

Inbreeding depression in an autotetraploid herb: a three cohort field study

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Summary

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- Autotetraploids are predicted to have reduced inbreeding depression relative to diploids. However, recent theory and information on genomic changes following autopolyploidy suggest that inbreeding depression may be closer to diploids.
- In three consecutive years, self and outcross pollinations were conducted on autotetraploid *Campanulastrum americanum*, seeds were planted into native sites, and biennial offspring were followed through seed production.
- Inbred individuals had lower germination rates, reduced survival, were smaller, and flowered later, producing fewer fruits with fewer seeds. Inbred offspring had 6% of the cumulative fitness of outcross offspring. Although performance varied substantially among cohorts, inbreeding depression for cumulative fitness was relatively constant, with δ ranging only from 0.92 to 0.95.
- *C. americanum*, like many outcrossing species, expressed very high amounts of inbreeding depression. This supports the hypothesis that inbreeding depression of some autotetraploids may be similar to that of diploids. Furthermore, few studies have measured temporal variation in inbreeding depression. Constant inbreeding depression given a sixfold range in cohort performance suggests that inbreeding depression may be relatively robust to environmental variation experienced by natural populations.

Key words: autotetraploid, cumulative fitness, environmental variation, field study, inbreeding depression, polyploidy, temporal variation.

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Introduction

Polyploidy is very common in angiosperms, with up to 70% of taxa having polyploid ancestry (Masterson, 1994). Classical theory suggests that polyploids will have reduced inbreeding depression relative to diploids. There are two rationales for this expectation. The first argues that polyploid establishment is more likely to occur if inbreeding depression is modest. When a novel polyploid arises within a diploid population, it is a minority cytotype. Mating with other individuals in the population that have a different chromosome number will result in a high frequency of sterility (Burton & Husband, 2000). Thus, the ability to self-pollinate increases the likelihood of successful reproduction and establishment (Rodriguez, 1996; Baack, 2005), especially if inbreeding depression is modest

(Rausch & Morgan, 2005). Modest inbreeding depression may be found because the genetic load of neopolyploids is lower than that of a comparable diploid if tetraploids are better able to mask single copy mutations (Otto & Whitton, 2000). Furthermore, early-stage polyploids typically experience very small effective population sizes and consequently it is likely that inbreeding will continue for a number of generations. During this establishment bottleneck, recessive deleterious alleles may be purged by selection, resulting in taxa with reduced genetic load and inbreeding depression (Lande & Schemske, 1985). In summary, the process of establishment is expected both to favor taxa with limited inbreeding depression and to result in a further reduction of inbreeding depression.

The second rationale for reduced inbreeding depression in polyploids relative to diploids is explicitly genetic. Like

diploids, inbreeding depression in the selfed progeny of autopolyploids is thought to be the result of the expression of deleterious recessive or partially recessive alleles in the homozygous form. However, unlike diploids, the rate of progression toward full homozygosity proceeds slowly. Specifically, following a single generation of selfing, the frequency of heterozygotes is expected to drop by 50% in diploid taxa but only by 17–21% in autotetraploid taxa (Bever & Felber, 1992). This is largely the result of an increase from a single heterozygous genotype in diploids to three heterozygous genotypes in tetraploids. Therefore, following a single generation of selfing, polyploid taxa should express less inbreeding depression as a result of recessive deleterious alleles than diploid taxa (Lande & Schemske, 1985; but see Ronfort, 1999). This expectation was upheld in a study comparing diploid and tetraploid populations of *Chamerion* (= *Epilobium*) *angustifolium*; polyploid populations had a 26% reduction in inbreeding depression relative to diploid populations (Husband & Schemske, 1997).

In contrast to these theoretical expectations, serial generations of selfing in autotetraploid crops often yield greater inbreeding depression than predicted. In fact, magnitudes of inbreeding depression in these cases more closely resemble diploid expectations than those estimated for recessive deleterious alleles in a tetraploid (Busbice & Wilsie, 1966). These conflicting empirical findings can, however, be accommodated by several extensions to the genetic theory. First, the age of the polyploid taxa must be taken into consideration. In contrast to neopolyploids, the genetic load of established polyploids where populations approximate a mutation-selection equilibrium is expected to be greater than diploids with a comparable selfing rate (Ronfort, 1999), although double reduction may slightly decrease this load (Butruille & Boiteux, 2000). Also, it has long been noted that polyploids become increasingly diploid-like over time (Grant, 1981). Second, both the genetic load maintained for a given amount of selfing and the magnitude of inbreeding depression depend on the degree of dominance (Ronfort, 1999). Because tetraploids have three types of heterozygotes, partial dominance results in a more complex set of possible responses. For example, under partial dominance, the proportion of AAaa and AAAa genotypes is expected to increase initially following selfing while the frequency of Aaaa decreases (Ronfort, 1999). Finally, empirical work on crops suggests that the number of different alleles at a locus may be important, as a loss of allelic diversity can explain greater than expected magnitudes of inbreeding depression (Bennett, 1976; Bingham *et al.*, 1994). However, it is difficult to know to what extent results from agricultural plants can be extrapolated to natural populations given that the genomes of crops are likely to reflect recent selection and possible genetic mixing with other varieties.

