXED in SAS (SAS 1999).

### Physiological Traits

Three physiological traits—leaf photosynthetic rate at a controlled light level and CO<sub>2</sub> levels (*A*), leaf N, and leaf carbon isotope ratio (δ<sup>13</sup>C)—were measured on recently matured leaves. These traits were sampled both before flowering (preflowering) and after fruiting began (postfruiting). Measurements of *A* were made with a Li-Cor 6400 with a 6400-02B LED light source (Li-Cor, Lincoln, NE). The chamber conditions were reference CO<sub>2</sub> 400 ppm, ambient humidity, and leaf temperature. For preflowering measurements, the light levels were 1000 μmol m<sup>-2</sup> s<sup>-1</sup> (light saturated) for both shade and light treatments. Preflowering measurements were taken over two consecutive days; the light treatment for the first block was measured on the first day, and both the light and shade treatment for the second block were measured on the second day. For postfruiting measurements, the light levels were set to reflect maximum light levels received during growth in each treatment and to minimize any potential for photoinhibition (300 and 900 μmol m<sup>-2</sup> s<sup>-1</sup> for the shade and light treatments, respectively). Postfruiting measurements were taken in a single day with the two light treatments measured first, followed by the two shade treatments. Leaf area was determined by scanning the portion of the leaf that was inside the Li-Cor chamber and then using WinFOLIA (Regent Instruments, Quebec) to determine the area. Because plants in the light and shade treatments were measured sequentially at different light levels, comparisons between genders for *A* can be made only within each light treatment and time period (preflowering or postflowering). Gender differ-

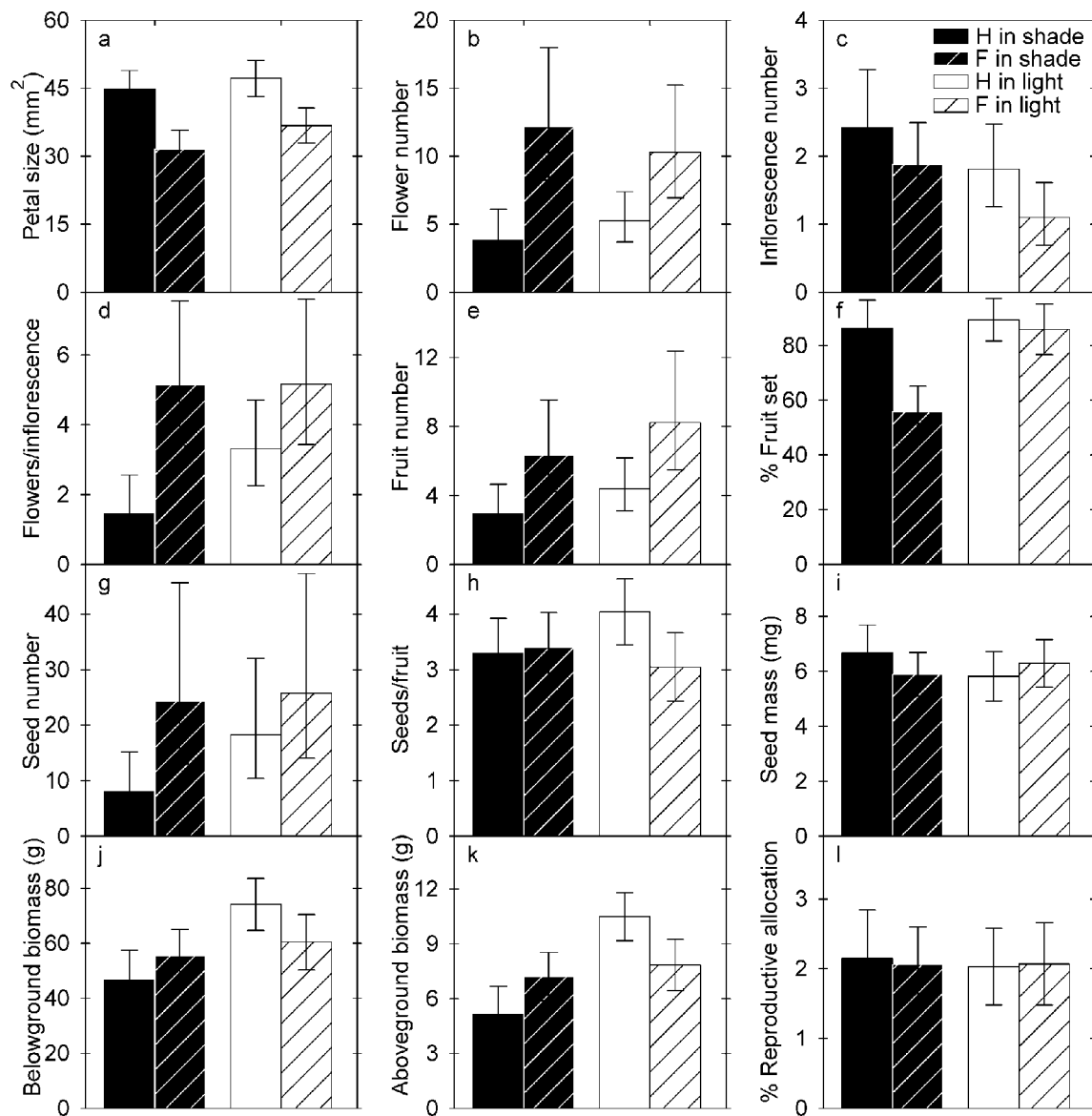
ences in *A* were tested using a *t*-test for each light treatment and time (preflowering and postfruiting) separately.

