

# EFFECTS OF STRESS AND PHENOTYPIC VARIATION ON INBREEDING DEPRESSION IN *BRASSICA RAPA*

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Received September 4, 2007

Accepted December 22, 2007

Stressful environments are often said to increase the expression of inbreeding depression. Alternatively, Crow's "opportunity for selection" (the squared phenotypic coefficient of variation) sets a limit to how much selection can occur, constraining the magnitude of inbreeding depression. To test these hypotheses, we planted self- and cross-fertilized seeds of *Brassica rapa* into a factorial experiment that varied plant density and saline watering stresses. We then repeated the experiment, reducing the salt concentration. We observed considerable inbreeding depression, particularly for survival in the first experiment and growth in the second. Both stresses independently depressed plant performance. Families differed in their amounts of inbreeding depression and reaction norms across environments. Outcrossed progeny were sometimes more variable. Stresses had small and inconsistent effects on inbreeding depression and, when significant, tended to diminish it. Levels of phenotypic variability often predicted whether inbreeding depression would increase or decrease across environments and were particularly effective in predicting which traits display the most inbreeding depression. Thus, we find little support for the stress hypothesis and mixed support for the phenotypic variability hypothesis. Variable levels of phenotypic variation provide a parsimonious explanation for shifts in inbreeding depression that should be tested before invoking more complex hypotheses.

**KEY WORDS:** Among family differences, environmental variance, genetic load, inbreeding depression, opportunity for selection, phenotypic variance, reaction norm.

Inbreeding depression has been of strong interest to evolutionary biologists since the age of Darwin (1876). He sought to understand why plants and animals had so many adaptations to promote outcrossing. Inbreeding depression is a key variable that is known to strongly affect the evolution of mating systems, particularly in plants and hermaphroditic animals that have the option of self-fertilization (Stebbins 1950; Jain 1976; Lande and Schemske 1985; Charlesworth and Charlesworth 1987; Waller 1993). However, other variables including pollen discounting and associations among loci also strongly affect how mating sys-

tems evolve (Holsinger 1988; Charlesworth and Charlesworth 1990; Charlesworth et al. 1990; Uyenoyama and Waller 1991; Uyenoyama et al. 1993). Inbreeding depression is also of fundamental interest to those developing inbred lines for practical use in plant and animal breeding, both because it is related to the heterosis that results from crossing inbred lines and because breeders must maintain fitness to sustain lines (Lynch and Walsh 1998). Finally, conservation biologists are concerned with how inbreeding within and among small populations can increase the risk of population extinction (Avisé and Hamrick 1996; Lynch 1996; Landweber and Dobson 1999; Bijlsma et al. 2000; Hedrick 2001; Frankham et al. 2002; Keller and Waller 2002).

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Population geneticists interpret inbreeding depression to represent the expression of either overdominance (superior heterozygote fitness) or dominance (reduced fitness in homozygotes expressing deleterious and at least partially recessive alleles). Recent models for the evolution of inbreeding load have tended to emphasize selection acting against deleterious recessive mutations under various genetic and demographic circumstances (Lynch and Gabriel 1990; Lande et al. 1994; Lynch et al. 1995; Charlesworth and Charlesworth 1999; Wang et al. 1999; Whitlock 2002).

Observed levels of inbreeding depression vary widely according to the character or life-history trait measured, habitat, environmental conditions, or even the year of study (Lynch and Walsh 1998). For example, in *Drosophila* fertility consistently displays more inbreeding depression than viability (Knight and Robertson 1957; Simmons and Crow 1977). Fitness traits also tend to exhibit more inbreeding depression than do morphological traits in animals, perhaps reflecting larger directional dominance in fitness-related traits (DeRose and Roff 1999). Such differences in the expression of inbreeding depression among environments could reflect either variation in the number of mutations expressing deleterious effects or the average effect of those mutations (Kondrashov and Houle 1994).

Those seeking to test whether inbreeding depression is great enough to consistently favor outcrossing often seek to compare observed estimates of this quantity to some absolute figure (e.g., a selfed fitness of 0.5 relative to outcrossed progeny). Similarly, those seeking to test whether selection efficiently purges deleterious recessive alleles from inbred populations often compare quantitative estimates of inbreeding depression among more and less inbred species or populations (Husband and Schemske 1996; Byers and Waller 1999; Crnokrak and Roff 1999; DeRose and Roff 1999). These comparisons implicitly assume that inbreeding depression assumes a relatively fixed value that reflects the amount of genetic load present within a population or species. If, instead, inbreeding depression responds sensitively to environmental conditions, comparisons among populations and taxa become problematic.

Inbreeding depression is known to vary across environments. Conventional wisdom holds that stressful environments (e.g., field conditions) act to increase the expression of inbreeding depression, perhaps because weaker inbred individuals succumb more readily to stressful conditions. We define "stress" as any condition that reduces growth and/or survival. Harsh environmental conditions do appear to magnify inbreeding depression in some studies (e.g., Wright 1922, 1977; Dudash 1990). Langridge (1962) and Pederson (1968) proposed a specific model to explain heterosis for heat and drought stress wherein alleles coding for enzymes tolerant of stress tend to be dominant to alleles more sensitive to these conditions. Inbreeding then exposes these more sensitive enzymes in the homozygotes. Under this hypothesis, we might

also expect inbred progeny to be more susceptible to environmental variation (less "buffered") than outbred progeny, an idea promoted by Lerner (1954).

Environmental stress does often act to increase the expression of inbreeding depression. In reviewing 34 studies on this topic, Armbruster and Reed (2005) noted that the estimated number of lethal equivalents expressed increased 69% (from an average of 0.85 to 1.45) when comparing performance in benign and stressful environments (see also Crnokrak and Roff 1999). Supporting examples include field studies of song sparrows (Keller et al. 1994) and field mice (Jimenez et al. 1994). Among plants, increased competition, disease, harsher field conditions can all magnify inbreeding depression (Dudash 1990; Wolfe 1993; Eckert and Barrett 1994; Norman et al. 1995; Koelewijn 1998; Ramsey and Vaughton 1998; Bijlsma et al. 1999; Cheptou et al. 2000; Meagher et al. 2000). This may place small populations exposed to both inbreeding and heightened stress at particular risk (Hedrick 1994; Kristensen et al. 2003).

Despite this general trend, inbreeding depression responses vary considerably within and among studies, often depending on the particular trait or environment studied. In fact, inbreeding depression increased in response to stress in 25% of the cases reviewed by Armbruster and Reed (2005). In a wild song sparrow population, Marr et al. (2006) noted that inbreeding depression for hatching success increased in response to rain stress, but decreased for laying date in colder years and two other fitness traits were unaffected by stress. Hedrick (1994) found no increase in inbreeding depression in *Drosophila* populations subjected to increased temperature or heavy metals. Dahlgaard and Hoffmann (2000) noted that inbred *Drosophila* showed reduced resistance to chemical and desiccation stress, whereas resistance to heat stress was unaffected by inbreeding. García-Dorado et al. (1999) also noted the environmental specificity of viability mutations in *Drosophila*. Nutrient stress increased inbreeding depression in *Cucurbita pepo* ssp *texana* but which traits were affected varied widely among years (Hayes et al. 2005). Inbreeding depression did not respond to plant density in *Impatiens* (Waller 1984). In *Raphanus sativum*, water stress did not affect the level of inbreeding depression (Nason and Ellstrand 1995). In *Lobelia*, Johnston (1992) found no systematic difference in the expression of inbreeding depression in the field versus the greenhouse. *Clarkia tembloriensis* displayed more inbreeding depression in the greenhouse than the field (Holtsford and Ellstrand 1989). Fertilizer treatments to reduce nutrient stress increased inbreeding depression in *Schiedea* (Norman et al. 1995). In conclusion, Keller and Waller (2002) cautioned against generalizing about the effects of stress and Lynch and Walsh (p. 273 1998) noted that "few of the data bearing on the subject come from well-designed experiments."

