

REPORT

Evolution of plant resistance and tolerance to frost damage

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Abstract

Plant defence against any type of stress may involve resistance (traits that reduce damage) or tolerance (traits that reduce the negative fitness impacts of damage). These two strategies have been proposed as redundant evolutionary alternatives. A late-season frost enabled us to estimate natural selection and genetic constraints on the evolution of frost resistance and tolerance in a wild plant species. We employed a genetic selection analysis (which is unbiased by environmental correlations between traits and fitness) on 75 paternal half-sibling families of annual wild radish [*Raphanus raphanistrum* (Brassicaceae)]. In an experimental population in southern Ontario, we found strong selection favouring plant resistance to frost, but selection against tolerance to frost. The selection against tolerance may have been caused by a cost of tolerance, as we provide evidence for a negative genetic correlation between tolerance and fitness in the absence of frost damage. Although we found no evidence for the theoretically predicted trade-off between frost tolerance and resistance among our families, we did detect negative correlational selection acting on the two traits, indicating that natural selection favoured high resistance combined with low tolerance and low resistance coupled with high tolerance, but not high or low levels of both traits together. There were few genetic correlations between the measured traits overall, but frost tolerance was negatively correlated with initial seed mass, and frost resistance was positively correlated with resistance to insect herbivory. Periodic episodes of strong selection such as that caused by the late-season frost may be disproportionately important in evolution, and are likely becoming more common because of human alterations of the environment.

Keywords

Additive genetic variance, Brassicaceae, cost of tolerance, genotypic selection analysis, paternal half-sibling design, plant temperature stress, *Raphanus raphanistrum*, wild radish.

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INTRODUCTION

Beginning with Darwin, evolutionary biologists have sought to understand how natural selection produces adaptations. One goal is to observe on-going evolution and to be able to predict future responses to natural selection. Because adaptive traits are likely to have multiple functions and may be energetically and ecologically costly, the net benefits of each trait and the response to selection in real environments are complex.

In response to any biotic or abiotic stress, organisms may evolve adaptations that provide resistance or tolerance. Resistance traits reduce the level of damage by the stressor, while tolerance traits reduce the negative fitness impact for a

given amount of stress. For example, plant resistance to herbivory may be provided by toxic chemicals that have the effect of reducing insect damage, whereas tolerance may be provided by root storage, which allows regrowth following damage (Strauss & Agrawal 1999). Theory developed by workers studying herbivory has predicted that plants should show a trade-off, or negative correlation, between levels of resistance and tolerance (Van Der Meijden *et al.* 1988; Fineblum & Rausher 1995). The logic behind this predicted trade-off is that natural selection for resistance results in low levels of attack, and hence reduced selection for tolerance. Conversely, organisms with a high level of tolerance should not experience selection for resistance, because attack does not reduce fitness (i.e. the organisms are tolerant). In

addition, costs associated with resistance and tolerance should constrain their maximal expression and will contribute to negative correlations between resistance and tolerance (Van Der Meijden *et al.* 1988; Simms & Triplett 1994; Fineblum & Rausher 1995). Despite these well-developed theoretical predictions, empirical evidence for natural selection favouring higher levels of either resistance or tolerance, but not both traits, remains sparse (Pilson 2000). Such negative correlational selection is predicted for any pair of redundant strategies, including resistance and tolerance to biotic and abiotic stressors.

Here we present empirical evidence supporting two of the major theoretical tenets of theory developed to explain the evolution of resistance and tolerance: fitness costs of tolerance and negative correlational selection acting on resistance and tolerance. We took advantage of a hard late-season frost that damaged natural communities and our experimental population of wild radish [*Raphanus raphanistrum* (Brassicaceae)] in southern Ontario, Canada, to study the evolution of resistance and tolerance to abiotic stress. Although understanding the genetics of plant defence against frost continues to be a goal of breeding programs (Raymond *et al.* 1992; Ogren 1999; Kole *et al.* 2002) and molecular analyses (Artus *et al.* 1996; Thomashow 1999; Smallwood & Bowles 2002), relatively little is known about variation and selection on defence against frost in natural populations (Daday 1965; Artus *et al.* 1996; Inouye 2000). Our results not only illustrate the utility of periodic intense selection events, such as catastrophic frosts, for evaluating major theoretical predictions in evolutionary ecology, but also potential genetic constraints and fitness costs of frost tolerance that may be encountered in agricultural breeding (Hsieh *et al.* 2002).

MATERIALS AND METHODS

Raphanus raphanistrum (Brassicaceae) is a widely distributed, self-incompatible annual plant found in disturbed sites on six continents; the plant was introduced to our study area well over 100 years ago and is naturalized in the community. We bred paternal half-sib families of *R. raphanistrum* from a single wild population in upstate New York (Conner & Via 1993), with the initial goals of studying the evolutionary ecology of floral morphology and plant defence against herbivores (Conner & Via 1993; Agrawal 1998; Agrawal *et al.* 1999; Conner 2002). To examine genetic variation in traits of wild radish, we crossed each of 75 randomly chosen sire plants with three unique randomly chosen dam plants, resulting in 225 full-sib families nested within 75 paternal half-sib families. We removed seeds from dried siliques and weighed each of approximately 10 seeds singly from each of the full-sib families. Initial seed mass was measured because of its strong potential effect on life-history traits of this

species (Mazer 1987). Seeds were planted on May 1, 2001 in 150 mL peat pots filled with unfertilized Pro-Mix soil (Red Hill, PA, USA).