After *A* measurements, each leaf was harvested, dried at 60°C, ground into a fine powder, and analyzed for leaf N and leaf δ<sup>13</sup>C (Carlo Erba NA 1500, CHN combustion analyzer coupled to a Finnigan Delta Plus mass spectrometer) at the Analytical Chemistry Laboratory, University of Georgia. Leaf δ<sup>13</sup>C provides an integrated measure of leaf intercellular CO<sub>2</sub> concentration (*c<sub>i</sub>*) over the lifetime of the leaf. Integrated *c<sub>i</sub>* is, in turn, a relative measure of seasonally integrated photosynthetic water use efficiency, provided that leaf temperatures and photosynthetic characteristics are similar (Farquhar et al. 1989; Ehleringer et al. 1992). Greater (i.e., less negative) leaf δ<sup>13</sup>C reflects greater water use efficiency. Leaf N and leaf δ<sup>13</sup>C were analyzed using a repeated-measures ANOVA with time (preflowering and postfruiting), gender, light treatment, and their interactions as main effects.

## Results

### Reproductive and Biomass Traits

Female and hermaphroditic plants did not differ for most of the reproductive and biomass traits measured, with only three exceptions: petal size, total number of flowers, and total number of fruits. Females produced flowers that were 26% smaller in area than did hermaphrodites (table 1; fig. 1a). Females produced, on average, a similar number of flowers per inflorescence as the hermaphroditic plants but a higher total number of flowers (table 1; fig. 1b, 1d). Females also produced a higher total number of fruits than did hermaphrodites, but fruit set and seed set did not differ by gender



**Fig. 1** Reproductive and biomass traits (back-transformed least squares means  $\pm$  1 SE) for each gender ( $H$  = hermaphrodite and  $F$  = female) and treatment (shade or light). Genders differed significantly in petal size (*a*), total flower number (*b*), and total fruit number (*e*). Plants grown in shade had less aboveground biomass (*k*). The remaining eight traits did not differ between genders or between light treatments. Statistics for these analyses are provided in the text and in table 1.

(table 1; fig. 1*e*, 1*f*, 1*h*). Although females produced more flowers and more fruits, they did not produce more seeds or heavier seeds (table 1; fig. 1*g*, 1*i*). For biomass measurements, genders did not differ for belowground biomass, aboveground biomass, or reproductive allocation (table 1; fig. 1*j*–1*l*).

Analysis of the inflorescence number revealed a significant interaction between gender and the covariate, vegetative biomass, suggesting that the gender differences in inflorescence number changed over the range of plant sizes. More specifically, females had fewer inflorescences than hermaphrodites did when at smaller plant size (at twenty-fifth percentile, 47.6 g:  $t_{22} = 2.91$ ,  $P = 0.008$ ), but at larger plant sizes, there were no gender differences (fiftieth percentile, 60.9 g:  $t_{22} = 1.72$ ,  $P =$

0.099; seventy-fifth percentile, 83.0 g:  $t_{22} = -0.93$ ,  $P = 0.363$ ). As a result, the slope of the relationship between rhizome size and number of inflorescence was positive for females ( $P = 0.002$ ) but indistinguishable from 0 for hermaphrodites ( $P = 0.255$ ).