Genomic studies have recently provided a tremendous breakthrough in our understanding of polyploid evolution. In many ways, the first step toward this richer understanding of polyploidy was the recognition that most polyploid taxa have

multiple origins, enhancing the probability of establishment and the genetic diversity of new polyploid species (Soltis *et al.*, 2004). More recently, rapid genomic rearrangements have been shown to occur following genome duplication in some, but not all, polyploidy lineages (Wendel, 2000; Soltis *et al.*, 2003). This rearrangement may be associated with the elimination of chromosome- or genome-specific sequences and, over longer periods of time, with genomic downsizing (Soltis *et al.*, 2003). Further work has demonstrated substantial gene silencing, the result of both genetic and epigenetic mechanisms following gene duplication, providing molecular insight into the black box of 'diploidization' (Wolfe, 2001; Adams & Wendel, 2005; Scannell *et al.*, 2006). With an understanding of the molecular evolution of polyploids comes the knowledge that regions of the genome may differ as to whether they are effectively polyploid or diploid. While many of these studies have been conducted on allopolyploids or recently synthesized polyploids that have not undergone generations of selection (Song *et al.*, 1995), these mechanisms of evolution are also likely to be found in autopolyploid taxa.

Genomic studies of polyploids suggest that we may need to alter our expectations of the magnitude of inbreeding depression following selfing in polyploid species. In particular, autotetraploid genomes are likely to demonstrate diploid-like inheritance in regions of the genomes that have experienced gene silencing, and tetrasomic inheritance in other areas. Indeed, in a recent review, Ramsey & Schemske (2002) found that tetrasomic inheritance, although predominate, is not ubiquitous even in neopolyploids. This calls into question whether the general rules developed on the assumption of tetrasomic inheritance (e.g. < 50% reduction in heterozygosity following selfing relative to diploids; Bever & Felber, 1992) can be generally applied. Furthermore, multiple origins and the suggestion that most if not all plant taxa have undergone at least one round of genome doubling (Soltis *et al.*, 2003), suggest the age of a polyploid taxa may be an important factor in determining the impact of inbreeding relative to a diploid. At this time, genetic theory of polyploid inbreeding and data from molecular evolution studies far surpass our empirical knowledge of the consequences of inbreeding in polyploid taxa. In particular, for natural autopolyploids there are only four studies to date (Husband & Schemske, 1997; Inoue *et al.*, 1998; Vange, 2002; Galloway *et al.*, 2003), and none has been conducted in a natural environment. Inbreeding depression is frequently stronger in natural environments (Dudash, 1990), and studies are necessary under these conditions to determine the extent to which inbreeding depression is expressed and therefore may influence mating system evolution in autopolyploids.

Here we present results for a study that evaluated inbreeding depression in *Campanulastrum americanum*, a highly outcrossing autotetraploid herb ($t_m = 0.94$ (t_m , multilocus outcrossing rate); Galloway *et al.*, 2003). Hand self and outcross pollinations were conducted in a natural population in three sequential years. The biennial offspring were followed from germination

through seed production permitting lifetime estimates of inbreeding depression. The three cohorts grew in years with a sixfold range in overall performance, permitting an evaluation of whether resource availability influences the expression of inbreeding depression.

Materials and Methods

Study species

Campanulastrum americanum (Campanulaceae) Small (= *Campanula americana* L.) is a monocarpic autotetraploid herb (Gadella, 1964; Galloway *et al.*, 2003). It is insect-pollinated and flowers from mid-July through late August. The primary pollinators are bumblebees foraging for nectar and halictids collecting pollen (Galloway *et al.*, 2002). Flowers are self-compatible but protandrous. Pollinators visit frequently and, as a result, the male phase is brief, with most pollen removed within 3 h of anthesis, leaving flowers functionally neuter (Evanhoe & Galloway, 2002). The stigmatic lobes open the next day and saturation of the stigma with pollen typically occurs in 4–6 h (Evanhoe & Galloway, 2002). Between one and 90 flowers may be open at the same time (Galloway *et al.*, 2002). Geitonogamous selfing may occur when pollinators visit a female-phase flower following a male-phase flower on the same plant. However, most seeds produced are outcrossed because of cryptic self-incompatibility when outcross pollen is present (Kruszewski & Galloway, 2006).

The study was conducted in population of *C. americanum* near the Mountain Lake Biological Station (Rt 700, Giles Co, VA, USA). This population has been the site of previous studies of pollination (Evanhoe & Galloway, 2002; Galloway *et al.*, 2002) and mating system (Galloway *et al.*, 2003; Kruszewski & Galloway, 2006). Seeds may germinate in the fall or in the spring. Seedlings grow as rosettes until the onset of winter. The following spring rosettes bolt and flower. Because winter induces flowering, fall-germinating seeds grow as (winter) annuals while spring-germinating seeds are biennials.