As stress provides an imperfect and inconsistent predictor of how levels of inbreeding depression can shift with changes in

environmental conditions, it behooves us to seek alternative explanations for how shifts in environmental conditions affect the expression of inbreeding depression. One alternative termed here the phenotypic variation hypothesis views inbreeding depression as merely another form of selection bounded by the level of phenotypic variation present in the population. Under this hypothesis, environments that accentuate phenotypic variation enhance the opportunity for selection and thus inbreeding depression. Crow (1958) introduced this idea and proposed a quantitative “index of the opportunity for selection” to describe how variation in viability and fecundity impose an upper limit to selection (Crow 1989):

$$I = \text{Var}/\bar{w}^2$$

where *Var* refers to the variance in fitness and  $\bar{w}$  is mean fitness. This quantity equals the coefficient of variation squared for a trait and is scaled to the mean, providing a proportional and dimensionless response that can be easily compared among traits and studies. The actual response via selection will always be less than this quantity as the heritability can never exceed one. Houle (1992) stressed the importance of phenotypic variation in limiting responses to selection and introduced the term “evolvability” to refer to the additive genetic component of Crow’s index. Under this view, whether stress enhances or diminishes inbreeding depression depends on whether stress enhances or diminishes phenotypic variation (as estimated via  $CV^2$  within each environment) and thus how much selection can act.

The “phenotypic variability” hypothesis stands as a distinct alternative to the stress hypothesis in accounting for differences among traits and environments in levels of inbreeding depression. Under the stress hypothesis, we expect stress to increase the selection differential between inbred and outbred progeny. However, this could occur either by increasing the selection coefficient against deleterious mutations or by increasing the level of phenotypic variation in the more stressful environment. We therefore considered effects of stress on phenotypic variability to potentially provide a more parsimonious explanation for the observation that stress frequently increases inbreeding depression.

Here, we test the stress versus phenotypic variation hypotheses in the outcrossing annual plant *Brassica rapa* by assessing how well each predicts shifts in the magnitude of inbreeding depression across traits and environments. We impose both biotic and abiotic stresses in a factorial design. We vary planting density to impose competition, a biotic stress. We impose an osmotic stress by using a saline solution to water the plants. We test the phenotypic variability hypothesis by comparing levels of inbreeding depression among environments and traits in relation to average levels of phenotypic variation ( $CV^2$ ) within inbred and outbred groups within each treatment cell. These experiments are part of a larger project designed to test how effectively inbreeding and pop-

ulation bottlenecks act to purge the genetic load and thus reduce inbreeding depression (Waller et al., unpubl. ms.).

## Methods

### SOURCE OF SELF- AND CROSS-FERTILIZED SEEDS

We used standard “Wisconsin Fastplants” seed stock (c1–33), a fast cycling variety of *B. rapa* derived from wild plants but selected for compact rapid growth and early flowering and fruiting over 10 generations of mass selection (Williams and Hill 1986). The gametophytic self-incompatibility system present in this and most species in the Brassicaceae acts to maintain genetic diversity and limit inbreeding (Nasrallah and Nasrallah 1989), allowing higher levels of inbreeding load to accumulate. These “Fastplants” have been used in several other genetic and ecological studies (Lascoux et al. 1994; Gurevitch et al. 1996; Mitchell-Olds 1996; Lascoux and Lee 1998).

To overcome self-incompatibility, we treated stigmas with a dilute saline solution (2% wt/vol.) 5–10 min before pollination (Monteiro et al. 1988). We also applied saline to the outcrossed stigmas to control for any possible physiological effects on the resulting seed. Outcrosses were randomized by randomizing the position of plants before pollination and mating adjacent plants. We alternated selfing and outcrossing on successive open flowers within the same maternal plant marking the pedicels to identify cross type. After the plants matured, we collected the dry siliques, noting maternal plant and seed type. As seed size varies and seemed likely to affect seedling performance, we estimated seed size and used this as a covariate in analyses of other fitness components. Seeds from each capsule were mounted in rows on sheets of paper using removable Scotch(tm) tape and scanned at 600 dpi on a flat-bed scanner. We used “NIH Image” (developed at the U.S. National Institutes of Health and available at <http://rsb.info.nih.gov/nih-image>) to estimate seed area using the “particle analysis” subprogram. Seed area was normally distributed with a mean of 2.03 mm<sup>2</sup> ( $N = 103$ , SD: 0.519, CV: 25.6%) and linearly related to seed weight ( $r = 0.77$ ,  $N = 103$ ).

### EXPERIMENTAL DESIGN

We compared the growth and fitness of selfed (S) and outcrossed (O) progeny in two experiments conducted in controlled climate greenhouses attached to the University of Wisconsin-Madison Biotron. In both experiments, we varied plant density (a biotic stress) and saline stress (an abiotic stress) in a factorial design. We planted Experiment 1 in July 1998, growing plants in either half-strength Hoagland’s solution or Hoagland’s plus 0.5% saline at three densities: 12, 24, and 48 plants per 10.6 × 16 cm flat (corresponding to a peak density of about 2800 plants / m<sup>2</sup>). There were four flats with 12 plants, two with 24, and one flat with 48 plants in each of three blocks except that a shortage of seed

required us to omit two 12 plant flats from block no. 3. These flats contained a total of 203 S and 203 O seed derived from eight maternal parents. Block no. 2 was assigned to receive the saline treatment whereas blocks nos. 1 and 3 received only dilute Hoagland's. At this osmotic level, the germination rate was under 25% and subsequent survival to maturity was poor (<20%). As these densities limited the usefulness of the density treatment, we repeated the experiment using a lower saline concentration.

Experiment 2 (July 1999) was similar except that we included more families (16), reduced the saline to 0.3% to reduce mortality, and planted only two densities (8 and 32 plants per flat). Because most smaller seeds failed to germinate in Experiment 1, we also set a minimum threshold seed size (1 mm<sup>2</sup> in area). We planted roughly equal numbers of selfed (S) and outcrossed (O) seeds from each maternal plant across the 2 × 2 design, resulting in 96 S and 96 O plants in each of the two density treatments (total *N* = 384). These were distributed over 24 low-density and six high-density flats. One of the high-density flats in the saline treatment experienced very low emergence leading us to eliminate it from further analyses.

To compare S and O performance, we measured % emergence, time to emerge (Experiment 2 only), seedling height at day 16, the number of flowers produced, and final plant size (biomass). Plants were cut at soil level and dried at 70°C for at least 24 h before weighing to the closest milligram. In Experiment 1, half the plants were randomly harvested at either day 21 or day 28. As there was significant growth of the surviving plants during the week between harvests, we multiplied the early biomass values by the ratio in mean weights between the two sampling periods within each saline-density treatment block whenever this value was greater than one (range: 1 – 6.74). In Experiment 2, all plants were harvested on day 29. In both experiments, we also calculated an overall measure of plant success (fitness) to incorporate the effects of both survival and final plant size. It consisted of survival (0 or 1) × log<sub>e</sub> (1 + biomass in cg), and thus ranged from 0 for plants that did not emerge or survive to the logarithm of plant mass. Biomass is closely related to the number of flowers produced in this annual.

## DATA ANALYSES

We analyzed the binary variables (emergence and survival to the end of the experiment) using contingency tables, chi-square analyses, and log-linear models solved iteratively. We estimate inbreeding depression using the familiar formula

$$\delta = 1 - W_S / W_O,$$

which estimates inbreeding depression as the decline in the mean fitness of self-fertilized progeny relative to randomly outcrossed

progeny. When variables were log transformed, inbreeding depression was calculated as 1 minus *e* raised to the difference in logged values. We evaluated normality and homogeneity of variances for residuals using descriptive statistics and box plots. All were close to normally distributed with homogeneous variances. We estimated standard errors (SEs) for these estimates of inbreeding depression using the SEs of the S and O within-cell means, summing the squares of these SE values (for the logged trait variables—the variance of a difference between independent variables being equal to the sum of the variances), taking the square root to obtain a SE' for the mean inbreeding depression, and calculating (1 – antilog (difference in means ± 1 SE')) to estimate a confidence interval (M. Clayton, pers. comm.).

