The Effect of Multiple Environmental Stresses on the Expression of Inbreeding Depression in *Mimulus ringens*

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ABSTRACT

Inbreeding is the mating of biological relatives that causes an increase in homozygosity. Inbreeding may lead to inbreeding depression is the reduction in fitness between inbred and outbred individuals due to deleterious alleles in homozygous form. Environmental stressors known to magnify the expression of inbreeding depression. Though much research has been focused on the effect of single stress factors on the expression of inbreeding depression, few studies have looked at the effect of multiple factors. In this study, I conduct two experiments which address the effects of multiple environmental stresses on the expression of inbreeding depression in the perennial plant Mimulus ringens. In the first experiment, I use herbivory and the amount of water available as stressors. Using a mixed-model ANOVA, I find a significant 4-way interaction between maternal line, cross, herbivory, and water treatment in branch number. In the second experiment, I use interspecific competition with the invasive purple loosestrife (Lythrum salicaria) and intraspecific competition with varying densities of M. ringens as the two environmental stresses. I find significant 2-way interactions between cross and each environmental factor, as well as two marginally significant 3-way interactions between cross, density, and competition in biomass and leaf length. The 3-way interaction trend shows that as stress increases, inbreeding depression actually decreases. The results of this study show a trend that multiple stresses may interact with cross, though the effect on the expression on inbreeding depression depends heavily on the types of stresses present as well as the maternal line. These results have implications for conservation, as species of concern may suffer from inbreeding depression and

multiple environmental stresses simultaneously, though this effect is highly influenced by maternal factors.

INTRODUCTION

Inbreeding is the mating of biological relatives that leads to an increase in homozygosity. Inbreeding depression, or the reduction in fitness between inbred and outbred individuals due to deleterious alleles in homozygous form, has long been recognized as a common phenomenon in numerous species (Darwin 1876). Inbreeding depression is often attributed to either overdominance (heterozygote advantage) or deleterious, recessive alleles in homozygous form (*e.g.* Lande and Schemske 1985; Campbell 1986). Species differ widely in their magnitudes of inbreeding depression; inbred individuals are inferior to outcrossed individuals in some species, whereas such differences in fitness are small or absent in others (e.g. Holsinger 1991). Theoretical and empirical work suggests that species that have evolved selfing mating systems have lower magnitudes of inbreeding depression due to the purging of deleterious alleles (Charlesworth and Charlesworth 1979; Lande and Schemske 1985; Charlesworth and Charlesworth 1987; Carr and Dudash 1996; Husband and Schemske 1996; Bijlsma et al. 1999).

A wide variety of levels of inbreeding and inbreeding depression are observed across populations within a species (Husband and Schemske 1996; Keller and Waller 2002; Steets and Ashman 2004). Environmental stress has also been identified as a key cause of variation in inbreeding's effect on individuals and populations. In a recent review, Armbruster and Reed (2005) found that inbreeding depression is magnified when individuals are subjected to an environmental stress factor in 48% of surveyed studies.

Furthermore, the authors found that in stressful environments there was a 69% increase in the number of lethal haploid equivalents, which are the decline in fitness for inbred individuals (Morton et al. 1956). The intensity of stress may be influenced by the type of experiment conducted. Empirical studies show varying magnitudes of inbreeding depression across experiments conducted in a greenhouse, garden plot, natural habitat (Dudash 1990), or semi-natural environment (Kristensen et al. 2008). Furthermore, these effects vary across maternal lines within a population due to genetic variability (e.g. Carr and Dudash 1997; Reed et al. 2003).

One explanation for such magnification of inbreeding depression centers on an individual's alleles, which are of course, derived from the maternal line. Different loci affect survival in different environmental contexts (Fry et al. 1998; Bijlsma et al. 1999; Dahlgaard and Hoffmann 2000). As inbred individuals are exposed to a variety of stressful environments, different loci may act to reduce fitness (Bijlsma et al. 1999; Dahlgaard and Hoffmann 2000; Armbruster and Reed 2005). In some cases lethal alleles may be triggered by the presence of some environmental factor, thereby causing inbreeding depression (Vermeulen et al. 2008).

One common environmental stress factor that has been shown to magnify the effects of inbreeding depression is competition for resources with other individuals. A review of the literature shows that inbred individuals may display enhanced inbreeding depression in the presence of intraspecific competition (Haag et al. 2002; Cheptou and Schoen 2003, Waller 1984; Schmitt and Ehrhardt 1990, Bijlsma et al. 1999) or interspecific competition (Cheptou et al. 2000). However, it is worth noting that several

68 studies (e.g., Willi et al. 2007) found no effect of competition on inbreeding depression,
69 highlighting the variation of inbreeding's effects across different species.
70 Herbivory is another type of biological interaction can affect the expression of
71 inbreeding depression. The presence of herbivorous spittlebugs (*Philaenus spumarius*) is
72 known to enhance inbreeding depression in *Mimulus guttatus* in both greenhouse (Carr
73 and Eubanks 2002) and natural habitats (Ivey et al. 2004). However, herbivory in this
74 system has also been shown to reduce inbreeding depression in some traits, such as
75 flower number and selfing rate (Ivey and Carr 2005).
76 Finally, abiotic factors such as water deprivation have been shown to influence
77 the expression of inbreeding depression. For instance, drought is known to increase the
78 magnitude of inbreeding depression in the perennial *Lychnis flos-cuculi* (Hauser and
79 Loeschcke 1996).
80 Despite the numerous studies that have looked at inbreeding depression in
81 stressful environments, to date, few have examined the effects of multiple stressors on the
82 expression of inbreeding depression. Because different types of stress affect the purging
83 of different alleles (Dahlgaard and Hoffmann 2000), a combination of stressors may
84 further reduce the fitness of inbred individuals, though one would expect this effect to be
85 variable across maternal lines. In this study I devise 2 experiments to analyze the effect of
86 multiple stressors on inbreeding depression in the perennial wetland plant *Mimulus*
87 *ringens* (Phrymaceae). In the first experiment, I consider the biotic effect of herbivory
88 and the abiotic effect of water deprivation to investigate whether the stressors interact to
89 further enhance inbreeding depression. Because of the variation in inbreeding depression
90 (e.g. Carr and Dudash 1997; Reed et al. 2003), I consider how maternal line interacts

with herbivory and water deprivation to enhance inbreeding depression. In the second experiment, I investigate the effect of 2 levels of competition, intraspecific competition and interspecific competition with an invasive species, on the magnitude of inbreeding depression.

Although the two experiments use different stresses, I predict similar results. Specifically, I hypothesize in both experiments that (1) the environmental stresses imposed should magnify the expression of inbreeding depression. I expect the stresses in the first experiment, herbivory and water, to magnify the expression of inbreeding depression. Similarly, I expect the interspecific and intraspecific competition stresses to increase the expression of inbreeding depression in the second experiment. Although some studies have found the opposite effect of stress on inbreeding depression (e.g. Willi et al. 2007), previous work by Griffin et al. (in prep) suggests that stressed *M. ringens* individuals exhibit greater inbreeding depression than unstressed individuals. I further hypothesize that (2) the combination of environmental stresses should interact to further increase the magnitude of inbreeding depression. Individuals in the first experiment which are subjected to both the herbivory and the water stresses should display the highest level of inbreeding depression, as should those individuals in the second experiment that experience the highest levels of interspecific and intraspecific competition. Finally, I hypothesize that (3) inbreeding depression should vary across maternal lines. Because of genetic variability within populations, I expect some maternal lines to display more inbreeding depression that others. Furthermore, I expect inbreeding depression among maternal lines to vary in response to stressful environments.

MATERIALS AND METHODS

Study System

Mimulus ringens (common name: square-stemmed monkeyflower) is a perennial herb native to wetlands across North America. It reproduces sexually and vegetatively through stolons. *M. ringens* is capable of both outcrossing and self-fertilizing, and has been found to exhibit inbreeding depression (Bell *et al.* 2005, Griffin et al. in prep). This trait, along with its easily manageable size and life-cycle, makes *M. ringens* an ideal model plant for this study.