Production of experimental seed

Inbred and outcrossed seeds were created in the field in three separate years, resulting in three cohorts of experimental seed (Fig. 1). Before flowering each year, individuals were haphazardly selected for hand pollination (25, 30 and 45 in cohorts 1, 2 and 3, respectively). Hand pollinations were conducted daily. Neuter flowers were bagged each morning. In the late afternoon, when the bagged flowers had entered the female phase, flowers were hand-pollinated with copious amounts of pollen from the same plant ('self') or from two random plants in the same population ('outcross'). Between eight and 10 crosses of each type were conducted on each plant over approx. 2 wk. At least 50 pollen donors were used in outcross pollinations. Therefore, on average, outcross seeds on a

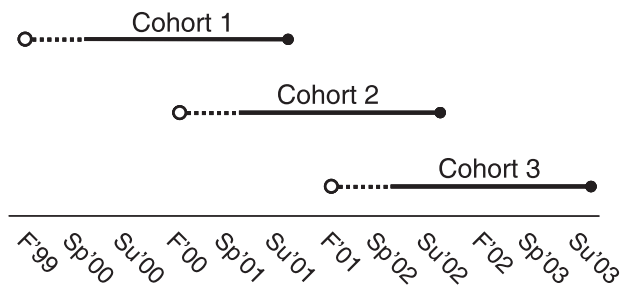


Fig. 1 Schematic of experimental design. A cohort of self and outcross *Campanulastrum americanum* seeds was planted in the fall in each of three consecutive years (open circles). Seeds germinated the following spring (solid lines), flowered their second summer and were harvested following fruit production (closed circles).

maternal plant were half-sibs. Bags were removed the following day when the corollas had wilted indicating successful pollination. Unpollinated control flowers did not set fruit, indicating that the bagging was effective. Fruits from hand-pollinated flowers were collected when the seeds were mature.

Experimental design

Seeds were planted in the natural population within 2 wk of the final fruit collection (Fig. 1). Seeds were selected from a subset of maternal plants with the highest seed production (15 in cohort 1, 20 in cohort 2, and 30 in cohort 3); seeds from a single maternal plant constitute a 'family'. Seeds were planted into 15 blocks (14 in cohort 3). The same blocks were used for all cohorts and were located under the forest canopy throughout the home population in sites within several meters of natural *C. americanum* plants. A single replicate of outcross and of inbred seeds from each maternal plant was planted in each block. In cohort 1, 15 replicates of each mating treatment for each family were planted, one in each block (15 families \times two mating treatments per family \times 15 blocks = 450 replicates). In cohort 2, seeds of each family were planted into 12 or 13 blocks (20 families \times two mating treatments per family \times 12.5 blocks = 500 replicates). In cohort 3, 15 replicates of each mating treatment were also planted for each family and these were evenly divided across the 14 blocks (30 families \times two mating treatments per family \times 15 replicates = 900 replicates).

A group of seeds was planted for each replicate because we expected a small germination percentage (Galloway, 2001). In cohorts 1 and 2, 20 seeds were planted in each replicate for most families. Owing to insufficient seed in cohort 1, five families had 15 or 17 seeds per replicate for one or both mating treatments. In cohort 3, only five seeds were planted per replicate to reduce the need to thin plants following germination. In total, 8215 seeds were planted in cohort 1, 10000 in cohort 2, and 4500 in cohort 3. Seeds were planted

in thick-walled plastic tubes (3.8×15 cm) sunk into the ground 10 cm apart in rows with 15 cm between each row. The tubes were filled with local soil and a 1.5 cm layer of potting mix was placed on top to prevent germination from the seed bank (K. L. Stuble & L. F. Galloway, unpublished). Seeds were sown on the soil surface of each tube approx. 2 wk after harvest and covered with < 0.5 cm of potting mix. In cohort 3, 3.8×15 cm cardboard sleeves were used instead of plastic tubes. The sleeves were placed adjacent to one another, but otherwise the same procedures were used. After planting, the blocks were surrounded by plastic netting to prevent deer herbivory.

Germination, survival and juvenile size were scored for all replicates. Seeds were censused for emergence every 2 wk after planting until snowfall, and again from snowmelt through May. Seedling survival was also scored at each census. Seedlings were thinned two times in the spring, first to two seedlings per replicate, and then to one. Both times seedlings near the center were retained. Most seeds germinated in the spring and plants therefore grew as biennials. Survival was censused monthly from June through November. For censuses in June through September in the first two cohorts, the number of leaves and the length of the longest leaf were recorded. Juvenile size was estimated by multiplying the number of leaves by the length of the longest leaf ('rosette size'). Survivorship was also censused monthly following snowmelt the second summer, but size was not measured as size estimates on bolting individuals confound developmental stage. Using monthly census information, we calculated survivorship for the first summer (rosette), over the winter, and during the second summer through initiation of flowering (bolting). At the onset of flowering, we censused plants every 3 days and recorded the date of first flower. Plants were harvested when flowering ceased and we quantified above-ground biomass and fruit number. We also collected an early fruit before it dehisced and counted the seeds (typically the top fruit on the plant).

We estimated cumulative fitness for each mating treatment by calculating the number of seeds produced for each seed planted. This was done by multiplying proportion germination by proportion survivorship by mean fruit number by mean seeds per fruit. Rainfall during the 5 yr the experiment was conducted was below average, particularly during the late summer and fall, and a number of plants died of drought (data not shown). As a consequence of limited lifetime survival, cumulative fitness was calculated using mean values for a mating treatment across a cohort.