If the development of outcrossed progeny is more canalized and buffered than the development of selfed progeny, we would expect selfed progeny to show greater variability than outcrossed progeny. Alternatively, if mortality truncates the distribution of selfed progeny more than outcrossed progeny (Armbruster and Reed 2005), we would expect selfed progeny to show reduced variability. We computed the relative variability of selfed and outcrossed progeny groups and compared their variances using a simple *F*-test.

We used mixed general models (analysis of variance [ANOVA] and analysis of covariance [ANCOVA]) to assess differences in fitness among the saline and density stress treatments (fixed effects), among families (random effect), and between the S and O progeny (fixed effect). All analyses were done using JMP version 6.0.3 (SAS Institute, www.jmp.com) using the restricted maximum likelihood option (REML) for estimating random effects. We report results primarily for survivorship and final plant biomass as biomass is closely related to height (*r* = 0.703), flower number (*r* = 0.901), and other fitness components. To control for differences in starting resources and possible maternal effects, we included seed area as a covariate in Experiment 1. In Experiment 2, we only used seeds above a threshold area to ensure viability. Because seed areas differed only slightly between the seed types (self: 2.91 vs. outcross: 2.78) and had no significant effect on any dependent variable, we dropped seed area from the analyses in Experiment 2.

Main effects for the treatments were usually significant. We therefore used the least square means for each seed type within treatment cells, adjusted for the effects of all other factors (including family), to estimate the amount of inbreeding depression using the formula above. This estimates inbreeding depression as the decline in fitness of self-fertilized progeny relative to randomly outcrossed progeny, controlling for treatment effects. As we consider faster emerging seedlings to be fitter than slower emerging seedlings, we reversed *W<sub>S</sub>* and *W<sub>O</sub>* in the formula for emergence date. When the effects of the stresses were independent, we

evaluated  $\delta$  across each stress separately (using within-cell means controlling for the other stress). We also evaluated variation in inbreeding depression across families (Johnston and Schoen 1994) (treated as a random effect) and within families across treatments to assess whether stress affects inbreeding depression differently among families.

We also used our estimates of inbreeding depression to assess the genetic load, conventionally expressed as the number of "lethal equivalents" (Morton et al. 1956) per gamete ( $B$ ) or zygote ( $2B$ ). If mutations have independent (multiplicative) effects, we expect the logarithm of survivorship (or other fitness component) to decline linearly with increases in  $F$ , the inbreeding coefficient. We converted our  $\delta$  values to estimates of  $B$  using the formula (Keller and Waller 2002)

$$B = -2 \times \ln(1 - \delta).$$

A significant interaction between seed type and either stress treatment allows us to reject the null hypothesis that the intensity of inbreeding depression does not depend on environmental conditions. Any significant increase in the degree of inbreeding depression under more stressful conditions would support the stress hypothesis.

#### TESTING THE PHENOTYPIC VARIABILITY HYPOTHESIS

To test whether levels of phenotypic variability predict levels of inbreeding depression better than stress, we calculated the opportunity for selection (the phenotypic  $CV^2$ ) within each treatment cell for each trait. Note that we calculated the  $CV^2$  within the S and O progeny groups separately within each combination of stresses before averaging these two values within each of the four (or six) treatment cells. We then tallied the number of times shifts in the expression of inbreeding depression matched the sign of shifts in phenotypic variation in moving between more and less stressful environments. This tests the conjecture that increases or decreases in  $CV^2$  serve to predict increases or decreases in levels of inbreeding depression. We also used mixed model analyses of covariance to compare how levels of  $\delta$  for each trait depended on the two stresses (treated as fixed effects), their interaction, family (treated as a random effect), and mean within-cell  $CV^2$  values (a covariate computed as the average between the selfed and outcrossed  $CV^2$  values within each family and stress cell). Finally, we tested how mean values of phenotypic variation among traits covaried with mean levels of inbreeding depression observed for those traits across environments. To do this, we averaged  $CV^2$  values across all the separate stress cells to estimate a mean variability for each trait and used these to predict mean levels of inbreeding depression (also averaged across stress cells). This regression tests whether amount of inbreeding depression for a trait reflects the levels of phenotypic variation that exist for that trait.

## Results

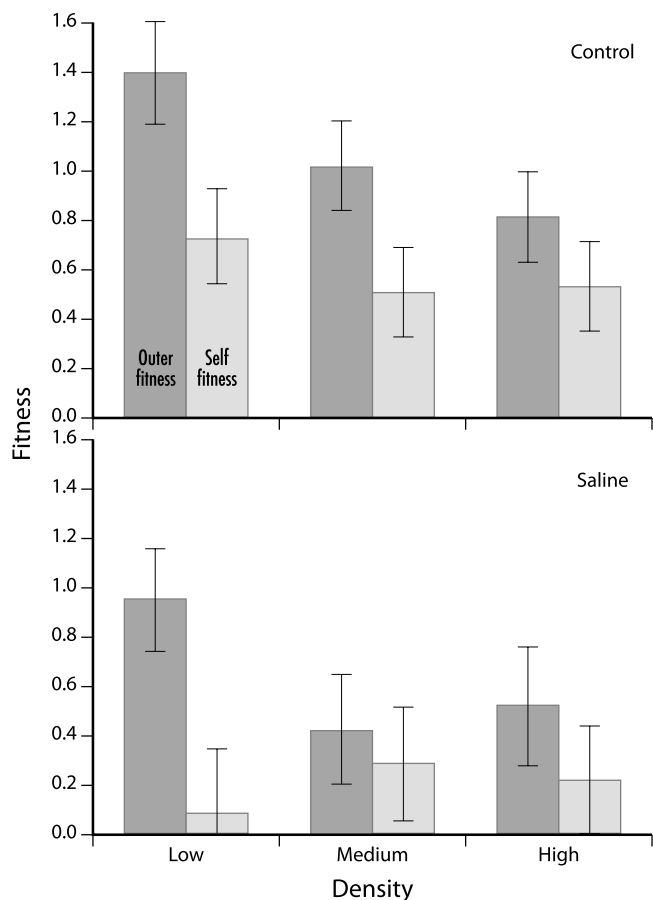
### EFFECTS OF STRESSES

Both increased density and saline watering depressed plant performance, as intended (Table 1). In Experiment 1, the saline stress had profound effects on survival and growth (Fig. 1, Table 2), depressing viability by 62%, height by 48%, and biomass by 81%. In contrast, saline watering did not affect seedling emergence (chi-square = 2.142,  $P = 0.343$ ). The density treatments had much less effect on seedling performance, probably reflecting reductions in density due to high mortality. Only 124 (30.5%) of the 406 seeds planted emerged and survived long enough to have their heights scored on day 16. Nevertheless, density significantly affected overall fitness (Tables 1 and 2). Larger seeds emerged and survived to day 16 at significantly higher rates ( $t = 8.651$ ,  $P < 0.001$ ) but seed area did not affect subsequent fitness traits. Interactions between the saline and density treatments had no significant effects on any of the response variables, suggesting these stresses had independent effects.

