Evolutionary Ecology Research, 2010, **12**: 617–632

Phenotypic plasticity and inbreeding depression in *Mimulus ringens* (Phrymaceae)

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ABSTRACT

Hypothesis: The mating system (outbred or inbred) a plant utilizes could influence the degree of phenotypic plasticity exhibited by the progeny. We predicted that progeny of outbred parents would display greater phenotypic plasticity when grown under soil moisture stress than progeny of selfed individuals.

Organisms: Seventeen families (8 outbred and 9 selfed) of mixed-mating wetland species, *Mimulus ringens*, were grown along a soil moisture gradient in the field and in the greenhouse.

Times and places: Field and greenhouse experiments were conducted at the University of Virginia's Blandy Experimental Farm, Boyce, Virginia, USA.

Analytical methods: Both fitness and morphological characters were measured for three soil moisture levels. All data were analysed using mixed-model analysis of variance or analogous accelerated failure time models. Two-way factorial analyses of variance were performed for each character to test for breeding (fixed) and water (fixed) effects individually and for their interactive effect across blocks (random). A difference in levels of plasticity was defined by a significant interaction effect between breeding and water.

Results: Inbreeding had little effect on phenotypic plasticity in the field or greenhouse. Plasticity in corolla width showed opposite patterns in inbred and outbred plants and growth rate showed greater plasticity in outbred plants in the greenhouse. There was little evidence of inbreeding depression among inbred or outbred *M. ringens*.

Keywords: inbreeding, *Mimulus ringens*, mixed-mating, phenotypic plasticity, water stress.

INTRODUCTION

The sessile nature of plants has made their survival contingent upon the ability to change their phenotype in response to spatial or temporal environmental heterogeneity. When populations are exposed to stressful environmental conditions such as low soil moisture, a reduction in the ability to be plastic could lead to decreased fitness for certain genotypes.

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The ability and extent to which an individual genotype is phenotypically plastic has been repeatedly shown to be heritable (Bradshaw, 1965; Scheiner, 1993; Briggs and Walters, 1997). Although numerous studies have examined how plasticity might evolve under natural selection, few have examined evolutionary changes that might occur through inbreeding.

The degree of inbreeding varies considerably across plant species and populations (Vogler and Kalisz, 2001). Individual offspring of mixed-mating species may be a product of one of three possible sexual reproductive methods, depending on the reproductive behaviour of the parent plant(s). An individual could be a result of two unrelated parents (outcrossing) or a product of two genetically related parents (biparental inbreeding) (Kelly and Willis, 2002). Offspring that are products of self-fertilization contain only a subset of the genetic material of their parent and, on average, are 50% less heterozygous than the parent (Charlesworth and Charlesworth, 1987). High levels of inbreeding could result in decreased heterozygosity leading to a decreased tolerance to environmental stress (Pigliucci and Schlicting, 1998; Keller and Waller, 2002) and inbreeding depression (Charlesworth and Charlesworth, 1987).

The 'developmental instability hypothesis' (Pederson, 1968) predicts that individual plants with low heterozygosity are less developmentally stable and will be more phenotypically sensitive to stressful environments. This could be due to the loss of gene product diversity or the increased expression of deleterious alleles, or both. Although this would suggest that inbreeding could result in increased plasticity, this may not result in increased fitness. For example, Carr and Eubanks (2002) found significantly greater phenotypic plasticity in inbred *Mimulus guttatus* when plants grown with and without herbivores were compared, but this effect was manifested as reduced tolerance to herbivory, potentially leading to decreased fitness, and was not interpreted as an adaptive change in plasticity. Other studies have highlighted a lack of response in plasticity from inbreeding. Schlichting and Levin (1986) grew *Phlox drummondii* under six different environmental conditions and found no systematic effect of inbreeding on phenotypic plasticity. Similarly, Quisenberry and Kohel (1971) found no effect of heterozygosity on phenotypic stability in *Gossypium* spp. grown in five different environments.

Species that occupy ephemeral wetlands are particularly susceptible to rapid changes in soil moisture conditions. Ephemeral wetlands in Virginia typically dry during the summer months and can vary widely in water level from one year to the next. Therefore, it could be argued that high levels of plasticity are adaptive for wetland species to cope with these short-term environmental changes (Dunn and Sharitz, 1991).

In this study, we used field and greenhouse experiments with the mixed-mating *Mimulus ringens* to address the following questions: (1) Does inbreeding affect phenotypic plasticity in response to water availability in either field or greenhouse settings? (2) Do the *Mimulus ringens* used in this experiment show inbreeding depression?