Outcrossed and inbred *M. ringens* individuals were created from several natural, maternal lines. Fruits were collected in October 2006 from individuals at the Shaw Nature Reserve in Gray Summit, Missouri. Seeds were propagated in water in sealed Petri dishes and transplanted into the Washington University in St. Louis greenhouse. One individual from each maternal line was selected as a parent plant. Parent plants were hand-fertilized by applying the open anthers of one flower to the open stigma of another. Selfed seed was generated by either using two different flowers on the same individual, or applying the anthers of a flower to its own stigma. Outcrossed seed was generated by randomly selecting the two parent plants. Flowers selected to propagate outcrossed seed were emasculated so as to prevent selfing. Outcrossing was not reciprocal and individuals did not donate pollen to more than one fruit. Fruits were collected, dried in envelopes, and stored at 4 °C. Seeds derived from the same parent plant were considered to be from the same maternal line.

Experiment 1: Herbivory and Water Deprivation

Design

I studied the combined effects of artificial herbivory and water deprivation on the expression of inbreeding depression in *M. ringens*. *M. ringens* plants experiences low levels of insect herbivory in the field (personal observations). My experiment simulates leaf loss at a level higher than observed. Such herbivory might be possible in years for which insect populations surge or outbreak. The second stress used, water deprivation, simulates *M. ringens*'s natural habitat. *M. ringens* grows around the edges of ponds at the Shaw Nature Reserve. These ponds dry out during the summer, leaving the plants vulnerable to drought. The extent to which the soil dries out depends on how far away the plant is from the water. My experiment simulates water deprivation by experimentally watering plants in a dry common garden.

To investigate the effects of herbivory and water deprivation on inbreeding depression, I germinated selfed and outcrossed seeds from 15 maternal lines in water-filled Petri dishes. After propagation, I transplanted the seedlings into 2-inch 50-cell jiffy pots filled with Metro-Mix 360 potting mix (Sun Gro Horticulture). I allowed the plants to establish in the greenhouse for 1 week under clear plastic domes. After this period, I transplanted the potted *M. ringens* in June 2007 into a common garden in Washington University's Tyson Research Center in Eureka, Missouri, USA. The area used within the common garden was cleared and periodically weeded to reduce variation from other plant growth. The soil was primarily clay, which caused *M. ringens* to be extremely water deprived.

The experiment consisted of 2 crosses (selfed and outcrossed) × 2 water treatments (low and high) × 2 herbivory treatments (clipped and control) × 15 maternal lines × 5 replicates for a total of 600 individuals. Individuals were randomized in a 21 ×

29 plot array in which each individual was 0.5 m away from its closest neighbors. I transplanted each jiffy pot containing one *M. ringens* individual directly into the soil. Individuals would grow out of the jiffy pots into the soil during the course of the experiment. I watered the plants daily and replaced individuals as necessary for the first week to avoid transplant shock. Each plant was assigned a water treatment and an herbivory treatment. The high, or wet, watering routine consisted of watering individuals every two days and the low, or dry, watering routine every four days. The water treatment began two weeks after transplanting to allow for establishment. In the herbivory treatments, I removed leaves to simulate herbivores' effects on *M. ringens*. For plants subjected to this treatment, I removed ¾ of the leaves on each individual two weeks after establishment.

I measured fitness-related traits when harvesting individuals in September 2007. For each plant, I measured height, branch number, biomass, stolon number, and fruit number.

Data Analysis

I used a mixed-model analysis of variance (ANOVA) to examine the effects of maternal line, cross, herbivory, water, and their interactions on each response variable. Cross, herbivory, water, and their 2- and 3-way interactions were all fixed effects, while maternal line and its 2-, 3-, and 4-way interactions with the other factors were random effects in the analysis. I conducted two separate analyses, one with maternal line included as a random effect in the model and one in which all of the families are pooled and only the fixed effects are considered. For height, branch number, stolons, and biomass, all living individuals who were measured at the end of the experiment were used in the

ANOVA. There were not enough degrees of freedom to analyze fruit number with maternal lines, but I was able to include fruit number in the ANOVA without maternal lines. All data were natural log-transformed for all response variables to achieve normality.

The interactions within the ANOVAs tell us about the extent to which inbreeding depression is affected by the stressor. Evidence of magnified inbreeding depression due to an environmental stress factor comes from a significant interaction of cross and one of the stresses (herbivory or water treatment). If inbreeding depression is further magnified by multiple stresses as I predict, then the 3-way interaction between cross, herbivory, and water treatment should be significant. All analyses were conducted using SYSTAT 10.2 (SYSTAT Software Inc. 2002).

Of particular interest is the difference in fitness value between outcrossed and selfed individuals within groups. From each group within each maternal line, I calculated inbreeding depression using the formula

$$\delta = \frac{w_o - w_s}{w_o}$$

where w_s and w_o are the selfed and outcrossed back-transformed mean values of a given response variable The inbreeding depression coefficient δ is therefore a measure of the outcrossed advantage over selfed individuals. Positive values indicate inbreeding depression, and negative values indicate outbreeding depression. I found δ for each group (maternal × herbivory × water) in order to investigate how inbreeding depression varied due to the fixed effects across different families.

Experiment 2: Intraspecific and Interspecific Competition

Design

To measure the effect of multiple stressors on the expression of inbreeding depression, I set up a greenhouse experiment with a 3 × 3 factorial design with factors of cross (selfed and outcrossed), intraspecific competition (4 different densities of *M. ringens*), and interspecific competition (presence or absence of the invasive *Lythrum salicaria*).

Lythrum salicaria was obtained as rootstock from the University of Illinois at Urbana-Champaign. All individuals were propagated from 1-inch stem cuttings of one of three plant. This process reduced genetic variation across individuals; my goal was to reduce variation in the size and competitive ability of *L. salicaria* across replicates as much as possible.

Selfed and outcrossed seeds for this experiment were from 8 *M. ringens* maternal lines. I germinated these seeds in water-filled Petri dishes. Once the seedlings had emerged, I transplanted them into 6 inch pots filled with Metro-Mix 360 soil. Pots were randomized and assigned an intraspecific competition treatment which consisted of a density of 4, 16, 32, or 64 *M. ringens* individuals. Additionally, each pot was assigned an interspecific competitive treatment which consisted of presence or absence of a single *L. salicaria*. Inbred and outcrossed *M. ringens* individuals from the 8 families were then randomized within each of the 4 intraspecific competition treatments. Seedlings were planted in a 4 cm^2 square array in the bottom half of each pot. For pots in the *L. salicaria* present treatment, *L. salicaria* was planted in the opposite half of each pot. Pots were placed in large tubs of water to allow for soil saturation through the first month. After this period, pots were moved into bins and were bottom-watered. Because of the shading

effect of the purple loosestrife on other pots, I separated the 2 competition treatments into different bins.

I set up the pots in October 2008, and replanted those that died 2 weeks later. I estimated survivorship and measured height, leaf number, and longest leaf length after individuals had established. Height and leaf length were measured to the nearest 0.5 cm. In January 2009, I took final measurements on the same variables as well as biomass. However, I was unable to record data for the 64-density pots due to the uncertainty of the individuals' identities and therefore disregarded that intraspecific competition treatment. I created an additional composite variable which I defined as (LN height) / (LN leaf number). This composite factor is an indicator of how the shape of the individual is influenced by the treatment group and of what growth strategies individuals may employ in stressful conditions.

Data Analysis

Survivorship data were normally distributed and could be analyzed with traditional parametric statistics. However, the other response variables, height, leaf number, leaf length, biomass, and the composite variable for plant shape, could not be transformed to fulfill the assumption of a normal distribution, and were analyzed with non-parametric statistics. Analyses for Experiment 2 were conducted using R 2.8.1 (The R Foundation for Statistical Computing 2008) and the CAR-package (Fox 2007 #571). I assessed the effects of cross, intraspecific competition, interspecific competition and their interactions on percent survivorship using an ANOVA. For each of the other response variables, I created 1,000 random permutations of the natural log transformed (LN (data + 1)) data. For each permutation, I constructed a mixed-model ANOVA which was used

to create a null distribution of permuted F-values. From the null model, I calculated p-values as the proportion of permuted F-values greater than the observed data. For all of these analyses, I pooled maternal lines; a much larger experiment with more replicates for each maternal line would have been necessary to assess the role of maternal line, and its interactions with other factors on the response variables.