It is also possible that interactions could occur among selection at different stages of growth in a manner that could affect the likelihood of detecting inbreeding effects. For example, if the individuals that best resist stress by surviving also show superior performance in later fitness measures such as height or biomass, the fitness of surviving inbreds would be upwardly biased relative to what would be expected if all individuals survived until the end of the experiment. Thus conditions that generate higher mortality among inbred seedling than outbred ones could reduce apparent inbreeding depression for later traits, particularly if these trait are positively correlated with survival. This might explain the absence of inbreeding effects on height and biomass in Experiment 1 (Table 2). It might also account for the pattern in which

**Table 1. Average proportional declines in each trait as a function of the two stresses applied and inbreeding. Values are based on differences between least square means, adjusted for other factors, and significant interactions, calculated from the models fitted for Tables 2 and 3.**

| Experiment | Trait         | Depression in trait due to |         |            |
|------------|---------------|----------------------------|---------|------------|
|            |               | Saline                     | Density | Inbreeding |
| 1          | Viability     | 0.622                      | 0.383   | 0.532      |
|            | Height        | 0.482                      | 0.253   | 0.066      |
|            | Biomass       | 0.812                      | 0.430   | 0.167      |
|            | Total fitness | 0.601                      | 0.509   | 0.651      |
| 2          | Viability     | -0.017                     | 0.103   | 0.096      |
|            | Height        | 0.126                      | 0       | 0.177      |
|            | Biomass       | 0.214                      | 0.661   | 0.164      |
|            | Total fitness | 0.172                      | 0.260   | 0.327      |



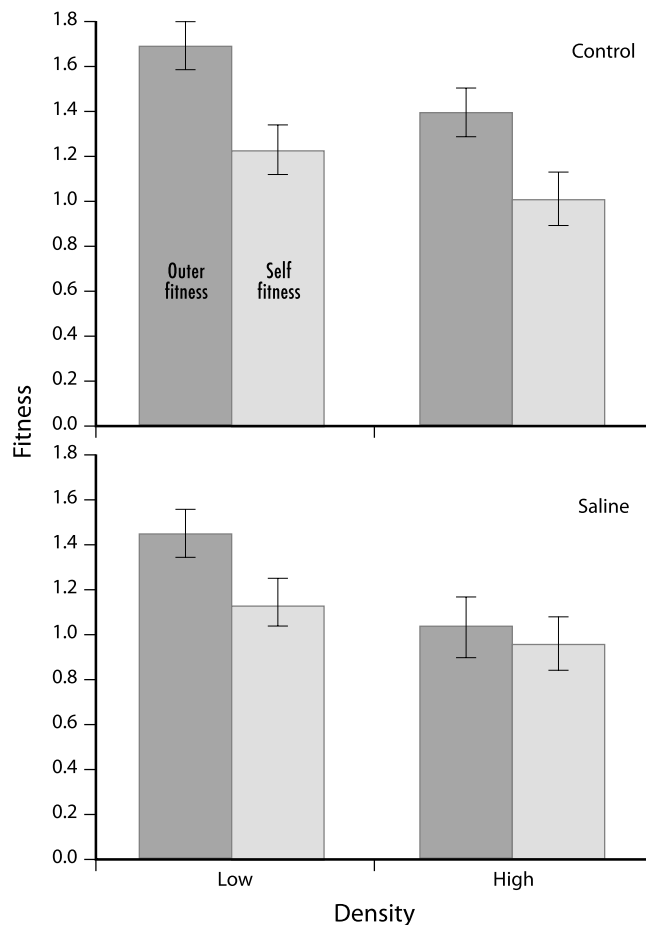
**Figure 1.** Selfed and outcrossed progeny fitness as a function of the saline and density stresses in Experiment 1. Values based on within-treatment cell least square means and standard error bars as fitted via the mixed model analysis of variance presented in Table 2.

inbreeding depression for biomass tends to vary inversely to the inbreeding depression for survival (Fig. 3A and B).

In Experiment 2, both saline watering and higher density significantly depressed plant performance (Tables 1 and 3). Reducing the concentration of salt to 0.3% boosted overall survivorship to 82.9% despite the fact that saline still curtailed growth, reducing plant biomass by an average of 21% (Table 1). Although the saline treatment accelerated emergence slightly (3.9 vs. 4.5 days,  $F = 3.91$ ,  $P = 0.049$ ), it had no effect on viability. Higher plant density depressed viability by 10%, did not affect height, and greatly depressed final biomass (Table 1). Again, there were no significant interactions between the two stresses, reflecting independent effects.

#### EFFECTS OF SEED SIZE

Although we observed no difference in mean seed area between the S and O seeds in Experiment 1 ( $t = 0.675$ ,  $P = .50$ ), seed size strongly affected emergence and survival to day 16 ( $F = 70.2$ ,  $P < 0.001$ ). Seed size also had the greatest effect on overall



**Figure 2.** Selfed and outcrossed progeny fitness as a function of the saline and density stresses in Experiment 2. Values based on within-treatment cell least square means and standard errors as fitted via the mixed model analysis of variance presented in Table 3. Labels and error bars as in Figure 2.

fitness ( $F = 42.5$ , Table 2). In the second experiment when we only planted seeds above a threshold size, S seeds were slightly (4.7%) larger than O seeds on average ( $t = 2.158$ ,  $P = 0.032$ ), but seed size did not significantly affect emergence, survival, height, biomass, or fitness.

#### INBREEDING DEPRESSION

Inbreeding depression was evident for most fitness components in both experiments (Table 1). In Experiment 1, inbreeding strongly affected survival (and thus overall fitness) but did not significantly affect the height or biomass of surviving plants (Table 2). Selfed seedlings suffered an overall 65% reduction in fitness relative to outcrossed seedlings, suggesting a load of 0.91 lethal equivalents. In Experiment 2, inbreeding effects also accumulated over plant lifetimes. Outcrossed seedlings emerged 1.5 days sooner (6.07 vs. 7.45 days,  $F = 6.018$ ,  $P = 0.015$ ) and tended to grow and survive better, resulting in 16–18% differences in height and weight and a 32% overall difference in fitness (0.33 lethal

**Table 2.** Results of multivariate general linear model analyses for Experiment 1. Values within the rows below “Predictor Variable” represent likelihood ratio Chi-square values from the logistic model (for the discrete dependent variable, survival), the % of variance accounted for (for the random family effect in the model), or *F* values from the mixed model analyses (for fixed effects). Fitness is the product of survival and log biomass in cg. The two seed types (S/O) by stress (saline or density) interactions (last rows) test the hypothesis that levels of inbreeding depression change in response to environmental stress.

|  | Survival | Dependent variables |               |          |
|--|----------|---------------------|---------------|----------|
|  |          | Log (height)        | Log (biomass) | Fitness  |
| <i>N</i>                               | 406      | 120                 | 113           | 406      |
| Overall adjusted <i>r</i> <sup>2</sup> | 0.34     | 0.39                | 0.40          | 0.36     |
| Predictor variable (levels)            |          |                     |               |          |
| Seed area (covar)                      | 36.89*** | 0.86 NS             | 0.31 NS       | 42.55*** |
| Family (8)                             | 43.84*** | 23.5 %              | 1.12 %        | 11.3 %   |
| Self/outcross (2)                      | 14.86*** | 0.67 NS             | 0.15 NS       | 16.16*** |
| Saline/water (2)                       | 9.60**   | 23.59***            | 41.44***      | 17.88*** |
| Density (3)                            | 4.68 AS  | 2.62 AS             | 1.72 NS       | 3.82*    |
| Family × S/O                           | 17.93**  | 0.78 NS             | 1.15 NS       | 3.17**   |
| S/O × Seed Area                        | 5.29*    | 5.18*               | 1.15 NS       | 10.53**  |
| S/O × Saline                           | 0.69 NS  | 1.18 NS             | 0.11 NS       | 0.18 NS  |
| S/O × Density                          | 4.45 NS  | 1.23 NS             | 0.20 NS       | 1.76 NS  |

NS, not significant  $P > 0.10$ , AS, almost significant  $0.05 < P < 0.10$ , \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .

**Table 3.** Linear model analyses for Experiment 2. Layout, tests, and abbreviations as in Table 2. Because seed area was not significant, it was dropped from these analyses. All S/O by stress interactions in this experiment reflect declines in inbreeding depression with increased stress.