The aboveground biomass was the only trait that was influenced by the light treatment (table 1; fig. 1*k*). It was significantly lower in the shade treatment than in the light treatment.

#### Physiological Traits

The physiological traits did not differ significantly between genders. Genders did not differ for  $A$  in any light level and time combination ( $t_{\text{shade, preflowering, df}=4} = 1.88$ ,  $P = 0.242$ ;

$t_{\text{light, preflowering, df=13}} = 0.18, P = 0.675$ ;  $t_{\text{shade, postfruiting, df=12}} = 4.18, P = 0.064$ ;  $t_{\text{light, postfruiting, df=13}} = 0.39, P = 0.544$ ; fig. 2a). Leaf N and leaf  $\delta^{13}\text{C}$  compared across light treatments and time periods revealed that leaf N increased from preflowering to postfruiting in both light treatments, but this pattern did not differ by gender; this is indicated by the absence of an interactive effect between time and gender (table 2; fig. 2b). Leaf  $\delta^{13}\text{C}$  became less negative from preflowering to postfruiting in the shade treatment and did not differ through time in the light treatment, but these patterns did not differ by gender (table 2; fig. 2c).

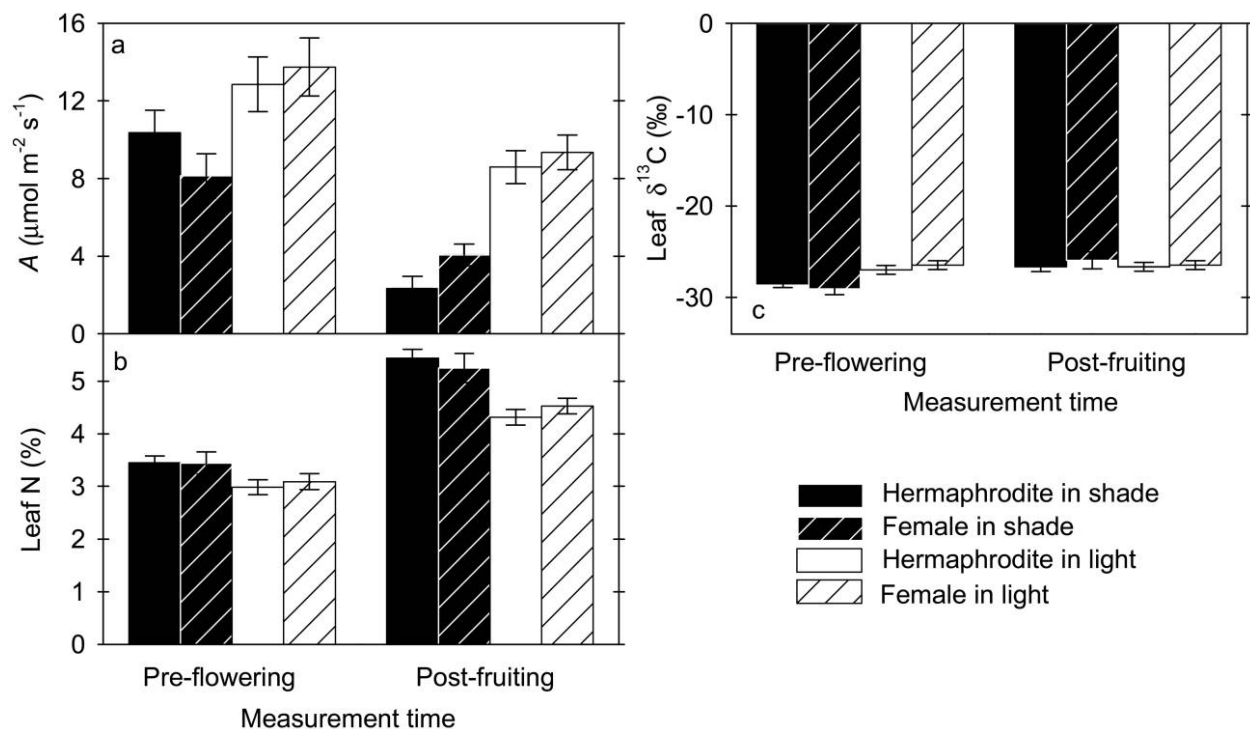
### Discussion

The goal of this study was to determine whether there were genetically based differences in reproductive or physiological traits between hermaphrodites and females that could explain the higher female seed fitness found in previous field studies (Ågren and Willson 1991; Chang 2006). We found few gender differences in reproductive and biomass traits, with three exceptions: petal size, total flower number, and total fruit number. Additionally, females and hermaphrodites did not differ in their photosynthetic rates, photosynthetic capacity inferred from leaf N and A, and seasonally integrated photosynthetic water use efficiency inferred from leaf  $\delta^{13}\text{C}$ . Combined, our results provide little support that the gender difference for seed production found in natural popu-

lations was from genetically based differences in these physiological traits.

The absence of differences in seed production and seed mass were unexpected given that *Geranium maculatum* females have been shown to have higher seed production (Ågren and Willson 1991; Chang 2006) and higher average seed mass (Chang 2006), a pattern also found in many other gynodioecious species (e.g., Kohn 1989; Olson 2001; Ramsey and Vaughton 2002; Schultz 2003; Shykoff et al. 2003). Though it is statistically nonsignificant, females produced approximately three times and 1.4 times, on average, as many seeds as hermaphrodites did in shade and sun treatments, respectively. The small sample size in this study may have contributed to the nonsignificant conclusion for this trait and can help explain the difference in the results of this and earlier studies. However, the minimal difference in the mean seed set and seed mass between genders and the higher fruit set in hermaphrodites, an opposite trend from the field results, would need additional explanations.