Statistical analysis

The performance of inbred and outcrossed individuals was compared using analysis of variance. A mixed-model ANOVA with cohort, mating treatment, and their interaction as fixed effects and family (nested within cohort), family \times mating treatment, and block as random effects was conducted on

proportion germination (arcsine square root-transformed), flowering date, fruit number ($\ln + 1$ transformed), seeds per fruit (square root-transformed), and above-ground biomass (\ln -transformed). Pairs of adjacent blocks were combined for the analysis of proportion germination. Analyses were conducted using PROC MIXED (SAS, 2003). Rosette size (square root-transformed) of individuals in the first two cohorts was analyzed using a similar model that had been adjusted to account for the repeated measurements on each individual.

Survivorship of inbred and outcrossed individuals was compared throughout the life cycle. Survival was determined over the first summer (rosette), the winter, the second summer (bolting until flowering), and the lifetime (seed to flowering). Lifetime survival underestimates true survival as it ignores seeds that remain in the seed bank (Wardle, 1998), but provides a comparative index of survival between the two mating treatments. Lifetime survival was small in cohorts 2 and 3, with few individuals living to adulthood. Therefore, it was not possible to include family or block in these analyses. Survival data was not collected in the spring for cohort 3, and therefore winter and second summer estimates could not be calculated. The influence of cohort, mating treatment, and their interaction on patterns of survival was conducted using a log-linear analysis assuming a binomial distribution and a logit-link (PROC GENMOD; SAS, 2003).

Reduction in fitness of selfed progeny relative to outcrossed progeny was estimated for each trait by δ , the coefficient of inbreeding depression. δ is equal to one minus the ratio of the fitness of self offspring to outcross offspring ($1 - (w_s/w_o)$). δ was calculated for groups of five adjacent blocks, resulting in three estimates per cohort because sample sizes within each cohort after the first summer were small. Although δ could be estimated for each family for germination and rosette size, the same technique was used for all variables to maintain similar statistical power in all tests. δ for overall fitness was calculated for each cohort. A t -test was used to determine if δ differed significantly from 0.

Results

Outcrossed *C. americanum* outperformed inbred individuals for most traits. Fewer selfed seeds germinated than outcrossed seeds and the mating treatment effect was constant across cohorts (Table 1, Fig. 2). Outcrossed individuals had greater survival over all three life cycle stages (Fig. 2). However the difference was not constant across the life cycle. Outcrossed individuals had a substantial survival advantage when bolting (summer 2), but only a modest, and not quite significant ($P < 0.06$), advantage over inbred plants for first summer and winter rosette stages of the life cycle (Table 2). Although the effect of the mating treatment varied in magnitude, the direction was consistent across these independent stages, and therefore cumulative lifetime survival differed strongly between inbred and outcrossed individuals (Table 2, Fig. 2). Outcrossed

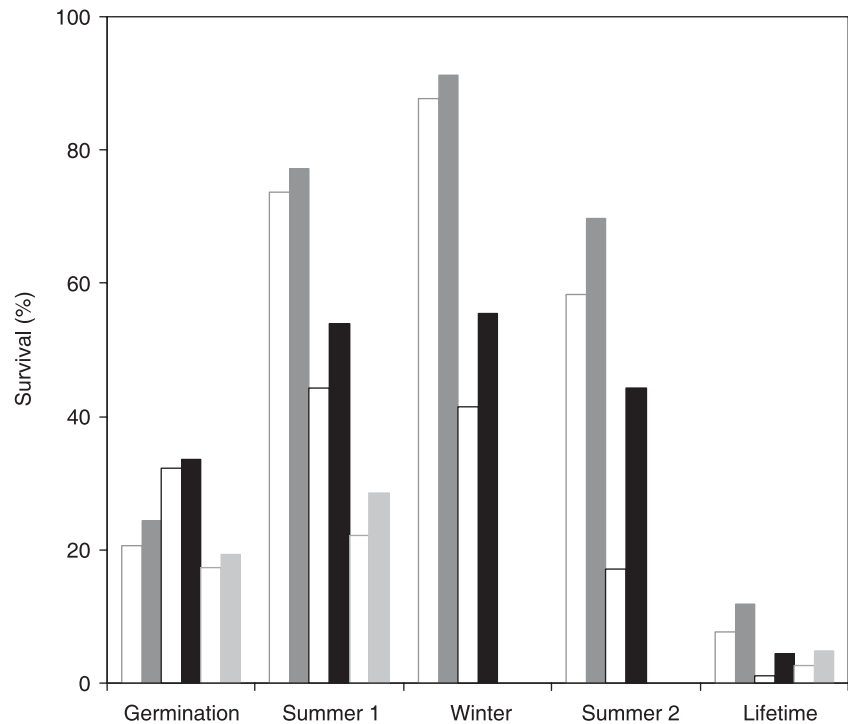


Fig. 2 Germination and survival of three cohorts of inbred (open bars) and outcrossed (closed bars) *Campanulastrum americanum* (cohorts 1–3, left to right). Survivorship is shown for the rosette stage (summer 1), over the winter, and through bolting until the initiation of flowering (summer 2). Lifetime survival is cumulative, including germination. Winter and second summer survivals are missing for cohort 3.