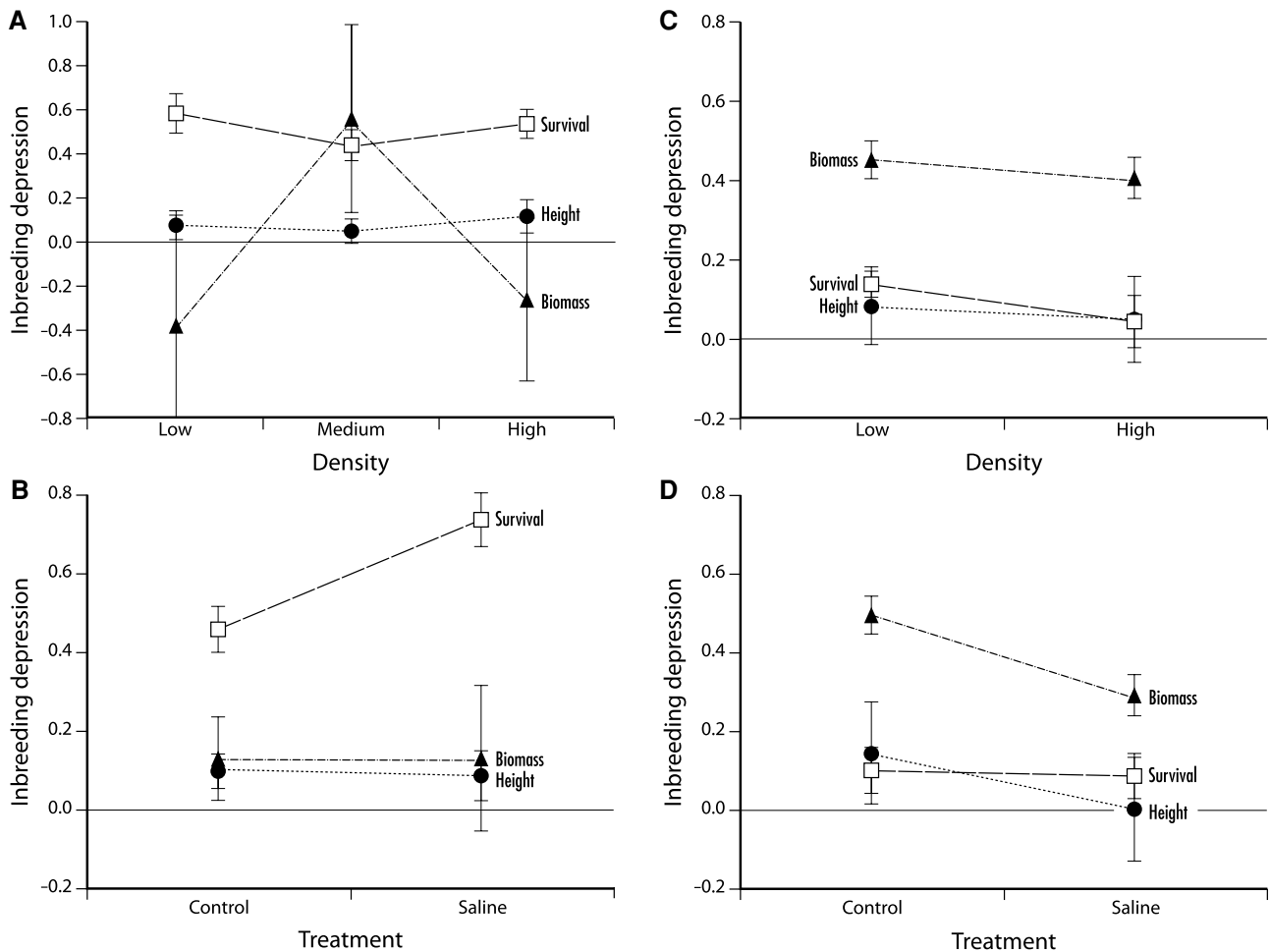
|  | Survival | Dependent variables |          |          |
|--|----------|---------------------|----------|----------|
|  |          | Log (height)        | Biomass  | Fitness  |
| <i>N</i>                               | 350      | 289                 | 291      | 350      |
| Overall adjusted <i>r</i> <sup>2</sup> | 0.33     | 0.25                | 0.47     | 0.24     |
| Predictor variable (levels)            |          |                     |          |          |
| Family (15)                            | 45.95*** | 26.8 %              | 14.2 %   | 12.5 %   |
| Self/outcross (2)                      | 0.00 NS  | 11.27***            | 40.49*** | 20.28*** |
| Saline/water (2)                       | 0.04 NS  | 3.35 AS             | 62.42*** | 7.46**   |
| Density (2)                            | 3.57 AS  | 0.16 NS             | 38.01*** | 17.19*** |
| Family × S/O                           | 19.92 NS | 0.89 NS             | 2.18**   | 1.16 NS  |
| S/O × Saline                           | 0.91 NS  | 7.62**              | 15.46*** | 3.32 AS  |
| S/O × Density                          | 2.13 NS  | 0.64 NS             | 4.75*    | 1.40 NS  |

equivalents). Inbreeding only depressed survival about 10% (Table 1), not enough to be significant (Table 3). Thus, both experiments produced substantial and statistically significant levels of inbreeding depression, ensuring that we could evaluate how stress and phenotypic variability affected its expression.

#### EFFECTS OF STRESS ON INBREEDING DEPRESSION

The density and saline stresses had mixed effects on the expression of inbreeding depression (interaction terms in Tables 2 and 3 and Fig. 3). In Experiment 1, the two stresses had various and contradictory effects on inbreeding depression. Inbreeding depressions

for survival and height were remarkably stable across the density treatments as were height and biomass across the saline treatments (Fig. 3A, B). The density stress increased then decreased,  $\delta$  for log biomass (Fig. 3A). The saline stress appeared to increase inbreeding depression for survival (from 46% to 73%, Fig. 3B). However, these effects were not significant (Table 2). Although the saline stress treatment was always significant, the density treatment had only minor effects on the fitness components (Table 2) and the two stresses had independent effects. The saline and density stress effects were also independent of seed type (S or O), meaning that they neither increased nor decreased inbreeding depression. We estimate 0.53 lethal equivalents per zygote in the



**Figure 3.** Response of estimated inbreeding depression values to biotic stress (A and C, plant density) and abiotic stress (B and D saline watering) in Experiments 1 (A and B) and 2 (C and D).

nonsaline environment versus 1.14 lethal equivalents with saline watering (based on the survival data).

In Experiment 2, increased density and saline watering had more consistent effects, generally decreasing observed levels of inbreeding depression (Fig. 3C, D). For example, the saline treatment reduced  $\delta$  for the biomass of surviving plants from 49% to 29% (interaction  $P < 0.001$ ) reflecting the fact that saline reduced the growth of outcrossed seedlings by 48% compared to only 36% for the (already smaller) selfed seedlings. The density stress also reduced  $\delta$  for biomass. Inbreeding depression for flower number also declined under the saline stress from 43% to 30% (interaction  $P < 0.05$ ) with salt reducing outcrossed flower number more than selfed flower number (32% vs. 23%). The decline in  $\delta$  for log height under the saline stress was also significant (Fig. 3D, Table 3). Log biomass and overall fitness showed almost significant declines under saline watering (Table 3). These results run counter to the stress hypothesis.