There are several possible reasons for the differences between results found here and those of previous studies. As mentioned earlier, field studies that measure reproductive traits in natural populations may confound the effects resulting from differences in the environment and selfing rates with effects resulting from genetically based gender differences. By growing plants in the greenhouse, we minimized the possibility of differences in gender microhabitat potentially experienced by field-grown plants, and this allowed us to focus on



**Fig. 2** Physiological measurements (least squares means  $\pm$  1 SE) for each gender, treatment (light or shade), and time (preflowering or post-fruiting). Genders did not differ in A (photosynthetic rate; a), leaf N (b), or leaf  $\delta^{13}\text{C}$  (c). For light treatment and time results for leaf N and leaf  $\delta^{13}\text{C}$ , see table 2 and text.

Table 2

**F Values from the Analysis of Leaf N and Leaf Carbon Isotope Ratio ( $\delta^{13}\text{C}$ ) Obtained Using a Repeated-Measures Analysis**

Source	df 1, df 2	Leaf N	Leaf $\delta^{13}\text{C}$
Gender	1, 11	.05	.21
Treatment	1, 11	19.03**	3.74
Gender $\times$ treatment	1, 11	.70	.09
Time	1, 8	297.87***	14.71**
Gender $\times$ time	1, 8	.05	.41
Treatment $\times$ time	1, 8	7.35*	11.33**
Gender $\times$ treatment $\times$ time	1, 8	.60	1.29

Note. Numbers in the degrees of freedom column are the numerators (df = 1) and denominators (df = 2) for the corresponding *F* values. There were no differences between hermaphrodites and females (gender) in either trait. Leaf N was different in the light and shade treatments (treatment). Both leaf N and leaf  $\delta^{13}\text{C}$  differed between preflowering and postfruiting (time).

\*  $P < 0.05$ .

\*\*  $P < 0.01$ .

\*\*\*  $P < 0.001$ .

the intrinsic gender differences. Additionally, by using only non-self-pollen to saturate the stigma, we controlled for the pollen source to keep the selfing rate at 0, and, therefore, we specifically examined differences between the genders that are not results of pollen limitation, inbreeding, or inbreeding depression. An absence of gender differences in seed number or seed mass following cross-pollination was also found by another greenhouse study with this species (Chang 2007). Combined, these results suggest that the gender differences in seed number and seed size found in natural populations (Ågren and Willson 1991; Chang 2006) might not be related to gender per se but are more likely to be related to other causes. One possible cause is the difference in selfing rate between genders. Preliminary results (A. C. Deen and S.-M. Chang, unpublished data) showed that the selfing rate of hermaphrodites in the source population of the rhizomes used in this study was as high as 40%, leading to a large difference between the selfing rates of females (effectively 0) and hermaphrodites. The combination of a high selfing rate and severe early inbreeding depression (Chang 2007) is a possible cause for the lower seed fitness of hermaphrodites found in natural populations.