METHODS

Experimental species

Mimulus [Phrymaceae, ex. Scrophulariaceae (Beardsley and Olmstead, 2002)] is a model genus for exploring the genetics of adaptation (Wu *et al.*, 2008). *Mimulus ringens* is an herbaceous perennial found in moist habitats across the central and eastern United States (Grant, 1924). *Mimulus ringens* produces two violet-light blue zygomorphic flowers at each node that open acropetally on the same day or within 2–3 days of each other. The hermaphroditic flowers

last 1–2 days (Karron *et al.*, 1995a, 1995b). *Mimulus ringens* is self-compatible but also outcrosses with the assistance of worker and queen bumblebees (*Bombus* spp.) (Karron *et al.*, 1995a, 1995b). *Mimulus ringens* can also reproduce asexually though rhizomes (personal observation).

Field experimental design

All field experiments were completed at Blandy Experimental Farm (BEF) in Clarke County, Virginia, USA (39°06′N, 78°06′W). Parent plants from a naturally occurring *M. ringens* population were hand-pollinated in the field at BEF on 16–17 August 2001. One day prior to anthesis, buds intended for outcrosses were emasculated, and buds intended for selfing were 'sham' emasculated (the calyx and corolla were torn with a forceps, but the anthers were not removed). The manipulated buds were then enclosed in bags made of polyester Pellonfi (Miami, FL, USA) interfacing to exclude natural pollinators. Pollen donors for cross-pollinations were chosen at random from the BEF population. Seeds were collected on 13 October 2001. Seeds were sown in the greenhouse on 3 March 2002, and individual seedlings were transplanted into one-gallon pots on 1 May 2002. Plants were cut at ground level at the end of the growing season and overwintered in the greenhouse.

Individual plants were moved to the BEF lath house in early spring 2003. To minimize cross-treatment genetic variation, each plant was clonally propagated by dividing underground rhizomes produced during the previous growing season. Each rhizome was buried under 4–5 cm of Wesco Professional Media III potting soil (Wetsel, Inc., Harrisonburg, VA, USA) in a one-gallon pot, placed in an open field, and allowed to grow for 3 weeks. On 1 July 2003, individual plants were randomly assigned a site within the BEF wetland (NPT, ARN, PAV1, PAV2) and water treatment (low, medium, high) along the gradient that ran parallel to the axis of the wetland. Each transect contained a clone of each genotype (17 in total; 8 outbred and 9 selfed) for a total of 51 plants per site. Before planting, the pre-existing vegetation in each 10-m transect was reduced to approximately 30 cm using a weed whip to establish a uniform light level around plants. All transects were enclosed with 1.2 m high 'square deal' Sierra field fencing to exclude animal disturbances.

Transect positions were selected based on the natural distribution of *M. ringens* at our field site to represent a realistic range of environmental stress. Medium and high transects reflected areas where wild *M. ringens* was commonly growing nearby our plots and low water transects represented areas where *M. ringens* was present but uncommon. Volumetric water content measurements were taken with a HydroSense (Campbell Scientific, Inc., Logan, UT, USA) soil moisture meter using a time-domain reflectometry measurement technique. Three measurements were taken per water treatment at each site approximately every 2 weeks after initial planting.

Greenhouse experimental design

Seeds were sown in the BEF greenhouse on 3 March 2003. On 2–3 May 2003, individuals were transplanted into half-gallon pots and divided into five replicate blocks. Plants were grown under sodium vapour lights on a 12-h photoperiod until 19 June 2003, at which time the lights were turned off and the plants received only natural light. Each block contained two full-siblings of 10 selfed genotypes and 12 outcrossed genotypes, for a total of 44 plants. One sibling was randomly assigned the 'high' water treatment, and the other the 'low' treatment.

Potted individuals were placed in a round plastic pot liner, 20.3 cm in diameter and 8.9 cm deep, in which water was added according to its treatment. The high water treatment aimed to reproduce soil moisture conditions representative of the most common soil moisture level in the field. The low water treatment aimed to create a stressful environment. In the high water treatment, the water level was maintained within 2 cm of the top of the plastic pot liner throughout the experiment. Plants in the low water treatment received 2 cm of water every 6 days. Volumetric water content measurements were taken approximately every 2.5 weeks after initial planting on a random sampling of 16 individuals per block using a HydroSense (Campbell Scientific, Inc., Logan, UT, USA) soil moisture meter using a time-domain reflectometry measurement technique.

Fitness characters measured

First flowering date

The number of days until first flower was calculated by counting from Day 0 (the day on which the plants were transplanted) until the date they first flowered. If an individual plant did not flower during the study period (77 days in the field study and 62 days in the greenhouse study), it was regarded as 'right-censored' in the failure time analysis (see 'Statistical analysis' below).