Next, I analyzed differences in group means for the factors and interactions that were significant ($p < 0.05$) or almost significant ($p < 0.10$) in the previous ANOVA. Analysis of group means tests for pairwise differences between treatment levels for each fitness response variable. For the significant interspecific × intraspecific competition interactions, I compared levels of intraspecific competition within a given interspecific competition. This method of comparison was more meaningful than comparing levels of interspecific competition across levels of intraspecific competition because of the especially strong effect of interspecific competition across every response variable ($p < .001$). Intraspecific competition was much more variable across all response variables in each group. Calculations were performed for the response variable height, leaf number, leaf length, biomass, and the composite variable for plant shape. Because the data were non-normally distributed, I generated permutations and found the permuted differences of the means for each group. Similar to construction of the permuted ANOVA, I compared these means to the observed means and generated a p-value. For groups involving cross, I compared the differences between outcrossed and selfed individuals. For groups without cross (i.e. intraspecific or interspecific × intraspecific), I compared different levels of intraspecific density within the same competition treatment. I generated means of each group using 1000 bootstraps of the data and created 95% confidence intervals from these

bootstraps as well. Finally, using group means, I calculated inbreeding depression (δ) as in Experiment 1 using the back-transformed means generated from bootstrapping.

RESULTS

Experiment 1

When maternal lines were included in the analysis, I found significant main effects of maternal lines, cross, herbivory, and water treatment in at least 1 fitness-related response variable (Table 1). Maternal lines differed in their fitness. On average, outcrossed individual had higher fitness than inbred individuals, indicating significant inbreeding depression. Plants in the high herbivory and low water treatments had lower fitness, suggesting that my experiment successfully manipulated stress for these plants. The only significant 2-way interaction was the maternal line × cross interaction for stolon number, suggesting that inbreeding depression of stolon number is dependent on the maternal line. No 3-way interactions were found for any of response variables, though marginally significant results were found for the cross × water interaction in branches and the maternal line × cross × water interaction in height. These results suggest that although the stresses altered fitness, they did not influence inbreeding depression. I found a significant 4-way interaction between maternal line, cross, herbivory, and water treatment for branches. I show this interaction by presenting δ for each combination of herbivory and water treatment in each maternal line (Figure 1). In this way, we can visualize how the two stresses influence the expression of inbreeding depression for each maternal line. For example, in family #27, inbreeding depression is highest in the most stressful combination of treatments (herbivory and low water) as expected. However, for families 13 and 23, inbreeding depression is lowest in the most stressful treatment (no herbivory

296 and high water), but is relatively high in all other treatments. When maternal lines are
297 excluded from the analysis, the results are qualitatively similar; main effects are
298 significant for most response variables, but interactions are not (Table 2).

299 **Experiment 2**

300 Interspecific competition was significant across all response variables, indicating
301 that the presence of *L. salicaria* reduced each fitness measure and altered plant shape for
302 the composite variable (Table 3). The composite variable response showed that the
303 proportion of height to leaf number decreased with *L. salicaria* presence, indicating that
304 the space between nodes was relatively smaller in this treatment. Similarly, increasing
305 intraspecific competition (*M. ringens* density) significantly reduced most of the fitness
306 response variables and altered plant shape (Table 3). In this case, however, the composite
307 variable increased with increasing density, resulting in a greater proportion of height to
308 leaf number, or a greater relative distance between nodes. Taken together, these main
309 effects show that the interspecific and intraspecific competition treatments caused stress
310 as expected. I only found a main effect of cross on leaf number (fewer leaves in inbred
311 compared to outcrossed individuals), indicating that there was little if any overall
312 inbreeding depression across all treatments (Table 3).

313 The effects of the intraspecific competition treatment were most apparent in the
314 presence of interspecific competition, as evidenced by significant interspecific ×
315 intraspecific interactions in five of the response variables (Table 3, Figure 2). Similarly
316 though to a much lesser extent, the cross × interspecific and the cross × intraspecific
317 interactions each had at least one significant response variable (Table 3). These results
318 indicate that inbreeding depression is magnified by both interspecific and intraspecific

competition stresses. The three-way interaction of cross × interspecific × intraspecific was marginally significant for biomass (Table 3, Figure 3) and leaf length (Table 3, Figure 4), but not significant for leaf number (Figure 5), height (Figure 6) and the composite variable for plant shape (Figure 7). This result suggests a trend that multiple stresses may interact with cross, although this effect is only significant in the benign environments with *L. salicaria* absence.

Analysis of the group means showed the break-down of these significant effects (Table 4). Because there were only two treatments in the interspecific competition treatment, I did not analyze group means. The effect of intraspecific competition between groups was significant in the height and composite response variables. However, height increased with increasing intraspecific competition contrary to my predictions. This result caused the composite variable to also increase with increasing intraspecific competition.

In the interspecific × intraspecific interaction (Table 4; Figure 2), most of the significant or marginally significant interactions occurred between intraspecific treatments in the groups with *L. salicaria*, further showing that the effect of intraspecific competition was dependent on the interspecific treatment imposed. However, the positive, significant group mean differences in Table 4 under the interspecific × intraspecific interaction indicate that those individuals in the more stressful treatments (greater intraspecific competition) performed better than those in less stressful treatments.

Interestingly though, I found the opposite effect in the cross × interspecific × intraspecific interaction. Only groups with *L. salicaria* absence and 4 or 64 intraspecific treatments exhibited significant differences between outcrossed and selfed individuals. However, the direction of these results contrasted with my predictions. Multiple stresses

seemed to decrease the expression of inbreeding depression in biomass (Figure 3b) and leaf length (Figure 4b).

DISCUSSION

In this study, I devised two separate experiments which examined how multiple stresses combine to magnify the expression of inbreeding depression. I predicted that inbreeding depression should increase with increasing stress. In general, I found weak support for this hypothesis. In Experiment 1, herbivory and water deprivation, while by themselves did not influence inbreeding depression, interacted with maternal line and cross in the mixed-model ANOVA for branch number. I found overall inbreeding depression in fewer fitness traits in Experiment 2 (Table 3) and a trend that inbreeding depression interacts with interspecific and intraspecific competition. However, this trend indicates that inbreeding depression *decreases* as environmental stress increases.

Experiment 1

One of the most striking results in this experiment was the high variation across maternal lines. Such variation is consistent with past studies in other species (Carr and Dudash 1997; Reed et al. 2003), and suggests that this source of variation might be of general importance. The significant 4-way interaction between maternal line × cross × herbivory × water treatment for branch number shows that the effects of the 2 environmental stress factors on the expression of inbreeding depression differs across families. Interestingly, none of the 15 maternal lines displayed the pattern of inbreeding depression that I initially predicted (Figure 1), in which single stress factors of herbivory and water would result in relatively intermediate levels of inbreeding depression, while the combination of the two stresses would yield the highest inbreeding depression. Only

365 in maternal lines 11 and 27 did the highest level of inbreeding depression occur in the
366 most stressful environment. The predicted pattern contrasts greatly with maternal lines
367 such as 28, in which inbreeding depression is reduced with added stresses, and maternal
368 lines such as 11, in which the combination of stresses results in outbreeding depression.
369 Clearly, the identity of the maternal line greatly influence the amount of inbreeding
370 depression, and the variability of inbreeding depression across treatments. This may be
371 attributed to maternal lines having different alleles to respond to different environments
372 (Fry et al. 1998). These alleles may be deleterious when triggered by an environmental
373 condition (Vermeulen et al. 2008). Though this study does not consider specific alleles, it
374 is evident that the variability of genetic content is important for the expression of
375 inbreeding depression.
376 The interactions of Experiment 1 are insignificant when maternal lines are
377 ignored (Table 2). As the results of the second ANOVA show, neither herbivory nor
378 water treatment influenced the amount of inbreeding depression displayed, despite the
379 success of each treatment in altering overall plant fitness. These results serve to
380 underscore the importance of maternal lines found in the mixed-model ANOVA (Table
381 1) on the expression of inbreeding.
382 **Experiment 2**
383 The results from Experiment 2 show that intraspecific and interspecific
384 competition each significantly interacts with cross (Table 3). However, in contrast to my
385 hypothesis, the analysis of the differences in group means (Table 4) suggests that
386 inbreeding depression is lowest in the most stressful environments. There was a trend (p
387 < 0.10) toward a 3-way interaction between cross and intraspecific competition and

interspecific competition for two of the six response variables. These results also suggest that inbreeding depression is lowest in the most stressful environments.