On May 3 (before seedlings emerged), we placed the peat pots in completely randomized positions in a ploughed old field at the Koffler Scientific Reserve at Jokers Hill in southern Ontario, Canada (44°03' N, 79°29' W, <http://www.zoo.utoronto.ca/jokershill>). Plants were not watered in the field. Natural germination frequently occurs during this time period in early spring, especially at sites in which the soil is disturbed. Germination was checked every three days. On the night of May 16, the evening before our first major census of the plots, we experienced an unseasonably hard frost that damaged many of our plants and the surrounding natural vegetation, and allowed us to examine the genetics and evolution of frost tolerance and resistance in our experimental population. Although the seeds were collected in Ithaca, NY, USA and planted outside of Toronto, ON, Canada (approximately 300 km apart), the dates for the last expected frosts are quite similar for the two locations (April 28 in Ithaca and May 9 in Toronto, Old Farmers Almanac, <http://www.almanac.com>).

We censused frost damage on 17 May and herbivore damage on 27 May. Frost damage was indicated by newly wilted and dead tissue. Proportion leaf damage values were assigned by visual estimation; we visually divided each leaf into four quadrants and scored each as damaged or undamaged. By summing over all quadrants and leaves we obtained a rough estimate of the proportion leaf area damaged over the entire plant. Resistance to frost and herbivory were each defined operationally as one minus damage (by the two respective sources). Flea beetles (Chrysomelidae) and lepidopterans were the primary herbivores. Herbivore damage is reported as per cent leaf area removed as above; because herbivore damage was measured 10 days after the frost event, we only assessed post-frost herbivory.

Frost tolerance was estimated for each paternal half-sib family as the slope of the regression of fitness on proportion frost damage, following standard practice (Fig. 1, see for e.g. Mauricio *et al.* 1997; Tiffin & Rausher 1999; Stinchcombe & Rausher 2002). There was no significant variance among sires in the curvature of this fitness-damage regression (sire \times damage² term, $P > 0.30$), so only linear estimates of tolerance were used. Because herbivore damage is measured with some error, it could be argued that use of Model II or major axis regression is warranted (e.g. Sokal and Rohlf 1995). In this approach, the perpendicular distance of each data point to the regression line is minimized, rather than just the vertical distance between each data point and the regression line. Although this is a potentially promising approach for estimating tolerance, we elected not to pursue it in this study because the analytical tools for evaluating the

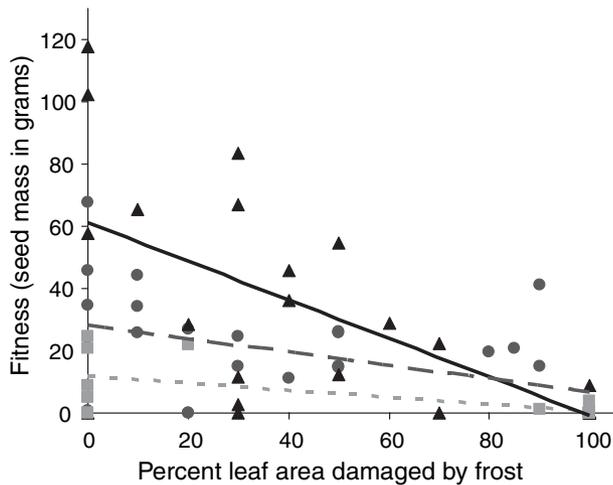


Figure 1 Plant tolerance to frost damage as depicted by the relationship between natural levels of leaf damage and plant fitness. Tolerance is defined as the slope of the regression of fitness on proportion frost damage. Genetic variation in frost tolerance is shown in three representative paternal half-sib families (triangles, circles and squares) out of a total of 75 employed in this experiment. The solid line is the regression through the triangles, dashed is through the circles and dotted is through the squares.

correlation between estimates of fitness in the absence of damage and major-axis regression slopes have not been developed. Examining this correlation is important in testing for costs of tolerance, and the analytical tools are well-developed for the ordinary least-squares approach (Mauricio *et al.* 1997, see below).

Through the season, we measured several traits that may correlate with frost resistance and tolerance: (1) number of leaves (May 27), (2) number of flowers 2 weeks after the first flower opened (June 23), (3) trichome density as the sum of trichomes on the tops and bottoms of 1.75 cm² leaf discs, destructively harvested and counted under a dissecting microscope (June 23), and (4) leaf toughness (June 23), measured by a force gauge penetrometer (Type 516; Chatillon Corp., NY, USA) that measures the grams of force needed to penetrate a surface. Two measures of toughness were taken for each leaf, one on each side of the mid-rib, and these data were averaged and used as a single data point per plant. Toughness was non-destructively measured on intact leaves. Germination time, leaf number and flower number were measured as general indications of plant growth and phenology, while trichome density and leaf toughness may be directly involved in resistance to frost or herbivores (Woodman & Fernandes 1991; Choong 1996; Mauricio & Rausher 1997; Lucas *et al.* 2000).