Despite the lack of gender differences in seed production and seed mass, we found that females produced smaller petals than did hermaphrodites, a pattern consistent with what was found in natural populations. This suggests that the petal size difference found in natural populations (Ågren and Willson 1991; Chang 2006) was a result not only of environmental conditions in the plants' microhabitat but, rather, was a result also of intrinsic differences between genders. This suggests that females spend fewer resources than do hermaphrodites in producing petals, pollen, and anthers; however, they do not produce a higher number of seeds or heavier seeds, as the resource reallocation hypothesis predicts. Combined, these findings lead us to conclude that there is no evidence that the energetic surplus in females had been reallocated to seed production, or at least not to a detectable degree. These results are in contrast to many other gynodioecy studies that found

resource reallocation (Ashman 1992, 1994, 1999; Eckhart 1992; Wolfe and Shmida 1997; but see Delph et al. 1999; Alonso and Herrera 2001). However, because *G. maculatum* typically produces a smaller number of flowers (five to 10; fig. 1a) than do most other gynodioecious species, it is possible that the amount of resources saved in females is too small to be detectable even if reallocated to seeds. In any case, it is unlikely that resource reallocation is the main mechanism for the increase in seed production and seed quality in *G. maculatum* females.

Comparisons of physiological traits between genders are scarce for gynodioecious species, and the results are generally inconsistent, with some suggesting that females have higher photosynthetic rates (Caruso et al. 2003) and others suggesting the opposite (Poot et al. 1996; Schultz 2003). When we grew *G. maculatum* in controlled environmental conditions, we found neither an increase in photosynthetic rate nor a decrease in allocation to photosynthetic tissues. These results are not surprising, considering that we did not find a gender difference in fruit set and seed set in this experiment. Nevertheless, the absence of gender difference in physiological traits found here does suggest that genders do not exhibit any genetically based differences in the traits measured. This conclusion is consistent with other physiological data collected on this species. Specifically, in a separate preliminary study, photosynthetic measurements taken in natural populations showed no differences in maximum *A* under standardized light-saturated conditions (S.-M. Chang, unpublished data). Additionally, leaf  $\delta^{13}\text{C}$  and leaf N from plants previously grown in the greenhouse did not show any gender differences (M. L. Van Etten, unpublished data). Combined, these results suggest that it is likely that female *G. maculatum* plants in natural populations increased their seed production (Ågren and Willson 1991; Chang 2006) not by the physiological mechanisms such as an increase in photosynthetic carbon gain but by other mechanisms such as habitat selection or less inbreeding depression. Additional studies that compare the microhabitat conditions and the selfing rate between genders will help to determine the causes of seed fitness differences between genders of this species.

Though we found no evidence to support physiological differences contributing to female fitness compensation in *G. maculatum*, results from this study do provide additional implications for the maintenance of gynodioecy in this species in a larger context. Our results speak to two of the other potential hypotheses for how females can increase their seed fitness: resource reallocation and inbreeding depression. First, even though females still produced no pollen and produced smaller petals than did hermaphrodites in our study, the lack of gender differences in seed number or seed mass found here provided no evidence for the hypothesis that females may use resources usually allocated to pollen production to increase the number or size of seeds. Second, our results show that genders differ in neither seed number nor seed mass following cross-pollination, while Chang (2007) found that self-pollination in hermaphrodites significantly reduced both seed production and seed mass. This suggests that the reduced seed number and seed size found in hermaphrodites in natural populations (Chang 2007) is likely to be a result of early-acting inbreeding depression following self-fertilization.