Total flower production

The number of persistent calyces or pedicels (from flowers removed for pollen studies) present at the end of the flowering season were counted as a measure of total flower production.

Pollen quantity

Anthers from all individuals were collected prior to anthesis from early (node 1, 2 or 3) and late (node 4, 5, 6 or 7) flower buds. Anthers were stored in glass vials or microcentrifuge tubes so that pollen sacs opened in the laboratory to minimize pollen loss during anther dehiscence. In preparation for analysis, the pollen was dried overnight in a drying oven at 40°C. After drying, 1 ml of 70% ethanol was added. The pollen suspension was then poured into a vial containing 24 ml of 70% ethanol and placed in a sonicator for 30 s. Pollen quantity was analysed using a Pacific Scientific HIAC Royco model 9703 Liquid Particle Counting System.

Morphological/physiological characters measured

Flower size

Corolla length and width data were collected as measures of flower size. Digital calipers were used to measure both early and late node flowers. Because plants flowered at different times, this method controls for the developmental stage of the plant.

Herkogamy

Some species produce anthers and stigmas of different lengths to avoid self-fertilization (Ennos, 1981; Dole, 1992; Robertson, 1992; Carr and Fenster, 1994; Barrett *et al.*, 1996). This spatial separation of male and female sexual organs prevents pollen from being deposited through gravity or

pollinator on the stigma of the same flower. *Mimulus ringens* outcrossing rates are positively correlated with anther–stigma separation (Karron *et al.*, 1997). Digital calipers were used to measure the distance between anthers and stigma for both early and late flowers.

Stomatal resistance (*field plants only*)

Stomatal resistance was measured using a LiCor LI-1600 Steady State Porometer (Lincoln, NE, USA) calibrated to local humidity and light availability conditions. A standard broad-leaf aperture was used on the sensor head. Measurements were taken for the Pav1 site on 9 September 2003, at approximately 15.30 EDT (20°C, relative humidity 78%, barometric pressure 768 mmHg, wind speed 0 $km \cdot h^{-1}$), and at the NPT site on 22 September 2003 at 15.30 EDT (24°C, relative humidity 69%, barometric pressure 768 mmHg, wind speed 0 $km \cdot h^{-1}$) (National Climatic Data Center, 2004). Stomatal resistance was not measured at the Pav2 and ARN sites or in the greenhouse.

Aboveground biomass

At the end of the growing season prior to senescence, biomass was harvested, dehydrated in a drying oven at 50°C for 4 days, and weighed.

Growth rate (*height/time*)

Field individuals were measured three times during the experiment and greenhouse individuals were measured twice during the experiment. All measurements were taken from the base of the stem to the highest point on the main stem. We determined a growth rate for each plant from the slope of the regression of height (dependent variable) on time.

Root and rhizome biomass (*greenhouse only*)

Greenhouse pots were removed from the water treatment on 25 August 2003 and the roots and rhizome were immediately washed of all potting soil and refrigerated. Rhizomes and roots (thinner, less rigid, descending from base of plant in all directions) were separated, dried in a drying oven at 50°C for 4 days, and weighed individually. The root-to-shoot ratio was calculated by dividing the belowground biomass by the aboveground biomass.

Statistical analysis

Except where noted, all data were analysed using SAS proc mixed (SAS 6.12). Differences in soil moisture were analysed using an analyses of variance (ANOVA) model. Two-way factorial ANOVAs were performed for each character to test for breeding (fixed) and water (fixed) effects individually and for their interactive effect. A difference in levels of plasticity will be evident by a significant interaction effect between breeding and water. The ANOVA models also included block as a random effect. Family was treated as a random nested effect within breeding treatment. Pair-wise comparisons were performed with a Tukey-Kramer test.

Soil volumetric water content data were analysed using a repeated-measures ANOVA to test for differences between treatments and through the duration of the growing season. A repeated-measures ANOVA was used to test for a time effect (early vs. late) on herkogamy, pollen quantity, corolla height and width. Because the effects of phenology were insignificant or small, a mean value for each of these characters was used to test for water, breeding, and water × breeding effects.

First flowering date was analysed using an accelerated failure-time model with SAS proc lifereg (Fox, 2001). The model included block, breeding, and water treatments, their two-way interaction, and family nested within inbreeding treatment. This analysis allowed consideration of right-censored data caused by the failure of some plants to flower before the end of the experiment. In both the field and greenhouse experiment, a log-normal survival curve was determined to be the best fit to the data using log-likelihood ratio tests.