Analysis of the differences in group means tells us that although some of the interactions were significant, the direction of these results contrasted with my predictions in some cases. Height, for instance, increased with increasing intraspecific competition. This may be attributed to individuals putting more resources into growth in an effort to capture the maximum amount of sunlight. Group mean differences for leaf number and biomass indicate that intraspecific competition interacts with cross when *L. salicaria* is absent, but not when *L. salicaria* is present. Biomass (Figure 3) only shows significant inbreeding depression in the most benign environment (no *L. salicaria*, low density), while leaf length (Figure 4) shows inbreeding depression in the low and high intraspecific competition treatments when *L. salicaria* was not present. These results suggest that increased stress may *reduce* the expression of inbreeding depression. This result may also be explained by the overpowering effect of the *L. salicaria* ($p < 0.001$ for all response variables). The large reduction in fitness traits for all individuals may have obscured my ability to see a difference between crosses.

The interspecific × intraspecific interaction, though not an indicator of inbreeding depression, is particularly interesting because it indicates that the two environmental treatments interacted for several aspects of fitness (Table 4; Figure 2). The intraspecific competition treatments significantly differed in their effects on fitness response variables in the presence but not in the absence of *L. salicaria*. Moreover, these cases show that as intraspecific competition increases, fitness also increases. The high density individuals in the intraspecific competition treatment performed better than those in lower densities.

411 This result may suggest some form of Allee effect is occurring with the presence of *L.*
412 *salicaria*. Higher densities of *M. ringens* might facilitate higher fitness in the face of
413 interspecific competition. This could perhaps be attributed to an allelopathic chemical
414 produced by *M. ringens* which, in greater quantity, alters the soil composition to favor
415 increased growth rate and fitness. Although no such chemical has been observed in this
416 species, allelopathy is frequently found in wetland plants (reviewed in Neori et al. 2000).
417 Higher densities of allelopathic species may facilitate establishment and growth, which
418 would explain the higher fitness in the 36-individual intraspecific treatment.
419 Lastly, analysis of plant shape provides insight into how individuals respond to
420 the stressful conditions imposed. Plant shape, represented by the composite variable, did
421 not appear to depend on cross ($p > 0.05$), but the interspecific ($p < .001$) and intraspecific
422 ($p < .001$) competitive treatments and their interaction ($p < .001$) did significantly
423 influence shape. The interspecific competition treatment caused individuals to grow
424 shorter in comparison to their leaf number, possibly indicating that individual's put more
425 resources into leaves to absorb additional sunlight rather than into height. The
426 intraspecific competition treatment showed that as the density of *M. ringens* increased,
427 individuals allocated more resources towards height than towards leaf number. This result
428 suggests that *M. ringens* individuals tried to outcompete one another for sunlight and
429 therefore grew taller relative to their leaf output.
430 **Conclusions**
431 In general, I find weak support for the hypothesis that multiple stresses increase
432 the expression of inbreeding depression in *M. ringens*. In a review of the literature,
433 Armbruster and Read (2005) found that 48% of the studies they reviewed showed

enhanced inbreeding depression; variation among studies is due to many factors, including the magnitude and type of stress imposed on individuals. My study considers four different stresses in two wholly different environments; therefore, the discrepancies in results may be attributed to the respective experimental designs. Experiment 1 was set in stressful soil and moisture conditions present in the common garden, but are absent in *M. ringens* native wetland habitat. In contrast, Experiment 2 was set in a relatively benign greenhouse environment. The environments and the different stresses imposed may have triggered different genes present in outcrossed and selfed individuals.

My results are a testament to the inconsistency in the literature regarding inbreeding depression and environmental stress. The results of the multiple stress interactions are not clear in either experiment. The water and herbivory treatment in Experiment 1 did little to affect inbreeding depression individually, though when taken together in the 4-way interaction, there was a significant effect for branch number. However, I cannot say from this analysis whether this interaction is in fact a magnification of inbreeding depression. Additional analyses comparing the group means would be necessary to conclude this hypothesis. In Experiment 2, the marginally significant 3-way interaction for leaf number and biomass suggests that these stressors may work together to affect inbreeding depression. However, the trend was in the opposite direction as expected, indicating that increased stress decreases the expression of inbreeding depression.

Implications for Conservation

The results of this study are relevant for future conservation work. As populations decrease in size, we may expect to find higher levels of inbreeding due to a smaller

457 mating pool. Inbreeding comes at high cost to individuals of historically outbreeding
458 species (*e.g.* Madsen et al. 1999; reviewed by Crnokrak and Roff 1999). Even
459 populations of species with a history of inbreeding may still have deleterious alleles
460 (Frankel and Soule 1981). Several studies have found that inbreeding depression creates
461 an obstacle to conservation efforts. A reduction in fitness may make species less
462 competitive against invasive congeners (Garcia-Serrano et al. 2008). Inbreeding
463 depression also accounts for a lower colonization rate in *Daphnia magna* (Haag et al.
464 2002) and lower population survival in the herb *Clarkia pulchella* (Newman and Pilsen
465 1997).

466 A recent theoretical study by Liao and Reed (2009) shows that an inbreeding ×
467 environmental interaction increases the extinction rate; thus, conservation biologists
468 should be concerned that new environmental stresses might affect the extinction risk of
469 species through increasing the magnitude of inbreeding depression. However, my results
470 suggest that new stressors in combination with one another might not magnify inbreeding
471 depression. Species of conservation concern might face new stresses in their future, such
472 as the introduction of new alien species or global climate change. These stresses might
473 have negative demographic consequences for these species and increase extinction risk.
474 However, my results for *M. ringens* indicate reason for optimism, in that new stresses and
475 multiple stresses are not likely to exacerbate the magnitude of inbreeding depression.
476 However, as the results from Experiment 1 show, any possible challenges to conservation
477 would be greatly influenced by the identity of the maternal line. Many endangered or
478 threatened species may have been initially reduced to low population numbers because of

these stresses; the additional challenge of overcoming an enhanced expression of inbreeding depression may be at least partially determined by the maternal lines.

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628

	Height	*Branch Number*	*Stolon Number*	*Biomass*
N	566	573	581	574
Multiple R	0.494	0.606	0.488	0.510
Squared multiple R	0.244	0.367	0.238	0.260
Mat	1.424	8.555***	1.723*	1.052
Cross	17.234***	8.594*	2.523	73.162***
Herb	16.881***	5.663*	16.642***	13.448**
Water	5.789*	0.275	0.000	4.234 MS
Mat × Cross	0.954	1.377	1.989*	0.526
Cross × Herb	0.136	0.001	1.208	0.080
Cross × Water	0.340	4.403 MS	0.094	0.043
Mat × Cross × Herb	0.674	0.803	0.604	1.111
Mat × Cross × Water	1.571 MS	1.039	0.638	1.036
Cross × Herb × Water	0.629	0.000	0.585	0.806
Mat × Cross × Herb × Water	1.029	1.916*	1.510	1.335

629

630 Table 1: Experiment 1 results from first ANOVA with factors family, cross, herbivory
631 treatment, and water treatment. All response variables are natural log transformed. MS
632 marginally significant $p < 0.10$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.
633

634

	Height	*Branch Number*	*Fruit Number*	*Stolon Number*	*Biomass*
N	566	573	140	581	574
Multiple R square	0.216	0.172	0.108	0.165	0.285
Squared multiple R	0.047	0.029	0.012	0.027	0.081
Cross	15.85***	8.812**	0.17	4.118*	36.915***
Herb	4.364*	3.355 MS	0.08	10.128**	9.874**
Water	5.815*	0.241	0.231	0	3.22 MS
Cross × Herb	0.101	0.024	0.215	0.618	0.166
Cross × Water	0.503	2.627	0.433	0.005	0.011
Herb × Water	0.513	1.805	0.069	0.843	0
Cross × Herb × Water	0.79	0.062	0.201	0.828	0.168