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### Literature Cited

- Abramoff MD, PJ Magelhaes, SJ Ram 2004 Image processing with ImageJ. *Biophotonics Int* 11:36–42.
- Ågren J, MF Willson 1991 Gender variation and sexual differences in reproductive characters and seed production in gynodioecious *Geranium maculatum*. *Am J Bot* 78:470–480.
- Alonso C, CM Herrera 2001 Neither vegetative nor reproductive advantages account for high frequency of male-steriles in southern Spanish gynodioecious *Daphne laureola* (Thymelaeaceae). *Am J Bot* 88:1016–1024.
- Ashman T-L 1992 Indirect costs of seed production within and between seasons in a gynodioecious species. *Oecologia* 92:266–272.
- 1994 Reproductive allocation in hermaphrodite and female plants of *Sidalcea oregana* ssp. *spicata* (Malvaceae) using four currencies. *Am J Bot* 81:433–438.
- 1999 Determinants of sex allocation in a gynodioecious wild strawberry: implications for the evolution of dioecy and sexual dimorphism. *J Evol Biol* 12:648–661.
- Caruso CM, H Maherali, RB Jackson 2003 Gender-specific floral and physiological traits: implications for the maintenance of females in gynodioecious *Lobelia siphilitica*. *Oecologia* 135:524–531.
- Case AL, T-L Ashman 2005 Sex-specific physiology and its implications for the cost of reproduction. Pages 129–157 in EG Reekie, FA Bazzaz, eds. *Reproductive allocation in plants*. Elsevier, Amsterdam.
- Case AL, SCH Barrett 2001 Ecological differentiation of combined and separate sexes of *Wurmbea dioica* (Colchicaceae) in sympatry. *Ecology* 82:2601–2616.
- Chang S-M 2006 Female compensation through the quantity and quality of progeny in a gynodioecious plant, *Geranium maculatum* (Geraniaceae). *Am J Bot* 93:263–270.
- 2007 Gender-specific inbreeding depression in a gynodioecious plant, *Geranium maculatum* (Geraniaceae). *Am J Bot* 94:1193–1204.
- Charlesworth B, D Charlesworth 1978 Model for evolution of dioecy and gynodioecy. *Am Nat* 112:975–997.
- Charlesworth D, FR Ganders 1979 Population genetics of gynodioecy with cytoplasmic-genic male-sterility. *Heredity* 43:213–218.
- Darwin C 1877 *The different forms of flowers on plants of the same species*. J Murray, London.
- Dawson TE, LC Bliss 1989 Patterns of water use and the tissue water relations in the dioecious shrub *Salix arctica*: the physiological basis for habitat partitioning between the sexes. *Oecologia* 79:332–343.
- Dawson TE, JR Ehleringer 1993 Gender-specific physiology, carbon isotope discrimination, and habitat distribution in boxelder, *Acer negundo*. *Ecology* 74:798–815.
- Dawson TE, JR Ehleringer, JD Marshall 1990 Sex-ratio and reproductive variation in the mistletoe *Phoradendron juniperinum* (Viscaceae). *Am J Bot* 77:584–589.
- Dawson TE, MA Geber 1999 Sexual dimorphism in physiology and morphology. Pages 175–215 in MA Geber, TE Dawson, LE Delph, eds. *Gender and sexual dimorphism in flowering plants*. Springer, Berlin.
- Delph LF, MF Bailey, DL Marr 1999 Seed provisioning in gynodioecious *Silene acaulis* (Caryophyllaceae). *Am J Bot* 86:140–144.
- Eckhart VM 1992 The genetics of gender and the effects of gender on floral characters in gynodioecious *Phacelia linearis* (Hydrophyllaceae). *Am J Bot* 79:792–800.
- 1999 Sexual dimorphism in flowers and inflorescences. Pages 123–148 in MA Geber, TE Dawson, LE Delph, eds. *Gender and sexual dimorphism in flowering plants*. Springer, Berlin.
- Eckhart VM, FS Chapin 1997 Nutrient sensitivity of the cost of male function in gynodioecious *Phacelia linearis* (Hydrophyllaceae). *Am J Bot* 84:1092–1098.
- Eckhart VM, J Seger 1999 Phenological and developmental costs of male sex function in hermaphroditic plants. Pages 195–213 in TO Vuorisalo, PK Mutikainen, eds. *Life history evolution in plants*. Kluwer Academic, Dordrecht.
- Ehleringer JR, SL Phillips, JP Comstock 1992 Seasonal variation in the carbon isotopic composition of desert plants. *Funct Ecol* 6:396–404.
- Farquhar GD, JR Ehleringer, KT Hubick 1989 Carbon isotope discrimination and photosynthesis. *Annu Rev Plant Physiol Plant Mol Biol* 40:503–537.
- Glaetli M, J Goudet 2006 Variation in the intensity of inbreeding depression among successive life-cycle stages and generations in gynodioecious *Silene vulgaris* (Caryophyllaceae). *J Evol Biol* 19:1995–2005.
- Graff A 1999 Population sex structure and reproductive fitness in gynodioecious *Sidalcea malviflora malviflora* (Malvaceae). *Evolution* 53:1714–1722.
- Klinkhamer PGL, TJ deJong, HW Nell 1994 Limiting factors for seed production and phenotypic gender in the gynodioecious species *Echium vulgare* (Boraginaceae). *Oikos* 71:469–478.
- Kohn JR 1989 Sex-ratio, seed production, biomass allocation, and the cost of male function in *Cucurbita foetidissima* HBK (Cucurbitaceae). *Evolution* 43:1424–1434.
- Lewis D 1941 Male-sterility in natural populations of hermaphrodite plants: the equilibrium between females and hermaphrodites to be expected with different types of inheritance. *New Phytol* 40:56–63.
- Littell RC, GA Milliken, WW Stroup, RD Wolfinger 1996 SAS system for mixed models. SAS Institute, Cary, NC.
- Lloyd DG 1975 Maintenance of gynodioecy and androdioecy in angiosperms. *Genetica* 45:325–339.
- Manicacci D, A Atlan, JAE Rossello, D Couvet 1998 Gynodioecy and reproductive trait variation in three *Thymus* species (Lamiaceae). *Int J Plant Sci* 159:948–957.
- Marshall JD, TE Dawson, JR Ehleringer 1993 Gender-related differences in gas-exchange are not related to host quality in the xylem-tapping mistletoe, *Phoradendron juniperinum* (Viscaceae). *Am J Bot* 80:641–645.
- Molina-Freaner F, SK Jain 1992 Female frequencies and fitness components between sex phenotypes among gynodioecious populations of the colonizing species *Trifolium hirtum* all in California. *Oecologia* 92:279–286.
- Olson MS 2001 Patterns of fruit production in the subdioecious plant *Astilbe biternata* (Saxifragaceae). *J Ecol* 89:600–607.
- Olson MS, AV Graf, KR Niles 2006 Fine scale spatial structuring of sex and mitochondria in *Silene vulgaris*. *J Evol Biol* 19:1190–1201.
- Poot P, J Pilon, TL Pens 1996 Photosynthetic characteristics of leaves of male-sterile and hermaphrodite sex types of *Plantago lanceolata* grown under conditions of contrasting nitrogen and light availabilities. *Physiol Plant* 98:780–790.