Phenotypic plasticity was measured as the variation of reactions for a specific character across the water treatments. To quantify a character's response to the water treatment, we calculated a coefficient of variation (CV) for each genotype and used a one-way analysis of variance to test for significant differences between outcrossed and selfed mean CVs.

RESULTS

Soil volumetric water

Mean field soil volumetric water content varied across water treatments ($F_{2,144} = 1681.56$, $P < 0.001$), with the high treatment averaging almost twice the volumetric content of the low treatment (Table 1). There were also differences between the beginning and end of the season ($F_{5,144} = 26.55$, $P < 0.001$). Mean soil volumetric water content ranged from 43.8% to 53.6% in the low treatment, from 64.2% to 74.2% in the medium treatment, and from 85.8% to 92.9% in the wet treatment. Mean volumetric water content in the greenhouse also varied across treatments ($F_{2,276} = 2306.34$, $P < 0.001$) and time ($F_{1,276} = 11.68$, $P < 0.001$). Water content ranged between 9.2% and 21.7% in the low treatment and between 65.6% and 72.8% in the high treatment.

Water effect

Mimulus ringens grown in the field exhibited significant differences in growth rate ($F_{2,16} = 5.18$, $P = 0.012$) and aboveground biomass ($F_{2,16} = 6.40$, $P < 0.008$) across the water gradient, suggesting plasticity for these characters (Table 1). Growth rate was highest in the medium treatment. Aboveground biomass was similar in both the low and medium treatments, but each was over 46% greater than the biomass in the high water treatment. The water treatment did not affect the remaining characters ($P > 0.05$; Appendix 1). In the field, early flowers were 4% wider ($F_{1,15} = 13.68$, $P = 0.002$) and 4% taller ($F_{1,15} = 10.89$, $P = 0.005$) than late flowers, although mean values were used to test for a water effect on these characters. Phenology did not affect pollen quantity ($F_{1,15} = 1.49$, $P = 0.242$) or herkogamy in the field ($F_{1,15} = 0.824$, $P = 0.05$).

In the greenhouse, phenotypic plasticity was observed for corolla length ($F_{1,13} = 13.28$, $P < 0.001$) and corolla width ($F_{1,13} = 13.89$, $P = 0.003$), which also differed for early and late flowers. Early flowers were 5% wider ($F_{1,13} = 4.8$, $P = 0.047$) and 8% taller ($F_{1,15} = 12.13$, $P = 0.004$) than late flowers, but there was no difference in herkogamy ($F_{1,13} = 3.2$, $P = 0.097$). Growth rate ($F_{1,23} = 9.47$, $P = 0.01$) and rhizome biomass ($F_{1,23} = 18.27$, $P < 0.001$) were also affected by the water treatment. These trait means were consistently greater in high water conditions (Table 2). Low water plants grew 31% slower and produced flowers that were about 6% smaller than high water plants. Low water plants also produced 18% less rhizome biomass. Water treatment did not affect first flowering date, total

Table 1. Means (and standard errors) for *Mimulus ringens* clones grown in the field under low, medium, and high water conditions

Trait	Low water	Medium water	High water
Soil volumetric water content (%)	47.7 (1.20)^{a}	68.3 (1.12)^{b}	87.8 (0.90)^{c}
Growth rate (cm · day^{-1})	0.699 (0.07)^{a}	0.849 (0.07)^{b}	0.730 (0.07)^{a,b}
Aboveground biomass (g)	21.01 (1.27)^{a}	20.85 (1.27)^{a}	14.22 (1.27)^{b}

Note: Treatment means were compared using the Tukey-Kramer method, and those with different superscript letters were significantly different ($P < 0.05$).

Table 2. Means (and standard errors) showing significant differences ($P < 0.05$) for *Mimulus ringens* clones grown in the greenhouse under low and high water conditions

Trait	Low water	High water
Soil volumetric water content (%)	13.7 (1.18)	68.2 (0.69)
Corolla length (cm)	18.21 (0.33)	19.23 (0.30)
Corolla width (cm)	24.46 (0.36)	25.36 (0.33)
Growth rate (cm · day^{-1})	0.267 (0.04)	0.386 (0.04)
Rhizome biomass (g)	0.80 (0.07)	0.98 (0.07)

flower production, herkogamy, pollen quantity, aboveground biomass, root biomass or root-to-shoot ratio ($P > 0.05$; Appendix 2).