635

636 Table 2: Experiment 1 results from second ANOVA (maternal lines pooled) with factors
637 cross, herbivory treatment, and water treatment. All response variables are natural log
638 transformed. MS marginally significant $p < 0.10$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

639

	Survivorship	*Height*	*Leaf Number*	*Leaf Length*	*Biomass*	*Composite*
Cross	0.021	0.723	6.126*	0.526	0.455	0.290
Inter-	239.859***	790.563***	347.330***	900.263***	921.491***	549.575***
Intra-	31.4380***	13.928***	4.978**	0.218	1.907	37.985***
Cross* × *Inter-	0.002	2.389	1.533	7.335**	5.579*	0.704
Cross* × *Intra-	6.423*	0.819	2.217	1.009	1.711	0.494
Inter-* × *Intra-	7.347**	2.122	16.627***	38.337***	42.873***	11.033***
Cross* × *Inter* × *Intra	1.851	1.809	1.334	2.368 MS	2.888 MS	0.456

640

641 Table 3: Experiment 2 likelihood ratio values for survivorship from ANOVA. F-values
642 from ANOVA performed with permutations for the 4 other natural log-transformed
643 response variables (height, leaf number, leaf length, and biomass) and the composite of
644 height and leaf number. MS marginally significant $p < 0.10$, * $p < 0.05$, ** $p < 0.01$, ***
645 $p < 0.001$

646

		Ln Height	Ln Leaf Number	Ln Leaf Length	Ln Biomass	Composite
Intra-	***36:16***	0.152**	0.024	–	–	0.216***
	36:4	0.211***	-3.832	–	–	0.068*
	16:4	0.059	-3.696	–	–	0.148***
Inter-* × *Intra-	***No L. salicaria 36:16***	–	-0.444	-0.569	-1.199	0.216***
	No L. salicaria 36:4	–	-0.169	-0.377	-1.926	0.068*
	No L. salicaria 16:4	–	-0.275	-0.192	-0.727	0.148***
	L. salicaria 36:16	–	0.251***	0.193*	0.547*	0.061**
	L. salicaria 36:4	–	0.110 AS	0.225**	0.499*	0.078***
	L. salicaria 16:4	–	-0.141	0.032	-0.048	0.017
Cross* × *Inter-	***No L. salicaria Out:Self***	–	–	0.048	0.548*	–
	L. salicaria Out:Self	–	–	-0.003	-0.116	–
Cross* × *Inter-* × *Intra-	***No L. salicaria, 4 Out:Self***	–	–	0.216***	1.396*	–
	No L. salicaria, 16 Out:Self	–	–	0.021	-0.217	–
	No L. salicaria, 36 Out:Self	–	–	0.106**	0.296	–
	L. salicaria, 4 Out:Self	–	–	-0.038	-0.002	–
	L. salicaria, 16 Out:Self	–	–	-0.008	0.021	–
	L. salicaria, 36 Out:Self	–	–	-0.053	-0.179	–

Table 4: Group mean differences for the 4 response variables and the composite. Only significant interactions ($p < 0.05$) from Table 3 shown, as well as the 3-way interaction for biomass which was almost significant. AS almost significant $p < 0.10$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

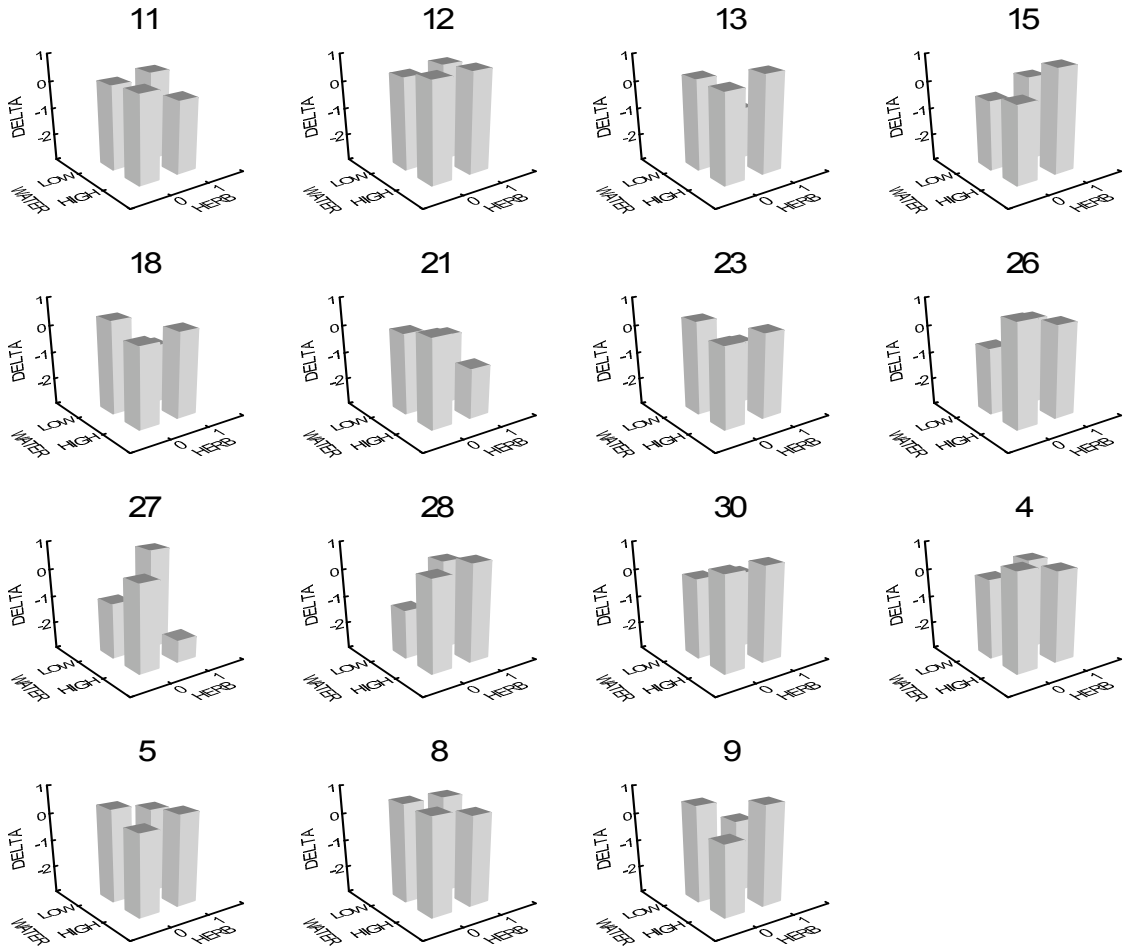


Figure 1: Inbreeding depression (δ) shown in the z-axis in each of the 4 herbivory × water treatment groups, grouped by maternal line. Inbreeding depression calculated using back-tranformed means of outcrossed and selfed individuals in each group. Herbivory level presented on the x-axis, 0 signifies no herbivory and 1 signifies herbivory. Water treatment (low or high) presented on the y-axis. Most benign environment (no herbivory, high water treatment) represented by the lower left bar of each graph. Most stressful environment (herbivory, low water treatment) represented by upper right bar of each graph.