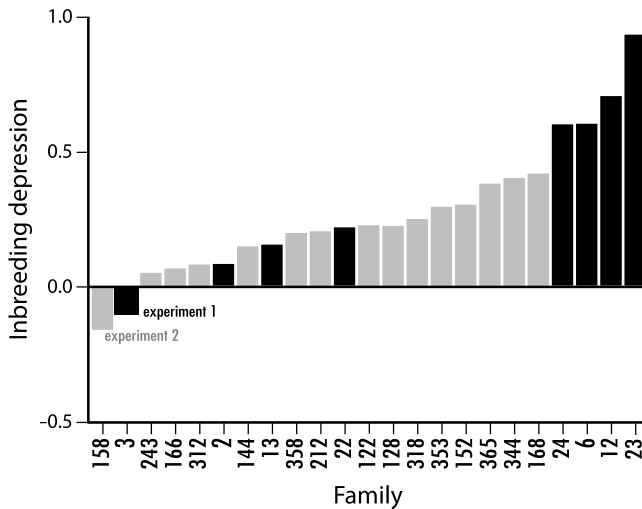
In summary, we find that when stress affects inbreeding depression it tends to reduce it. Stress effects were also inconsistent

in that whether stress increased  $\delta$ , reduced it, or left it unchanged depended on the trait measured, the stress applied, and the year the experiment was done.

### FAMILY EFFECTS

Maternal families varied widely in mean progeny performance for most fitness variables (Tables 2 and 3). Inbreeding depression also tended to vary over families particularly for the traits that showed the highest  $\delta$ , namely survival in Experiment 1 and biomass in Experiment 2.

High inbreeding depression is particularly evident in a few families in Experiment 1 (6, 12, 23, 24—Fig. 4), often reflecting poor selfed seedling emergence. Across both experiments,  $\delta$  varies widely as does the estimated number of lethal equivalents per zygote (range: 0 to 2.5, mean: 1.59). Our estimates of the genetic load ranged from 0 to 11.2 lethal equivalents per zygote across maternal families with means across families ranging from 1.8 to 5.2, depending on environmental conditions. Armbruster and Reed (2005) noted similarly strong lineage effects in many other



**Figure 4.** Distribution of inbreeding depression values over families in the two experiments: families 2–24 are from Experiment 1 and 122–365 from Experiment 2.

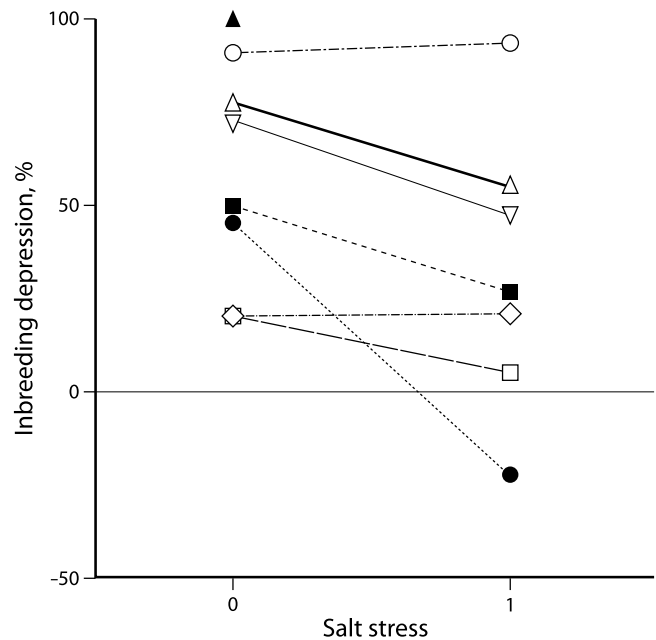
studies. Lascoux and Lee (1998) found a similar number of lethal equivalents (1.62 for germination, 2.96 for flowering) in this fast-cycling variety of *B. rapa*. They considered these values low for an outcrossed population and hypothesized that biparental inbreeding had purged some of the load. As they grew plants in individual pots, it is difficult to guess how stress or competition might have modified these estimates. Families also appear to differ in their norms of reaction for inbreeding depression (Fig. 5) with  $\delta$  tending to decline with increasing stress in most, but not all, families.

#### THE RELATIVE VARIABILITY OF S AND O PROGENY

We found some significant differences in the relative variability of S and O progeny but these were inconsistent across traits (Table 4). The variances of selfed progeny are only significantly higher for survival in Experiment 2, whereas outcrossed variances were higher for flower number, biomass, and almost height, in Exper-

**Table 4.** The ratio of self-fertilized to outcrossed progeny trait variances, averaged across all density by saline treatment cells in Experiments 1 and 2. Significance levels inferred from *F*-tests and symbolized as in Table 2.

| Trait          | Relative S/O Variance |              |
|----------------|-----------------------|--------------|
|                | Experiment 1          | Experiment 2 |
| Seed area      | 1.080                 | –            |
| Emergence date | –                     | 1.322 AS     |
| Height         | 0.594 AS              | 0.731 AS     |
| Flower number  | –                     | 0.459***     |
| Biomass        | 0.2386***             | 0.360***     |
| Log biomass    | 0.828                 | 0.983        |
| Survival       | 1.042                 | 1.545**      |



**Figure 5.** Reaction norms for inbreeding depression for overall fitness (the log of the product of survival and biomass) among families across the control (0) and saline stress (1) environments in Experiment 1. Families averaged 12.6 seeds of each type.

iment 2 and biomass and almost height in Experiment 1. Many of these differences parallel differences in the means, suggesting that these minor differences may reflect scaling effects. Neither S nor O progeny appear to exhibit consistently greater levels of quantitative genetic diversity (as inferred from among family differences).

#### TESTING THE PHENOTYPIC VARIATION HYPOTHESIS

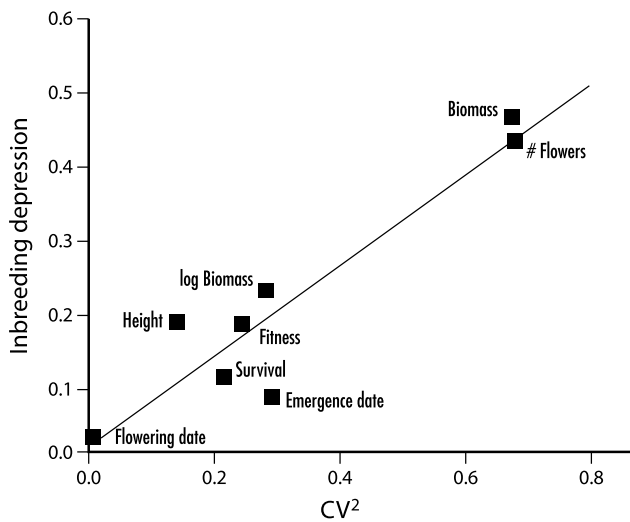
We found mixed support for the idea that levels of phenotypic variation (as estimated by Crow's index of the opportunity for selection) serve to predict levels of inbreeding depression in a given environment. The biotic and abiotic stresses did often affect the amount of variability observed for a given trait, as evidenced by significance in Bartlett tests for heterogeneity of variance over the environmental treatments. For example, in Experiment 2, there was significant heterogeneity in variance for survivorship, flower number, biomass, log biomass (all  $P < 0.001$ ), and emergence date ( $P < 0.05$ ), but not height. These results led us to evaluate whether shifts in  $CV^2$  for particular traits across stress treatment cells (calculated by averaging the separate S and O values) served to predict changes in inbreeding depression. In particular, we scored how often the shift in  $CV^2$  in response to the saline or density stress matched the sign of the corresponding shift in inbreeding depression. Each trait provided four such comparisons and we tallied these shifts for four traits (seed area, survival, height, and biomass) in Experiment 1 and seven traits (emergence day, survival, flowering date, height, flower number, biomass, and fitness)

**Table 5.** Summary of analyses of covariance of average inbreeding depression values across families within stress cells as a function of family, the stresses applied, and the index of the opportunity for selection ( $CV^2$ ). Mixed model analyses with family treated as a random variable, the stresses as fixed effects, and  $CV^2$  as a covariate. Values shown are estimated effect sizes for each variable (except family which shows the percent variance accounted for). Significance values as in other Tables.

| Trait analyzed  | Family  | Saline | Density | $CV^2$  | Saline $\times$ Density |
|-----------------|---------|--------|---------|---------|-------------------------|
| a) Experiment 1 |         |        |         |         |                         |
| Seed area       | 0.01 %  | -0.192 | 0.168   | -0.066  | 0.126                   |
| Height          | 0.01 %  | 0.356  | -0.172  | -0.570  | 0.251                   |
| Fitness         | 0.56 %  | -0.25  | 0.127   | -0.036  | 0.311                   |
| Log biomass     | 0.2 %   | -0.365 | -0.245  | 6.506   | 3.317*                  |
| b) Experiment 2 |         |        |         |         |                         |
| Emergence Date  | 16.69 % | -0.043 | 0.179   | -1.138* | -0.043                  |
| Height          | 0.00 %  | -0.236 | 0.068   | -0.034  | -0.287                  |
| Biomass         | 11.04 % | -0.237 | 0.074   | 0.053   | -0.005                  |
| Fitness         | 0.00 %  | 0.254  | 0.268   | 0.126   | -0.407                  |

in Experiment 2. The signs matched on 34 of these 44 comparisons, suggesting that the null hypothesis of independent changes is highly improbable ( $P < 0.0001$ , Fisher's exact test).