Breeding effect

Inbreeding main effects were seen in only one trait in field-grown *M. ringens*. Outcrossed plants had 18% greater herkogamy than selfed plants (mean ± S.E.: 1.95 ± 0.082 mm vs. 1.65 ± 0.074 mm; $F_{1,15} = 8.23$, $P = 0.01$). The breeding treatment affected only first flowering date in greenhouse plants (see Appendix 2 for all other traits). Inbred plants tended to flower earlier ($\chi^2 = 7.91$, d.f. = 1, $P = 0.0049$) with a median time to flowering of 42.0 versus 62.0 days in outbred plants. With so many 'negative' hypothesis tests, one might be concerned about a high Type II error rate. However, our sample sizes in both the field and greenhouse experiments are quite high ($n \geq 26$ for all comparisons, and usually ≤ 40) and our standard errors are usually quite low relative to the means. Inbreeding seems to have a very limited effect on most of these character means.

Breeding × water effect

Inbreeding effects on plasticity would be revealed by a significant breeding × water interaction. For *M. ringens* grown in the field, only corolla width showed differences in plasticity between inbred and outbred plants ($F_{2,27} = 4.93$, $P = 0.008$; Table 3). Corollas on outbred plants became increasingly larger from low to high water, but corollas on selfed plants became smaller along this gradient. Inbreeding did not affect plasticity in the remaining characters ($P > 0.05$; Appendix 1).

Table 3. Breeding (outcross, selfed) × water interaction (low, medium, high) least-squares means (medians and standard deviations presented for first flower) for field-grown *Mimulus ringens*

	Outcross/low			Outcross/medium			Outcross/high		
Character	mean	S.E.	*n*	mean	S.E.	*n*	mean	S.E.	*n*
First flower (days)	55.0	13.1	28	52.0	13.5	29	56.0	13.2	30
Total flowers (*n*)	37.140	1.404	28	33.454	1.397	29	28.484	1.399	30
Corolla height (mm)	16.236	0.558	16	16.905	0.554	17	16.736	0.547	17
Corolla width (mm)	24.079	0.722	16	25.145	0.720	17	25.272	0.709	17
Herkogamy (mm)	1.863	0.110	16	1.9767	0.110	17	2.0086	0.109	17
Pollen quantity (*n*)	140 178	25 542	17	148 404	26 116	17	159 262	25 079	16
Growth rate ($cm \cdot day^{-1}$)	0.662	0.081	28	0.822	0.083	29	0.699	0.080	30
Stomatal resistance ($s \cdot cm^{-1}$)	1.287	0.740	12	1.14	0.738	13	1.452	0.733	14
Aboveground biomass (g)	18.865	1.296	28	19.136	1.294	29	12.854	1.294	30
	Selfed/low			Selfed/medium			Selfed/high		
Character	mean	S.E.	*n*	mean	S.E.	*n*	mean	S.E.	*n*
First flower (days)	56.832	13.4	30	58.286	13.5	33	52.121	12.7	32
Total flowers (*n*)	36.286	1.358	35	33.971	1.348	36	35.276	1.358	35
Corolla height (mm)	16.798	0.510	24	16.295	0.517	23	16.479	0.500	26
Corolla width (mm)	25.321	0.673	24	24.477	0.681	23	24.563	0.662	26
Herkogamy (mm)	1.632	0.095	24	1.632	0.099	23	1.676	0.092	26
Pollen quantity (*n*)	157 559	24 154	22	142 128	24 717	21	157 937	21 747	26
Growth rate ($cm \cdot day^{-1}$)	0.735	0.077	35	0.875	0.077	36	0.762	0.078	35
Stomatal resistance ($s \cdot cm^{-1}$)	1.471	0.222	17	1.741	0.732	18	1.919	0.734	18
Aboveground biomass (g)	23.399	1.286	35	22.727	1.286	36	15.740	1.289	35

Growth rate of greenhouse plants showed a strong breeding × water interaction ($F_{1,23} = 7.45$, $P < 0.01$). The growth rate of outbred plants in the high water was 83.3% greater than the low water treatment compared with only 4.9% in selfed plants (Table 4). Final aboveground biomass did not show a similar pattern, however. The remaining characters did not show a breeding × water effect ($P > 0.05$; Appendix 2).

There were no differences between outcrossed and selfed CVs for any of the characters measured (Table 5). Levels of plasticity varied among character traits. For example, greenhouse CVs for herkogamy were more than three times higher than corolla height and width CVs.

DISCUSSION

Phenotypic plasticity

The response of *Mimulus ringens* to differences in soil volumetric water content was plastic for a number of characters. When significant, the plasticity generally seemed to reflect lower resource limitation and greater investment into characters that could increase fitness.