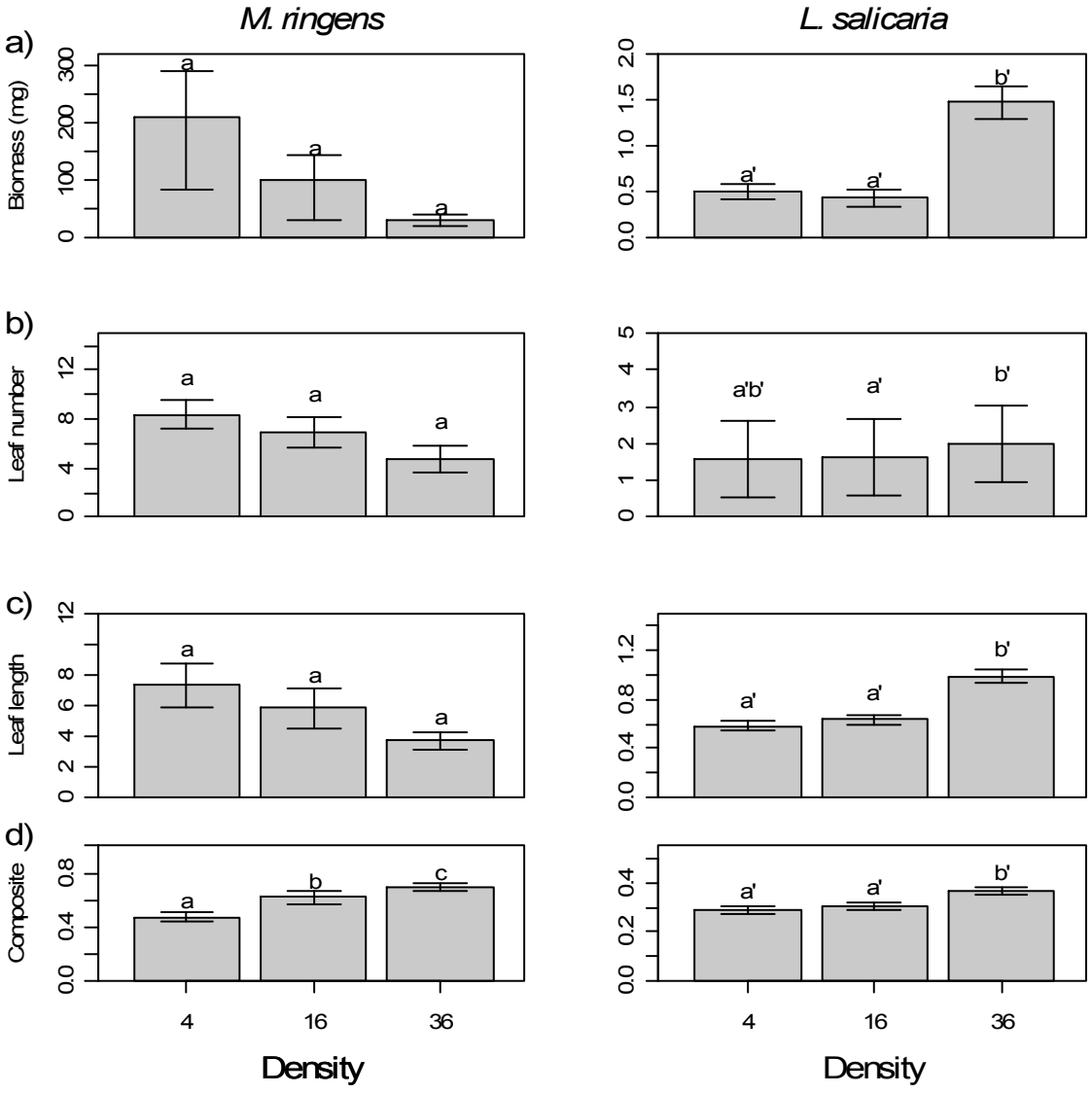
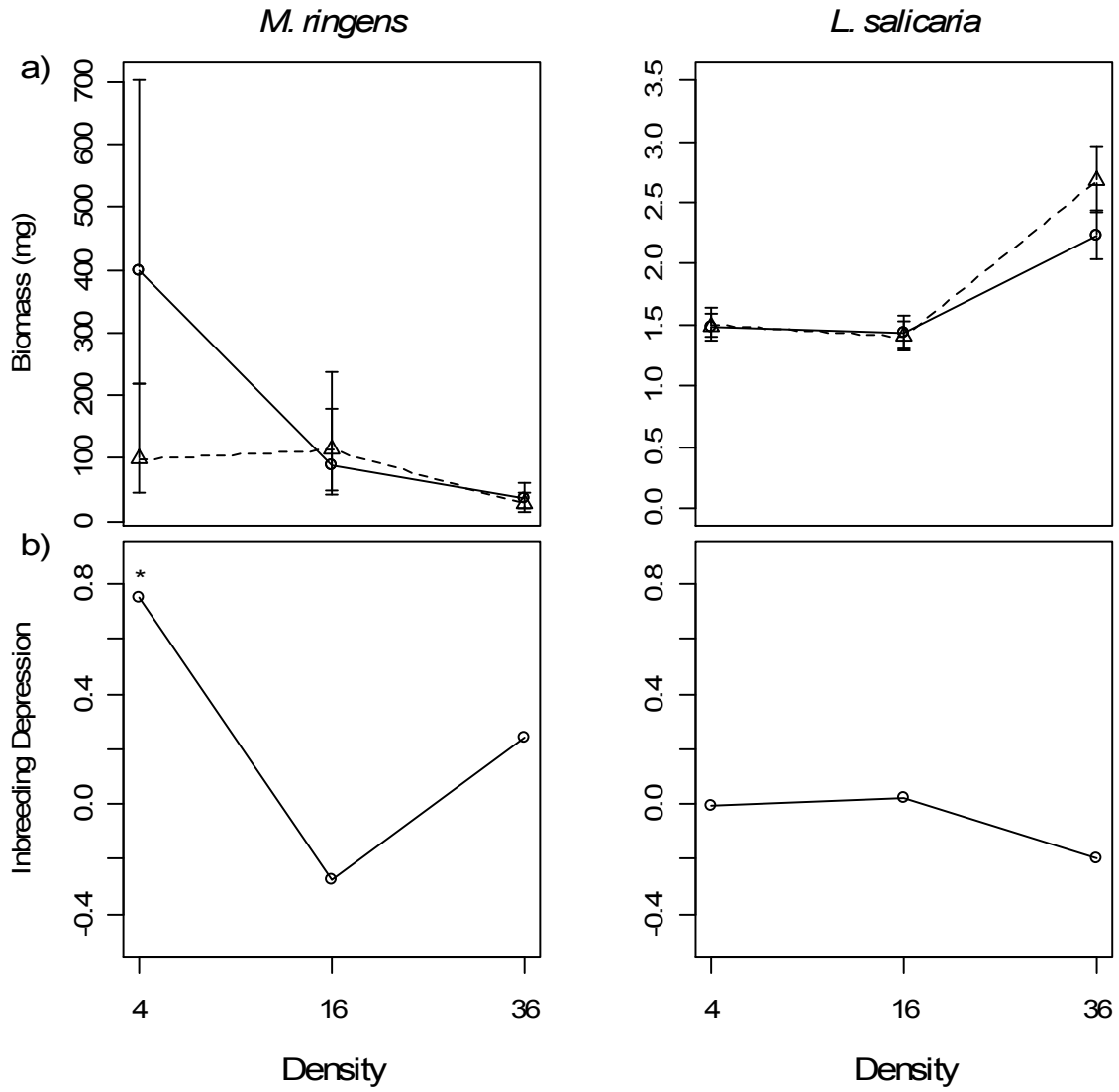


Figure 2: Interspecific × Intraspecific interactions for biomass (2a), leaf number (2b), leaf length (2c) and the composite response variable [LN(height + 1)/LN(Leaf Number + 1) (2d). Error bars show 95% confidence interval. Interspecific competition treatments (vs. *M. ringens* or vs. *L. salicaria*) shown in separate plots due to different y-axis scales.