- Poot P, T VandenBroek, JMM Van Damme, H Lambers 1997 A comparison of the vegetative growth of male-sterile and hermaphroditic lines of *Plantago lanceolata* in relation to N supply. *New Phytol* 135:429–437.
- Ramsey M, G Vaughton 2002 Maintenance of gynodioecy in *Wurmbea biglandulosa* (Colchicaceae): gender differences in seed production and progeny success. *Plant Syst Evol* 232:189–200.
- SAS 1999 SAS user's guide: statistics. SAS Institute, Cary, NC.
- Schultz ST 2003 Sexual dimorphism in gynodioecious *Sidalcea hirtipes* (Malvaceae). I. Seed, fruit, and ecophysiology. *Int J Plant Sci* 164:165–173.
- Schultz ST, FR Ganders 1996 Evolution of unisexuality in the Hawaiian flora: a test of microevolutionary theory. *Evolution* 50:842–855.
- Shykoff JA, SO Kolokotronis, CL Collin, M Lopez-Villavicencio 2003 Effects of male sterility on reproductive traits in gynodioecious plants: a meta-analysis. *Oecologia* 135:1–9.
- Stamp NE, JR Lucas 1983 Ecological correlates of explosive seed dispersal. *Oecologia* 59:272–278.
- USDA, NRCS 2007 The PLANTS database (<http://plants.usda.gov>, 2007). National Plant Data Center, Baton Rouge, LA.
- Wolfe LM, A Shmida 1997 The ecology of sex expression in a gynodioecious Israeli desert shrub (*Ochradenus baccatus*). *Ecology* 78:101–110.