Table 1 Analysis of variance of three annual cohorts of inbred and outcrossed *Campanulastrum americanum* grown in a natural population

Source	Num d.f.	Germination (%)	Flowering date	Fruit number	Seeds/fruit	Biomass
Cohort ^a	2	17.91***	28.21***	7.70**	7.50**	2.63+
Mating type ^a	1	8.00**	5.96*	13.23**	3.93+	13.51**
Cohort × mating type ^a	2	0.49	1.74	1.33	0.45	4.29*
Family(cohort) ^b	–	4.03***	0.42	0.21	0	0
Family(cohort) × mating type ^b	–	0	1.90*	0	0.82	0
Block ^b	–	1.58+	0.46	1.13	1.12	1.75*
Den d.f.	64		24	20	14	19

+, $P < 0.1$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

^aF-value.

^bZ-value.

plants were larger than inbred plants during the rosette stage (Table 3, Fig. 3). Adult vegetative and reproductive traits differed between inbred and outcrossed *C. americanum*. Inbred individuals were smaller, flowered later, and produced fewer fruits that tended to have fewer seeds resulting in lower cumulative fitness than outcrossed plants (Table 1, Figs. 4, 5).

There were substantial cohort effects for most traits. For example, survivorship in the first summer averaged 75% for cohort 1 but only 25% for cohort 3, whereas the mean biomass of plants in cohort 1 was, on average, half that of the other cohorts. Despite large differences among cohorts, there was little difference in performance of inbred and outcrossed individuals across cohorts, that is cohort × mating type was rarely significant. The influence of mating type differed between the cohorts only for biomass where outcrossed plants

outperformed selfed individuals to a greater degree for cohort 3 than for the other cohorts (Table 1, Fig. 4). There is a near-significant interaction between mating type and cohort for lifetime survivorship (Table 2); lifetime survivorship of selfed individuals was only 23% of that of outcrossed in cohort 2, but 65% of that of outcrossed individuals in cohort 1.

There was little evidence for genetic variation among families both in general and in response to the mating treatment. Only germination varied among families (Table 1). However, the effect of mating type varied among families for flowering date and rosette size (Tables 1 and 3), indicating genetic variation for inbreeding depression for these traits.

Although significant inbreeding depression was expressed throughout the life cycle, the magnitude of inbreeding depression was greater for reproductive traits than juvenile traits. Relative

Table 2 Log-linear analysis of survivorship of three cohorts of inbred and outcrossed *Campanulastrum americanum* through the rosette stage (first summer), over the winter, through bolting until the initiation of flowering (second summer), and over the lifetime (germination and vegetative survivorship)

Source	Cohort ^a	Mating type ^b	Cohort × mating type ^a
First summer	135.19***	3.78+	0.39
Winter	102.11***	3.75+	0.15
Second summer	34.04***	12.41***	2.47
Lifetime	83.10***	24.33***	5.90+

+, $P < 0.06$; ***, $P < 0.001$.

^a χ^2 d.f. = 2 except winter and second summer, where d.f. = 1.

^b χ^2 d.f. = 1.

Table 3 Repeated measures analysis of variance of rosette size for two inbred and outcrossed cohorts of *Campanulastrum americanum* grown in a natural population

Source	d.f.	F/Z
Cohort	1.33	35.48***
Mating type	1.33	13.43***
Cohort × mating type	1.33	0.21
Time	3.2315	370.74***
Cohort × time	3.2315	23.16***
Mating type × time	3.2315	0.73
Cohort × mating type × time	3.2315	0.92
Family(cohort)	–	0
Family(cohort) × mating type	–	1.84*
Block	–	2.31*

*, $P < 0.05$; ***, $P < 0.001$.

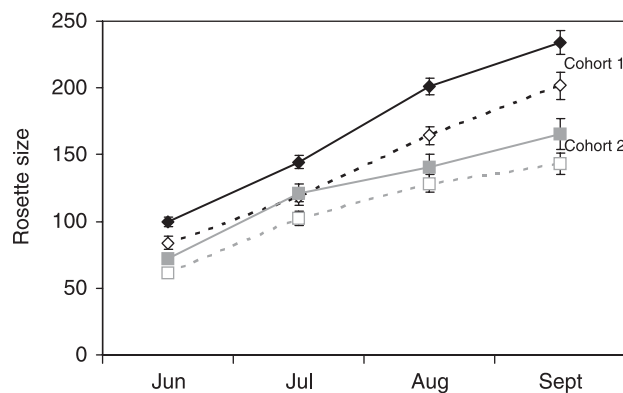


Fig. 3 Rosette size (\pm SE) of two cohorts of inbred (dashed line) and outcrossed (solid line) *Campanulastrum americanum* over their first summer. Rosette size is the number of leaves multiplied by the length of the longest leaf.