673
674 Figure 3: Group back-transformed means for 2a biomass and 2b inbreeding depression
675 (δ). In 2a, open circles and solid lines represent outcrossed individuals, and triangles and
676 dashed lines represent selfed individuals. Error bars show 95% confidence interval.
677 Interspecific competition treatments (vs. *M. ringens* or vs. *L. salicaria*) shown in separate
678 plots due to different y-axis scales. In 2b, * represents $p < 0.05$.
679

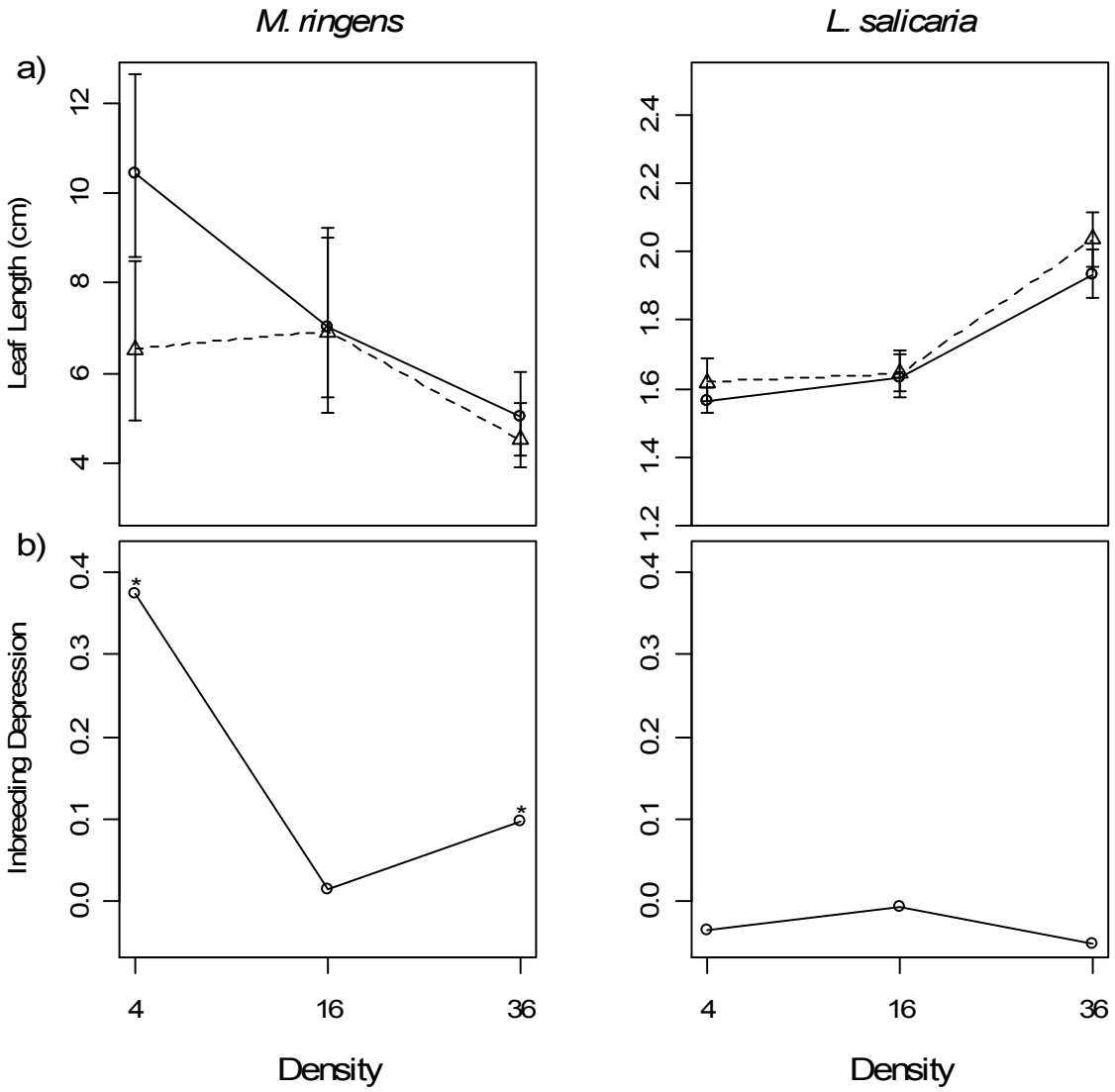
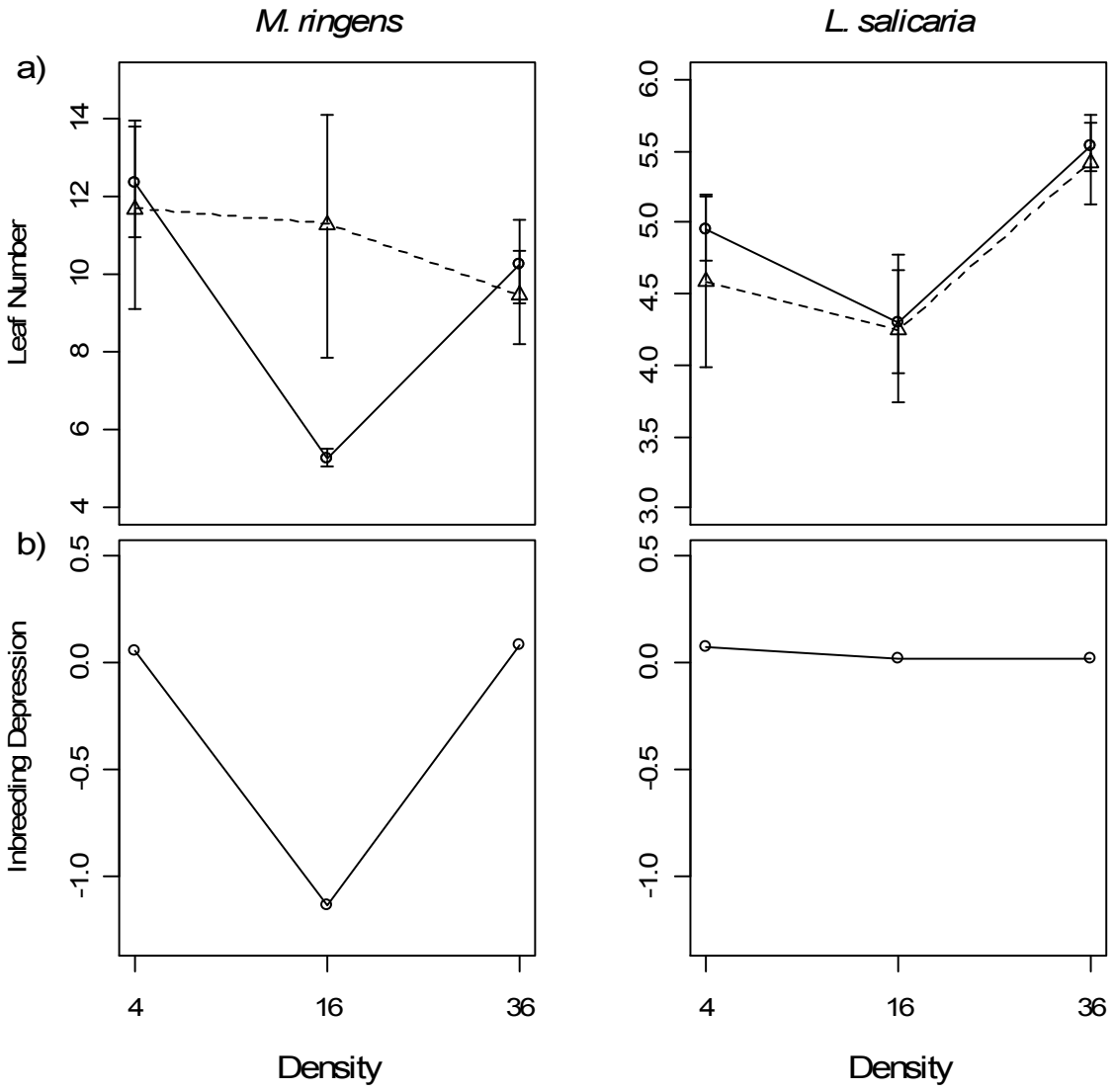


Figure 4: Group back-transformed means for 3a leaf length and 3b inbreeding depression (δ). In 3a, open circles and solid lines represent outcrossed individuals, and triangles and dashed lines represent selfed individuals. Error bars show 95% confidence interval. Interspecific competition treatments (vs. *M. ringens* or vs. *L. salicaria*) shown in separate plots due to different y-axis scales. In 3b, * represents $p < 0.05$.