Table 4. Breeding (outcross, selfed) × water interaction (low, high) least-squares means (medians and standard deviations presented for first flower) for greenhouse *Mimulus ringens*

	Outcross						Selfed					
	Low			High			Low			High		
Character	mean	S.E.	*n*	mean	S.E.	*n*	mean	S.E.	*n*	mean	S.E.	*n*
First flower (days)	63.0	15.3	61	43.0	15.3	62	42.0	15.6	51	41.5	15.2	48
Total flowers (*n*)	5.352	1.194	24	6.632	1.063	41	6.039	1.111	34	6.208	1.140	33
Corolla height (mm)	18.084	0.447	20	19.423	0.360	32	18.329	0.462	24	19.040	0.444	24
Corolla width (mm)	24.624	0.500	20	26.155	0.421	32	25.094	0.421	24	26.567	0.502	24
Herkogamy (mm)	1.657	0.121	20	1.576	0.098	32	1.490	0.123	24	1.592	0.120	24
Pollen quantity (*n*)	109 229	17 918	12	81 312	15 140	20	115 567	17 215	14	134 875	14 341	24
Growth rate ($cm \cdot day^{-1}$)	0.269	0.053	62	0.493	0.053	61	0.265	0.058	50	0.278	0.060	48
Aboveground biomass (g)	1.997	0.146	61	2.446	0.145	64	2.475	0.161	49	2.849	0.161	49
Root-to-shoot ratio	2.301	0.110	58	1.999	0.070	64	2.105	0.070	49	1.915	0.071	49
Root biomass (g)	2.642	1.143	61	3.148	1.143	66	3.750	1.161	49	3.388	1.167	50
Rhizome biomass (g)	0.686	0.089	58	0.893	0.088	65	0.918	0.099	49	1.064	0.100	50

Table 5. Coefficients of variation (CV) for *Mimulus ringens* characters measured in the field and greenhouse experiments

	Field/outcross		Field/selfed	
Character	mean CV	S.E.	mean CV	S.E.
First flower	12.71	2.58	17.81	2.16
Total flowers	96.73	10.87	92.60	9.59
Corolla height	10.35	1.25	11.10	1.19
Corolla width	7.70	1.36	10.84	1.26
Herkogamy	31.94	77.23	126.58	68.26
Pollen quantity	58.26	5.77	50.23	4.83
Growth rate	40.17	11.45	31.61	11.16
Stomatal resistance	31.26	4.55	32.66	3.95
Aboveground biomass	50.81	7.01	49.12	6.71
	Greenhouse/outcross		Greenhouse/selfed	
Character	mean CV	S.E.	mean CV	S.E.
First flower	15.87	3.37	17.90	3.14
Total flowers	95.13	8.82	81.76	9.29
Corolla height	9.56	1.29	10.07	1.56
Corolla width	6.24	0.85	7.40	1.03
Herkogamy	30.20	5.23	36.00	6.09
Pollen quantity	31.00	7.45	16.53	7.34
Height	60.24	8.59	79.43	9.89
Aboveground biomass	27.43	3.16	24.22	3.66
Belowground biomass	27.38	4.95	23.39	5.51
Root biomass	31.44	5.37	23.77	6.07
Rhizome biomass	32.53	3.13	26.40	3.46

Note: In all cases, there were no significant differences between outcross and selfed coefficients of variation.

Of these characters, only growth rate demonstrated a plastic response in both the field and greenhouse experiments. In the greenhouse growth rate was greater in the high water treatment, whereas in the field growth rate was greatest in the medium water treatment, dropping a bit (though not significantly so) in the high water treatment. Unexpectedly, field plants in the high water treatment had the lowest biomass. Upon planting, the medium and low water treatments were exposed to denser, mesic soil, while the wet plants were exposed to super-saturated soils. It was often difficult to get plants firmly anchored into the super-saturated soils, and this may have slowed the established strong root systems early in the season, putting them developmentally behind the other treatments.

In the greenhouse experiment, plasticity was observed in corolla size and rhizome biomass, with plants in the high water treatment producing larger flowers and more rhizome biomass. Because some pollinators, including *Bombus* spp., are attracted to larger flowers (Creswell and Galen, 1991; Schemske and Agren, 1995; Galen, 2000; Blarer *et al.*, 2002), this could result in increased outcrossing rates. If a higher rhizome biomass produced increased ramets in the

following growing season, this might give an individual a vegetative reproduction advantage.

Not all characters exhibited plastic responses. Of these, the most surprising was stomatal resistance. High treatment plants did not differ in stomatal resistance (smallest stomatal conductance) from other treatments yet had significantly lower biomass. Plants in the wet treatment were presumably not water limited, so we expected that they would have higher stomatal conductance, increased rates of photosynthesis, and thus higher biomass. However, the reduced biomass in the high water treatment plants may be due to reduced aerobic respiration resulting from low soil oxygen levels in saturated soils (Lambers *et al.*, 1998).