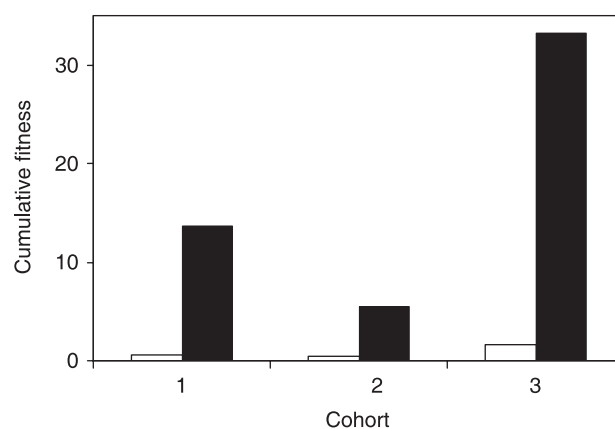


Fig. 5 Mean cumulative fitness of inbred (open bars) and outcrossed (closed bars) *Campanulastrum americanum* grown in nature.

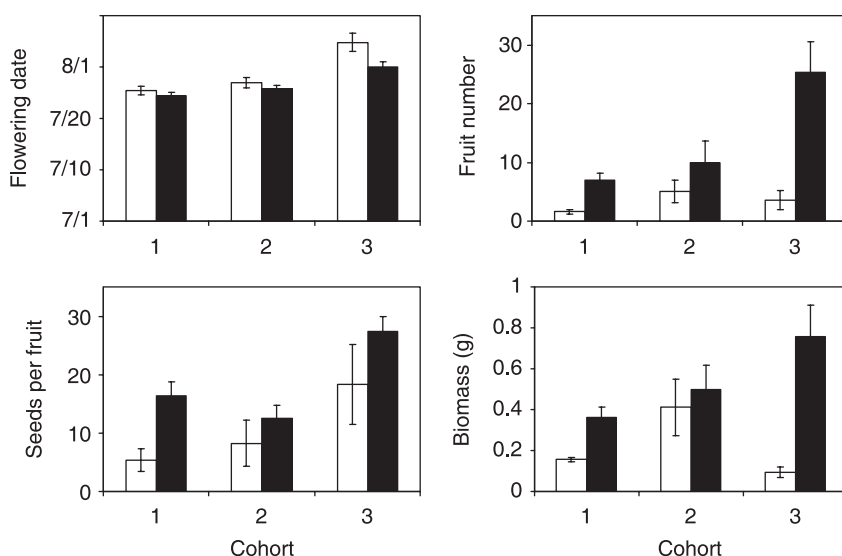
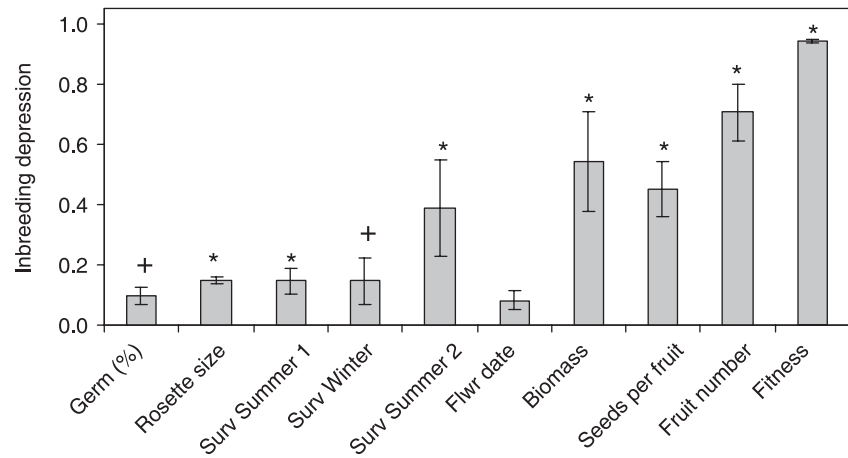


Fig. 4 Means (\pm SE) of adult vegetative and reproductive traits of inbred (open bars) and outcrossed (closed bars) *Campanulastrum americanum* grown in nature. Flowering date, fruit number, number of seeds per fruit, and above-ground biomass are shown for three cohorts.

Fig. 6 Inbreeding depression ($\delta \pm \text{SE}$) of *Campanulastrum americanum* grown in nature. Traits in which inbreeding depression differs significantly from zero ($P < 0.05$) are indicated with an asterisk (*); +, $P < 0.10$; d.f. for these t -tests is 5–8, except for fitness where d.f. = 3. Germ, germination; Surv, survival; Flwr, flowering.



to outcrossed plants, selfed individuals averaged a 15% reduction in germination, rosette size, and survivorship over the first summer and over the winter (Fig. 6). In contrast, the average inbreeding depression for reproductive traits was three times larger. The only exception was flowering date, which had no significant inbreeding depression. Incorporating the whole life cycle, inbred individuals performed very poorly, having on average 6% of the fitness of outcrossed plants (Fig. 6). Despite large cohort effects on many traits, the inbreeding depression of cumulative fitness was very constant over the three cohorts, ranging from only 0.92 to 0.95.