687
688
689 Figure 5: Group back-transformed means for 4a leaf number and 4b inbreeding
690 depression (δ). In 4a, open circles and solid lines represent outcrossed individuals, and
691 triangles and dashed lines represent selfed individuals. Error bars show 95% confidence
692 interval. Interspecific competition treatments (vs. *M. ringens* or vs. *L. salicaria*) shown in
693 separate plots due to different y-axis scales. Significance not tested for because leaf
694 number did not show a significant 3-way interaction.
695

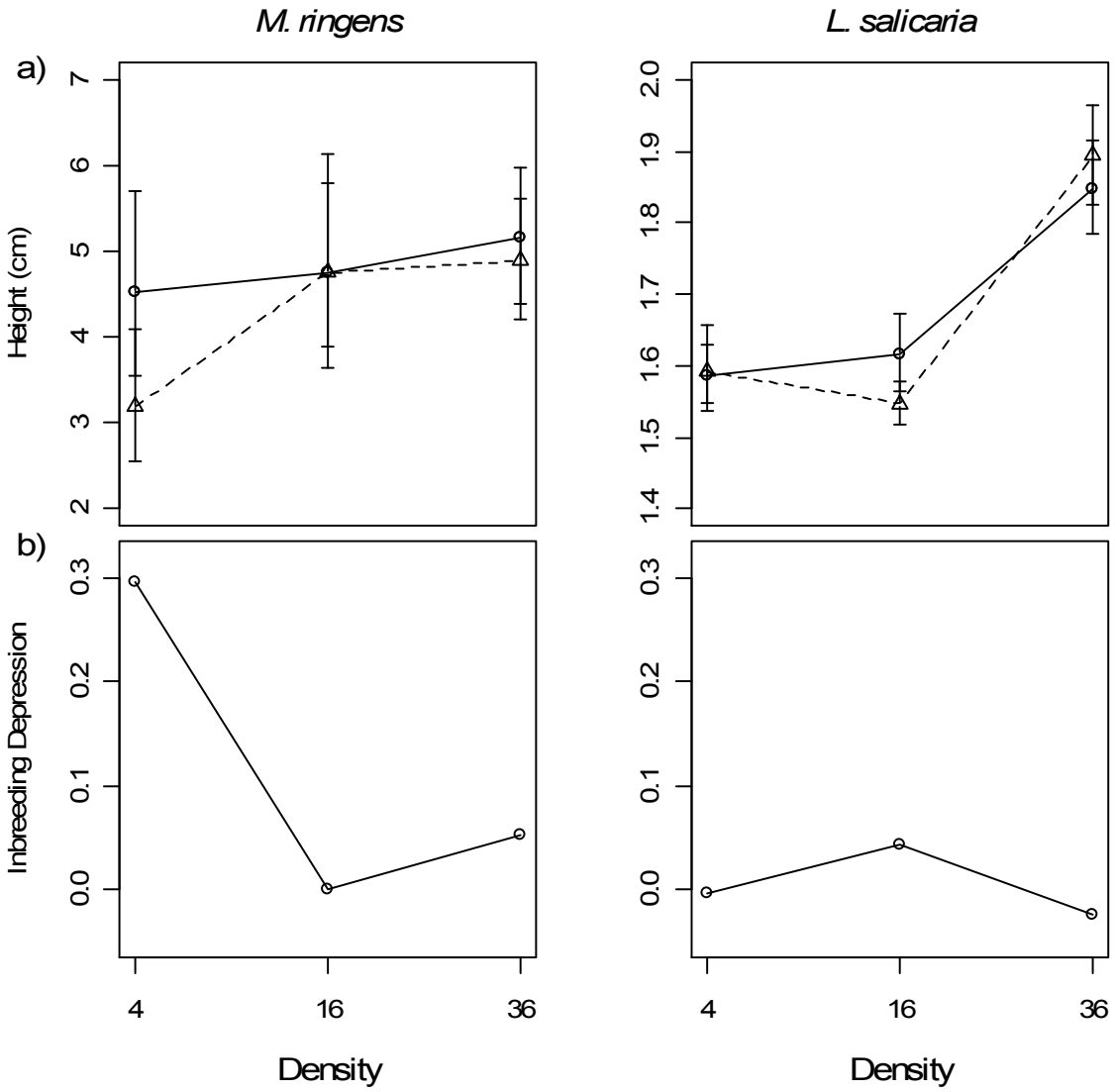


Figure 6: Group back-transformed means for 5a height and 5b inbreeding depression (δ). In 5a, open circles and solid lines represent outcrossed individuals, and triangles and dashed lines represent selfed individuals. Error bars show 95% confidence interval. Interspecific competition treatments (vs. *M. ringens* or vs. *L. salicaria*), shown in separate plots due to different y-axis scales. Significance not tested for because height did not show a significant 3-way interaction.

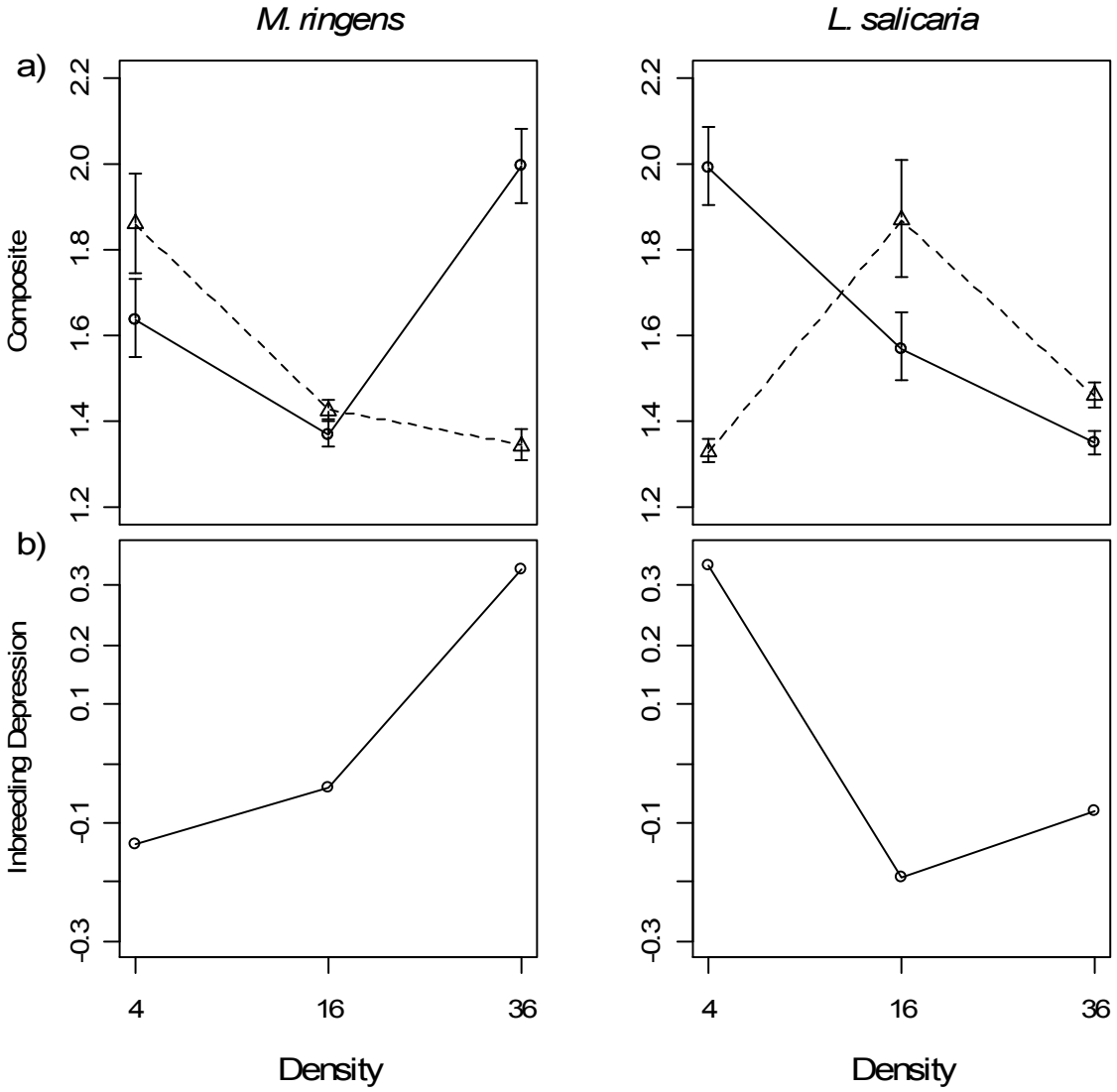


Figure 7: Group back-transformed means for 6a composite and 6b inbreeding depression (δ). In 6a, open circles and solid lines represent outcrossed individuals, and triangles and dashed lines represent selfed individuals. Error bars show 95% confidence interval. Interspecific competition treatments (vs. *M. ringens* or vs. *L. salicaria*) shown in separate plots. Significance not tested for because composite did not show a significant 3-way interaction.