Plants exhibit trade-offs by reducing the expression of one character to maintain or increase the expression of another character (Schemske and Agren, 1995; Rosales-Serna *et al.*, 2004; Zhang *et al.*, 2004). For example, *Populus davidiana* grown under drought stress showed a higher root-to-shoot ratio and water use efficiency than non-stressed plants (Zhang *et al.*, 2004). In the low water treatment plants, we would have expected to find higher belowground biomass to increase surface area available for water uptake. The *M. ringens* grown in the greenhouse, however, did not show a trade-off in the amount of above- and belowground biomass production between water treatments.

Inbreeding effects on plasticity

We found very little evidence for a difference in plasticity between selfed and outbred plants. We observed no differences between inbred and outbred plants in the coefficients of variation for any trait. In the field experiment, only corolla width showed an inbreeding × water interaction. In this case, there was essentially no difference in plasticity between selfed and outbred plants but rather a difference in the direction of their response to the water gradient. The most interesting effect was a more pronounced increase in growth rate of outbred greenhouse plants grown under high versus low water conditions relative to the growth rates of inbred plants under these conditions. Selfed and outcrossed plants had similar aboveground biomass in each water treatment, however, suggesting that the selfed plants may have adopted a slightly different architecture in the high water treatment.

The results of this experiment do not support the developmental instability hypothesis (Pederson, 1968), which suggests a negative relationship between heterozygosity and phenotypic plasticity. Our results are generally consistent, however, with those other studies that failed to see a relationship between inbreeding and phenotypic plasticity. Schlichting and Levin (1986) reported no differences in phenotypic plasticity for 12 traits in outbred *Phlox drummondii* compared with plants that had experienced three consecutive generations of inbreeding. Hauser and Loeschcke (1996) examined the drought stress response of *Lychnis flos-cuculi* that ranged from completely outbred to selfed for two consecutive generations. They found significant inbreeding × stress interactions for only one morphological trait (leaf length) and one fitness component (survival from day 55 to day 131) among the 13 morphological and fitness traits that they examined. In both of these cases, outbred plants showed less environmental sensitivity (less plasticity) than the more inbred plants, consistent with the developmental instability hypothesis.

Research on plant tolerance of herbivore and pathogen stress frequently has demonstrated differences between inbred and outbred plants consistent with the developmental instability hypothesis (Carr and Eubanks, 2002; Carr *et al.*, 2003; Hayes *et al.*, 2004; Ivey *et al.*, 2004; Stephenson *et al.*, 2004;

Hull-Sanders and Eubanks, 2005). In these studies, inbred plants typically show a greater reduction in biomass and fitness traits than outbred plants when under high herbivore or pathogen stress, although this effect is not universal (e.g. Núñez-Farfán *et al.*, 1996).

General inbreeding effects

We observed very few inbreeding effects in this population of *M. ringens*. Although we saw a reduction in herkogamy in selfed plants, the fitness components we examined were not altered by inbreeding, indicating a general lack of inbreeding depression. Days to flowering even showed outbreeding depression in the greenhouse population. The lack of general inbreeding effects likely affected our ability to detect inbreeding effects on plasticity. It is worth noting that similar studies (Schlichting and Levin, 1986; Hauser and Loeschcke, 1996) detected significant inbreeding depression for many of the characters examined, yet they too failed to find support for a consistent relationship between inbreeding level and plasticity.

Populations with an extended history of inbreeding typically show very low levels of inbreeding depression (Husband and Schemske, 1996) as a result of genetic 'purging.' As the level of inbreeding increases, the expression of deleterious alleles increases, and accumulated deleterious alleles will be purged from the population through natural selection (Barrett and Charlesworth, 1991; Byers and Waller, 1999). The resulting population will be characterized by individuals with lower inbreeding depression. The low (33%) outcrossing rate observed in other *M. ringens* populations (Karron *et al.*, 1997) may indicate a substantial opportunity for genetic purging in this species and may help explain our observations.

Another possible explanation for why we see a small difference between selfed and outbred plants is that the genotypes selected as parent plants to create the offspring might have been so closely related to each other (biparental inbreeding) that outcrossing plants offered only slightly greater heterozygosity than selfing. To increase the variation in genotypes used in inbreeding studies, future experiments should also include individuals from other populations that may have experienced different environmental pressures and pollinator patterns or identify unrelated mates using allozyme electrophoresis or microsatellites.