Discussion

There was substantial inbreeding depression in *C. americanum* grown under natural conditions. Inbred individuals had lower germination rates, became smaller rosettes and mature plants, and had reduced survival during bolting. Inbred plants also flowered later and produced fewer fruits with fewer seeds each. Differences in performance between inbred and outcrossed individuals translated into significant or near-significant inbreeding depression for all traits except flowering date. Inbreeding depression for cumulative fitness was very high, with inbred individuals having on average only 6% of the fitness of outcross plants in three separate annual cohorts. This number may even be an overestimate, as male fitness was not measured and often declines with inbreeding (Carr & Dudash, 1997; Good-Avila *et al.*, 2003; Hayes *et al.*, 2005a). Inbreeding depression is the result of the expression of recessive deleterious alleles following inbreeding (reviewed in Charlesworth & Charlesworth, 1999; Carr & Dudash, 2003). Selfing or mixed mating taxa have fewer recessive deleterious alleles as they are expressed by inbreeding and then removed by selection, whereas predominately outcrossing taxa are expected to maintain a substantial genetic load (Lande & Schemske, 1985). The high magnitude of inbreeding depression suggests that inbreeding is infrequent in *C. americanum*. Indeed, a previous study found this same

population of *C. americanum* is almost entirely outcrossing ($t_m = 0.94$; Galloway *et al.*, 2003). Although self-compatible, high amounts of outcrossing are found as a result of cryptic self-incompatibility where outcross sires are favored when flowers are pollinated with both self and outcross donors (Kruszewski & Galloway, 2006). The high magnitudes of inbreeding depression in *C. americanum* match theoretical predictions and studies of other predominately outcrossing taxa (reviewed in Husband & Schemske, 1996; also closely related *Campanula rapunculoides*, Vogler *et al.*, 1999).

The probability of establishment of a novel autotetraploid is enhanced by self-fertilization or mating with relatives who share the same cytotype (Rodriguez, 1996; Baack, 2005; Rausch & Morgan, 2005). *C. americanum* is completely self-fertile; hand outcross and self pollinations result in a near-equivalent number of seeds (cohort 1: $F_{1,21} = 1.02$, $P = 0.32$; see also Galloway *et al.*, 2003). In addition, many plants are large enough that, although protandrous, geitonogamy is common (Galloway *et al.*, 2002; Kruszewski & Galloway, 2006). Therefore self seeds will be produced if outcross pollen is not present. However, the consistently high magnitude of inbreeding depression suggest that the establishment of a population via self seed is unlikely. Cumulative fitness, estimated as the number of seeds produced per seed planted, was less than the replacement value of one for inbred individuals in two of the three cohorts (0.43 and 0.66 for cohorts 1 and 2, respectively) and only slightly above one for the third cohort (1.69). This low fitness of inbred seeds calls into question the effectiveness of *C. americanum*'s flexible mating system that allows for the production of outcross seeds when mates are available and self seeds when conspecifics are scarce.

While the high magnitudes of inbreeding depression found in this study suggest that the establishment of a new population via selfing is unlikely, these conditions were probably not present at the time of polyploidization. Neopolyploids will have a smaller genetic load than diploids if they can better mask single copies of deleterious alleles (Otto & Whitton, 2000), whereas the genetic load of an autotetraploid in mutation-

selection balance is expected to be greater than a comparable diploid (Ronfort, 1999). It is not known whether autotetraploids, including *C. americanum*, are typically in mutation-selection balance. However, it is likely that genetic load and hence inbreeding depression will increase with tetraploid age. There are no known diploid populations of *C. americanum*, indicating that this species is not a neopolyploid and in fact may be closer to mutation-selection balance than taxa with mixed cytotype populations (Husband & Schemske, 1998; Burton & Husband, 1999; Hardy *et al.*, 2000; Weiss *et al.*, 2002). Mixed cytotype populations in *Chamerion angustifolium* (Husband & Schemske, 1998; Husband, 2004), on the other hand, may indicate that tetraploids are young and therefore perhaps less likely to be in mutation-selection balance than *C. americanum*. If so, we might expect less inbreeding depression in tetraploid than diploid populations as found in empirical studies of *C. angustifolium* (Husband & Schemske, 1997). Therefore, while *C. americanum*'s current genetic load may be so large as to make establishment unlikely, it is probable that the load, and hence inbreeding depression, was more modest during the establishment of the species.

High magnitudes of inbreeding depression are not expected in an autotetraploid because the decrease in heterozygosity following a single generation of selfing is less than half that of diploid taxa (Bever & Felber, 1992). However, the increase in homozygosity following inbreeding may be too simplistic to predict inbreeding depression if the expression of inbreeding depression depends on allele dominance (Ronfort, 1999). On average, inbreeding depression is expected to be greater in diploids than autotetraploids; however, under certain conditions of dominance, inbreeding depression in a tetraploid may exceed that of a comparable diploid (Ronfort, 1999). Inbreeding depression in autotetraploids may also be similar to that expected in a diploid if the genome has been diploidized. New autopolyploids typically have tetrasomic inheritance, with segregation among the four alleles at a locus. However, over time, polyploid genomes become diploidized, expressing disomic inheritance. This process of diploidization is not well understood but likely involves gene silencing, changes in gene methylation, and the loss of some duplicated regions (reviewed in Wolfe, 2001; Soltis *et al.*, 2003; Adams & Wendel, 2005; see also Scannell *et al.*, 2006). Diploidization is an ongoing process such that older tetraploid taxa are more diploidized than neotetraploids. Although allozyme markers indicated tetrasomic inheritance in two of seven loci in *C. americanum* (Galloway *et al.*, 2003), other portions of the genome may be effectively diploid, contributing to the high magnitudes of inbreeding depression. High magnitudes of inbreeding depression were also found in a highly outcrossing autotetraploid aster (Inoue *et al.*, 1998), but less for partially selfing autotetraploid *C. angustifolium* (Husband & Schemske, 1997). The fact that *C. americanum* is not a new polyploid supports possible diploidization as a partial explanation for high magnitudes of inbreeding depression.