Despite this pattern of significant positive associations between shifts in  $CV^2$  and shifts in inbreeding depression among stress treatments, we detected few quantitative effects of  $CV^2$  on the expression of inbreeding depression within traits (Table 5).



**Figure 6.** Relationship between average levels of phenotypic variability ( $CV^2$ ) for a trait and the estimated average inbreeding depression ( $\delta$ ) observed for that trait in Experiment 2. The  $CV^2$  values are weighted averages of the separate S and O groups within each stress treatment cell averaged across all four cells. Equation of the fitted line: Inbreeding depression =  $0.0096 + 6.224 \times 10^{-5} \times CV^2$ ,  $r^2 = 0.863$ . The analogous line for five traits in Experiment 1 has  $r^2 = 0.942$ .

Crow's Index of the Opportunity for Selection was only significantly associated with variation in inbreeding depression for emergence date in Experiment 2, and this association was negative. Thus,  $CV^2$  proved to be a poor predictor of how much inbreeding depression would exist for a given trait across environments. These analyses are also consistent with the results shown previously in failing to demonstrate significant effects of the stress treatments on inbreeding depression. The only significant effect of stress was the saline  $\times$  density interaction term affecting inbreeding depression for log biomass in Experiment 1 (Table 5). However, both saline and density acted not to enhance inbreeding depression but rather to depress it in this case. Analyses of variance (not shown) also revealed that stresses generally had few consistent or significant effects on levels of phenotypic variability,

Despite these minor and inconsistent effects within traits, levels of phenotypic variation appear highly correlated with levels of inbreeding depression when these are compared over traits (Fig. 6). That is, a strong positive association exists between the average level of inbreeding depression observed for a trait and the average variability ( $CV^2$ ) observed for that trait (averaging between the selfed and outcrossed seedling groups and over stress treatments). That is, traits varied considerably in how much variation they expressed and this variation served to predict the average amount of inbreeding depression observed for a trait. In Experiment 2, the relationship between average Crow's index and average inbreeding depression over traits gave an  $r$  of 0.93. The analogous fit for five traits in Experiment 1 (seed area, survival, height, biomass, and log biomass) gave an  $r$  of 0.97. In a combined analysis of covariance (not shown),  $CV^2$ , Experiment, and  $CV^2 \times$  Experiment were all significant. Thus, traits that varied more displayed greater inbreeding depression, but this relationship changed between the two experiments.

## Discussion

### STRESS EFFECTS

Conventional wisdom has held that inbred progeny are more susceptible to stressful environmental conditions than cross-fertilized progeny, increasing inbreeding depression in harsh and stressful environments (see Introduction). Armbruster and Reed's (2005) review of 34 studies supports the view that stress often acts to increase inbreeding depression but emphasized that "accurate predictions regarding the response of specific populations or lineages to inbreeding under stress will often be difficult or impossible" (p. 238). Results presented here and elsewhere fail to support the stress hypothesis, however. Such inconsistency among studies and environments suggests that other factors beyond stress may act to affect inbreeding depression.

In this study, inbreeding depression showed little consistent relation to either the abiotic saline stress or the biotic density stress (Fig. 3; Table 5). Instead, stress often had little effect on inbreeding depression (e.g., height in both years) or contradictory effects between the two stresses (with saline stress decreasing, but density increasing,  $\delta$  for biomass) or between the two experiments (saline stress increasing  $\delta$  for survival in Experiment 1 but decreasing it in Experiment 2). Inbreeding depression for survival was about 40% in Experiment 1 and appeared to increase under saline stress to near 75%. However, this was the only trait observed to show an increase under stress and the trend was not statistically significant (Table 2). Other traits and overall trends showed stress having little effect or decreasing rather than increasing the expression of inbreeding depression (Fig. 3, Tables 3 and 5). Thus, we find little support for the stress hypothesis and none that is consistent or repeatable across stresses and experiments.

These inconsistent and contradictory results mirror the mixed reports in the literature regarding how environmental stress affects inbreeding depression (see Introduction). The failure to find support for the stress hypothesis in these experiments does not reflect poor experimental design or conditions. The experiments worked as designed. Both the abiotic and biotic stresses substantially diminished plant performance, as intended (Table 1). We also detected moderate to high levels of inbreeding depression for most traits. This was expected given that *B. rapa* is a historically outcrossing species with a functional genetic self-incompatibility system. Although the high saline-induced mortality in Experiment 1 may have limited the impacts of plant density, several density effects were still significant and we repeated the entire experiment at a lower salt concentration. This balanced the stresses nicely in Experiment 2, with both depressing fitness to a degree similar to inbreeding. Both the abiotic and biotic stresses also tended to act independently judging from the nonsignificant interaction terms (Tables 2 and 3). The exception was log biomass in Experiment

1 in which saline and density appeared to synergistically depress inbreeding depression (Table 5).

Other studies have also examined variation in inbreeding depression over environments that differ in levels of some environmental stress. Kondrashov and Houle (1994), for example, observed variation in the genetic load in mutation accumulation lines of *Drosophila melanogaster* with dramatic increases in load for populations exposed to crowded and nutrient starved conditions. Keller (1998) observed similarly wide variation among years in the expressed load in a field population of juvenile song sparrows. Such environmental dependence implies either increases in the magnitude of the deleterious effect per mutation or expression of more previously neutral mutations as deleterious under certain conditions. We cannot distinguish between these hypotheses with our results, but QTL mapping of plants in alternate environments (e.g., Mitchell-Olds 1995; Remington and O'Malley 2000) may eventually reveal more.

Stress could initially increase the expression of inbreeding depression by increasing the amount of selection and/or levels of phenotypic variation. However, increases in stress will eventually stunt or kill plants, often truncating the inbred distribution and thus diminishing the mean fitness difference between inbred and outbred progeny and thus inbreeding depression (as noted by Armbruster and Reed 2005). At the limit, a severe stress kills all inbred and outbred individuals, eliminating our ability to estimate relative fitness. Stress did increase mortality in Experiment 1 but inbreds did not suffer disproportionately high mortality. Still, inbreeding depression for other traits potentially correlated with viability could have been decreased, reducing the opportunity to observe enhanced inbreeding depression in those traits. The high early seedling mortality in Experiment 1 may thus have acted to reduce both the opportunity for selection and inbreeding depression for later traits like height and biomass (Table 2).

If stress generally diminishes inbreeding depression, stressful environments might exert selection that could favor a shift in plant breeding systems toward more selfing. For example, plants like *Impatiens capensis* (Waller 1980) and *Amphicarpea bracteata* (Schnee and Waller 1986) often produce a higher proportion of cleistogamous (obligately self-fertilizing) flowers under stressful conditions, a widespread phenomenon that has been termed "environmental cleistogamy" (Lord 1980; Clay 1982; Wilken 1982). Declines in inbreeding depression under more stressful conditions could thus provide an adaptive explanation for the pattern that cleistogamous plants usually produce more outcrossing flowers under conditions that favor growth. However, other explanations including pollinator availability and the higher relative time and/or material costs of outcrossing flowers under stressful conditions provide more parsimonious explanations for this pattern (Waller 1980).