CONCLUSIONS

Both the field and greenhouse portions of this study indicate that there is little or no difference between outcrossed and selfed *M. ringens* in phenotypic plasticity across different water treatments. In the one instance in which we observed a difference in plasticity between inbred and outbred plants, outbred plants showed greater plasticity (a larger increase in growth rate in high versus low water treatment). The inability to detect an effect of inbreeding on plasticity could be attributed to the fact that there is little evidence of inbreeding depression in the Blandy *M. ringens* population, but our results are generally consistent with other tests of the developmental instability hypothesis. Studies with results that support the developmental instability hypothesis seem to indicate a greater sensitivity of inbred plants to stress reflected in reduced fitness or performance rather than adaptive differences in morphological or physiological plasticity.

ACKNOWLEDGEMENTS

The authors would like to thank P. Lee, A. Priest, T. Kennedy, S. Hopkins, and summer 2003 REU students at Blandy Experimental Farm for field assistance. T'ai Roulston provided helpful discussion and feedback on manuscript drafts. This work was supported by NSF grants #DEB-0075225 to D.E.C. and #DIR-9512202 to M.A. Bowers and E. Connor.

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Appendix 1. Least-squares means (medians and standard deviations for first flower) for breeding treatments (outcross, selfed) and water treatments (low, medium, high) for field-grown *Mimulus ringens*

	Breeding						Water								
	Outcross			Selfed			Low			Medium			High		
Character	mean	S.E.	*n*	mean	S.E.	*n*	mean	S.E.	*n*	mean	S.E.	*n*	mean	S.E.	*n*
First flower (days)	54.0	13.4	87	53.0	13.4	106	57.0	13.2	63	53.0	13.8	65	50.0	13.1	65
Total flowers (*n*)	32.832	1.267	87	35.165	1.247	106	36.710	1.292	63	33.712	1.287	65	31.699	1.290	65
Corolla height (mm)	16.626	0.464	50	16.524	0.442	73	16.517	0.454	40	16.600	0.455	40	16.608	0.447	43
Corolla width (mm)	24.829	0.607	50	24.787	0.590	73	24.696	0.624	40	24.812	0.626	40	24.918	0.617	43
Herkogamy (mm)	1.949	0.082	50	1.647	0.074	73	1.747	0.075	40	1.804	0.076	40	1.842	0.073	43
Pollen quantity (*n*)	149 281	16 811	50	152 541	15 784	69	148 869	18 938	39	145 266	19 319	38	158 599	18 020	42
Growth rate ($cm \cdot day^{-1}$)	0.728	0.069	87	0.791	0.068	106	0.699	0.071	63	0.849	0.072	60	0.730	0.071	65
Stomatal resistance ($s \cdot cm^{-1}$)	1.295	0.726	39	1.710	0.725	53	1.379	0.728	29	1.443	0.728	31	1.686	0.728	32
Aboveground biomass (g)	16.679	1.266	87	20.304	1.263	106	21.010	1.271	63	20.854	1.271	65	14.224	1.272	65

Appendix 2. Least-squares means (medians and standard deviations for first flower) for breeding (outcross, selfed) and water treatments (low, high) for greenhouse *Mimulus ringens*

	Breeding						Water					
	Outcross			Selfed			Low			High		
Character	mean	S.E.	*n*	mean	S.E.	*n*	mean	S.E.	*n*	mean	S.E.	*n*
First flower (days)	62.0	15.4	123	42.0	15.3	99	46.0	15.9	112	42.0	15.2	110
Total flowers (*n*)	5.992	1.021	65	6.123	1.029	67	5.696	0.935	58	6.420	0.905	74
Corolla height (mm)	18.754	0.322	52	18.684	0.363	48	18.207	0.332	44	19.231	0.298	56
Corolla width (mm)	25.389	0.400	52	25.830	0.438	48	24.859	0.361	44	26.361	0.328	56
Herkogamy (mm)	1.616	0.078	52	1.541	0.088	48	1.573	0.087	44	1.584	0.078	56
Pollen quantity (*n*)	95 270	12 245	32	125 221	42 366	28	112 398	12 856	26	108 093	11 058	44
Growth rate ($cm \cdot day^{-1}$)	0.381	0.047	126	0.272	0.051	98	0.267	0.040	112	0.386	0.041	109
Aboveground biomass (g)	2.221	0.131	125	2.66	0.145	98	2.23	0.109	110	2.647	0.109	113
Root-to-shoot ratio	2.139	0.065	123	2.007	0.051	99	2.213	0.071	107	1.963	0.050	115
Root biomass (g)	2.884	1.127	127	3.565	1.148	99	3.148	1.107	110	3.266	1.107	116
Rhizome biomass (g)	0.789	0.084	123	0.991	0.094	99	0.802	0.070	107	0.979	0.070	115