Inbreeding depression increases throughout the life cycle in *C. americanum*. Although significant or near-significant inbreeding depression was found from germination through fruit production, the magnitude was greater for later life traits. With the exception of flowering date, inbreeding depression of reproductive traits was three times larger than that of vegetative traits. A similar pattern, although with more modest magnitudes of inbreeding depression, was found following selfing in plants from three *C. americanum* populations grown in the glasshouse (Galloway *et al.*, 2003). Small inbreeding depression for early life traits and larger inbreeding depression for later life characters is typical of habitually selfing taxa, whereas outcrossing species typically have high inbreeding depression both early and late in life (Husband & Schemske, 1996). The contrast of these patterns suggests that inbreeding depression in early development may be caused by lethals that are efficiently removed by selection, whereas inbreeding depression in later life traits may be a function of many slightly deleterious alleles (Husband & Schemske, 1996). The life cycle pattern of inbreeding depression in *C. americanum* more closely resembles selfing taxa. This may reflect inbreeding associated with small population sizes expected in the establishment of a novel polyploid or fluctuations in population size that occur in taxa of disturbed habitats. However, relatively high allelic polymorphism suggests this can only be a partial explanation (Galloway *et al.*, 2003). Alternatively, similar to differences in the genetics underlying inbreeding depression at different life stages, dominance patterns may differ among deleterious alleles for early and late life traits, which would result in different patterns of inbreeding depression. More studies of inbreeding depression in autotetraploids are needed to evaluate this hypothesis.

The expression of inbreeding depression was strikingly constant across variable environments. Self and outcross seeds were planted into their natural population in three separate years. Year-to-year environmental variation resulted in strong cohort effects for all traits. For example, the germination rate of cohort 2 was nearly double that of cohort 3, and the cumulative fitness of cohort 3 was six times that of cohort 2. Despite these large differences in performance among years, the effect of inbreeding only differed among cohorts for biomass and there was little variation in inbreeding depression among the three cohorts. A large cohort effect with little change in inbreeding depression is possible because the difference in fitness between self and outcross individuals ($w_o - w_s$) changed in proportion with the fitness of outcrossed individuals. Although $w_o - w_s$ did change among cohorts, transformation of the data before analysis reduced the magnitude of this variation in the analyses. These differences in performance between years are likely the result of temperature or rainfall rather than local environmental conditions, including nutrient availability, since seeds were planted in the same blocks in all years. This suggests that inbreeding depression is robust to annual changes in temperature and rainfall.

Constant inbreeding depression across variable environments contrasts with findings of a number of studies that demonstrate greater inbreeding depression in more stressful environments (reviewed in Armbruster & Reed, 2005; Hayes *et al.*, 2005b; but see Heschel *et al.*, 2005). The sixfold increase in cumulative fitness between cohorts 2 and 3 indicates that cohort 2 experienced more stressful conditions than cohort 3. However, the inbreeding depression for cumulative fitness was statistically indistinguishable between the cohorts. Several studies that have found greater inbreeding depression in stressful environments have compared plants grown in glasshouse and field conditions (Dudash, 1990; Koelewijn, 1998; Ramsey & Vaughton, 1998). Similar to these studies, glasshouse-grown *C. americanum* expressed substantially less inbreeding depression than field-grown plants ($\delta = 0.26$ flowers per seed planted; Galloway *et al.*, 2003). Plants in the glasshouse had a larger average biomass, suggesting that the natural environment was more stressful. It appears that the scale of performance difference between the glasshouse and field was substantial enough to result in differences in the expression of inbreeding depression often seen in other studies.

However, year-to-year variation in environmental conditions within a natural population, although changing lifetime performance sixfold, was not great enough to alter inbreeding depression. Few studies have evaluated temporal variation in inbreeding depression in a single population (Dole & Ritland, 1993; Cheptou *et al.*, 2000; Hayes *et al.*, 2005b). The most direct test found strong year effects on all traits but changes in the effect of inbreeding among years in only two of five traits when *Cucurbita pepo* ssp. *texana* was grown in cultivated fields in four sequential years (Hayes *et al.*, 2005b). Together with our findings, these results suggest caution is necessary when extrapolating variation in inbreeding depression found under experimental conditions to natural populations. Mating system evolution is determined by the magnitude of inbreeding depression expressed in natural populations, and therefore understanding the effect of temporal variation in natural environments on inbreeding depression is critical (cf. Cheptou & Mathias, 2001; Cheptou & Schoen, 2002).

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