## COMPARATIVE STUDIES

Researchers also use patterns in how inbreeding depression varies over environments and taxa to test general patterns or theories. Both evolutionary biologists and conservation geneticists seek to know whether field conditions exacerbate the inbreeding load (Hauser and Loeschcke 1996; Bijlsma et al. 1999; Crnokrak and Roff 1999). Those wishing to test whether purging is more likely to occur in more inbred taxa have also conducted meta-analyses to compare levels of inbreeding load among populations and species compiled from the literature (Husband and Schemske 1996; Byers and Waller 1999; Crnokrak and Barrett 2002). Although such studies and comparisons can be informative, they typically compile data without regard to environmental conditions or levels of phenotypic variation. The results presented here suggest that Crow's opportunity for selection could provide a more parsimonious explanation for the differences in inbreeding depression observed among studies and taxa. This point parallels the observation that differences in heritability among traits and studies may reflect differences in environmental effects rather than differences in genetic effects (Barton and Turelli 1989; Merilä and Sheldon 1999).

## AMONG FAMILY VARIATION IN INBREEDING EFFECTS

We observed genetic variation among families for the amount of inbreeding depression (Fig. 4). Similar results have been reported in *Impatiens* (Waller 1984, 1985; McCall et al. 1994), *Lychnis* (Hauser and Loeschcke 1996), *Plantago* (Koelewijn 1998), *Lo-belia* (Mutikainen and Delph 1998), *Clarkia* (Holtsford 1996), *Mimulus* (Dudash et al. 1997; Willis 1999), the flour beetle *Tribolium* (Pray and Goodnight 1995), and the snail *Physa* (Jarne et al. 2000). Variation among families in  $\delta$  could reflect variation in either the number of mutations across families or their average fitness effects. High inbreeding depression in some families and high inviability and loss of inbred lines in further experiments with this population of *B. rapa* (Waller et al., unpubl. ms.) suggest that lethal or sublethal mutations are segregating in many families. This also appeared to occur in *Mimulus guttatus* (Willis 1999). Such variance in the inbreeding load provides opportunities for among-line selection to purge inbred populations of some of their genetic load (Lande and Schemske 1985; Dudash et al. 1997) perhaps favoring different breeding systems. The variation in inbreeding depression observed over families in this study may also be inflated by using only a single pollen source for the outcrosses rather than a mixture of fathers. For this reason, we place more stock in the population averages than these individual family values.

We also observed variation among families in reaction norms—that is, how inbreeding depression varies in response to the saline stress (Fig. 5). These differences suggest that families differ not only in their genetic load but also in how this load is expressed under various environmental conditions. Such variance

among families in how inbreeding depression responds to stress could allow this trait to evolve (Via and Lande 1985; Schlichting 1986; Scheiner 1993; Pigliucci et al. 1995; Via et al. 1995), particularly in inbred lines where epistatic interactions might allow alleles at different loci to coevolve.

Lerner (1954) and others have speculated that inbred progeny express more phenotypic variation than outbred progeny because they lack the genetic homeostasis provided by allelic interactions at heterozygous loci. Quantitative genetic models predict that additive genetic variation for traits could either decrease or increase after inbreeding (Mitchell-Olds and Waller 1985). We did find higher selfed variance for survival and (almost) emergence date in Experiment 2 supporting the notion that selfed progeny may lack developmental stability. However, we more commonly observed outcrossed progeny to have higher variance (Table 4). This may support the idea that the lower survival of inbred progeny commonly truncates distributions of their trait values (Arbruster and Reed 2005). Rao et al. (2002) also failed to find evidence for increased variability or asymmetry in flowers or cotyledons from self-fertilized progeny in *Brassica cretica*, although some support for this idea exists in other species (e.g., mice—Falconer and Mackay 1996). Roy and Stanton (1999) found that various stresses all decreased fitness and increased asymmetry, but different stresses affected different organs and individual fitness was not strongly correlated with asymmetry.

## PHENOTYPIC VARIABILITY

If Crow's (1958) index of the opportunity for selection (the phenotypic  $CV^2$ ) sets an upper limit to selection and inbreeding depression, we expect shifts in inbreeding depression among environments to parallel shift in (within cell) variability. We found limited support for this idea working with particular traits. Qualitatively, the sign test demonstrated that increases or decreases in phenotypic variability often serve to predict corresponding shifts in inbreeding depression. However, quantitative analyses of variation in inbreeding depression over families and environments found that increases in  $CV^2$  had no significant power for predicting increases in inbreeding depression for particular traits (Table 5). In addition, the stress treatments had no consistent effects on  $CV^2$ . Thus, we have no clear evidence that the effects of stress on inbreeding depression are acting through the effect of stress on levels of phenotypic variation.

In contrast, we found strong support for the phenotypic variability hypothesis when comparing levels of inbreeding depression among traits (Fig. 6). Traits with more within-cell variation clearly express higher levels of inbreeding depression, supporting the idea that a trait's variability may limit the amount of inbreeding depression it can express. The strength and linearity of this relationship surprised us. The linearity supports the theory behind Crow's Index. The high coefficient of determination could also

reflect the fact that the traits differ considerably in variability and that the values plotted represent means across all environments and the two progeny groups.

In summary, we find no support for the idea that the biotic or the abiotic stresses applied in these experiments act to increase the expression of inbreeding depression. Instead, they sometimes acted to reduce it. We find somewhat better but still mixed support for idea that differences in levels of variation expressed among environments serve to predict differences in inbreeding depression. Within traits, stresses did not consistently increase or decrease  $CV^2$  and  $CV^2$  did not serve to predict inbreeding depression. However, among traits, average levels of phenotypic variability and inbreeding depression are highly correlated.

Crow's theory on the opportunity for selection provides a simple and theoretically justified quantity (the  $CV^2$ ) for making predictions about how levels of inbreeding depression may vary over traits and environments. Because it is dimensionless, it also provides a convenient metric for making comparisons among traits, environments, and studies. Because phenotypic variability provides a parsimonious explanation for variation in inbreeding depression, we encourage researchers studying how inbreeding depression varies among environments and traits to first consider how the differences in inbreeding depression they observe might be accounted for simply on the basis of differences in  $CV^2$ . Once such effects are taken into account (e.g., by regressing  $\delta$  on  $CV^2$ ), it may be possible to apply more refined tests for the stress and purging hypotheses by examining residuals from this relationship. It should also be possible to conduct meta-analyses of results from multiple studies (like Armbruster and Reed 2005) to assess the relative power of the stress and phenotypic variability hypotheses. We look forward to what such tests will tell us about the factors affecting variation in inbreeding depression.

#### ACKNOWLEDGMENTS

We dedicate this article to our friend and colleague, J. Crow, whose opportunity for selection only scarcely represents his many contributions to genetics. We gratefully acknowledge support from the NSF (award 9728855) and the Univ. of Wisconsin Graduate School. In addition, we thank P. Williams and the Wisconsin "fast-plant" consortium for advice on the care and feeding of *Brassica rapa*. These experiments depended on the able assistance of P. Mandrekar, R. Donicht, J. Steven, J. Stolt, J. Reid, J. Remfert, and the UW Biotron staff. M. Clayton and C. Ané provided statistical advice. We thank J. Crow, P.-O. Cheptou, L. Keller, A. Kondrashov, H. P. Koelwijn, D. Schoen, J. Willis, and an anonymous reviewer for providing useful suggestions on drafts of the manuscript. DW expresses gratitude to I. Olivieri at the Univ. de Montpellier II and P. Jarne at CEFÉ-CNRS for hosting the sabbatical stay that allowed the analyses presented here.

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Associate Editor: D. Schoen