At the end of the growing season when plants began to shed seeds, we harvested all fruits and dried them in paper bags. Lifetime fruit mass was measured as an estimate of female fitness. Lifetime fruit mass is a good predictor of the

number of seeds produced ($n = 624$, $r^2 = 0.80$, $P < 0.05$; combined data from Agrawal 1998; Agrawal *et al.* 1999).

Analyses

Genetic variances and heritabilities were calculated using restricted maximum likelihood (REML) with the Proc Mixed procedure of SAS (Littell *et al.* 1996). Statistical models included only sire and dam nested within sire (both random effects). Sire breeding values were also estimated with these models using best linear unbiased prediction (BLUP; refer chapter 6 of Littell *et al.* 1996). BLUP estimates are more accurate than sire family means because they use all available information, and thus are not biased by dominance and environmental effects as are family means (Shaw *et al.* 1995). Genetic variation for tolerance to frost was estimated in two ways. First, we used the sire by frost damage interaction term for differences in tolerance (slopes) among sires. In addition, we used a more powerful approach by first calculating a tolerance value for each full-sib (dam) family by regressing damage on fitness; we then determined if there was significant variation among these slopes by using these slopes as the response variable in a one-way ANOVA with sire as the main effect. Genetic correlations among the traits were estimated as Pearson product-moment correlations among the BLUP-breeding values ($n = 75$). Tolerance to herbivory was not analysed in the data set because a large amount of leaf area was damaged by frost (and other plants were killed), thus making it difficult to obtain meaningful estimates of herbivore damage, especially before the frost event, needed to estimate tolerance to herbivores.

Fitness costs of frost resistance and tolerance were estimated by evaluating the correlation between resistance or tolerance and fitness in the absence of damage. Operationally, this corresponds to evaluating the statistical significance of the correlation or covariance between the family mean level of resistance or slopes of damage on fitness (tolerance) and the intercepts from those regressions (an estimate of fitness in absence of frost damage) for the 75 paternal half-sib families. Estimating costs of tolerance by this method, however, introduces a statistical bias because the slope and intercept of an individual quantitative genetic family are estimated from the regression analysis using the same data (Mauricio *et al.* 1997). As such, an artefactual covariance exists between these two estimates that inflates the estimated covariance for tolerance. To address this problem, we used statistical methods that have been developed specifically for this problem (e.g. Appendix B of Mauricio *et al.* 1997 and Appendix B of Tiffin & Rausher 1999) to calculate and remove the artefactual covariance between tolerance and fitness in the absence of damage. To test the statistical significance of the corrected covariance

between these two terms we determined whether the 99% confidence limits for the corrected covariance, calculated from 1000 bootstraps, overlapped zero (Stinchcombe 2002; Agrawal *et al.* 2004).

To estimate natural selection on frost and herbivore resistance and tolerance traits, we used the regression approach developed by Lande & Arnold (1983), which provided biologists with the statistical tools needed to measure selection simultaneously on multiple traits. Estimates of phenotypic selection, however, may not be reflected in responses to selection because of biases introduced by environmental covariances (Rausher 1992; Stinchcombe *et al.* 2002). Where an environmental factor affects both a trait value and relative fitness (e.g. resource availability affects body size and fecundity), the causal link between phenotype and fitness is uncoupled and a bias may be introduced in the measurement of selection. One solution to the problem of environmental covariances in estimates of selection, which we use here, is to conduct similar analyses as those proposed by Lande and Arnold, but on additive genetic breeding values (Rausher 1992). Although this method is limited by sample size (i.e. the number of genetic families is the unit of replication, not the individual) and the ability to conduct controlled crosses (i.e. paternal half-sib analysis is the best way to estimate breeding values), it controls for environmental covariances, even when the sources of the environmental bias are unknown (Rausher 1992). To date, relatively few studies have employed such genetic selection analyses on paternal half-sibs (Rausher & Simms 1989; Tiffin & Rausher 1999; Scheiner *et al.* 2002).

Genetic correlations between the traits and fitness estimate the strength of total selection on breeding values, including both direct selection and indirect selection caused by genetic correlations (analogous to selection differentials on phenotypic data), and were estimated as Pearson product-moment correlations among the BLUP breeding values for the trait and fitness. Selection gradients measure the strength of direct, adaptive selection, after correcting for correlations among measured traits. We implemented Rausher's (1992) selection gradient method by using the BLUP breeding value estimates for fitness and all of the measured phenotypic traits. Relative values of the breeding value estimates of fitness were regressed on unstandardized breeding value estimates for the traits. Directional selection gradients were estimated in models containing the linear terms only, while quadratic selection gradients (which estimate curvature of the fitness function) were estimated as two times the quadratic regression coefficients in a model containing both linear and quadratic terms (Lande & Arnold 1983). These models also included one cross-product term between frost resistance and tolerance to examine the *a priori* hypothesis that selection favours specific combinations of

these traits because of their redundancy (see Introduction). Other cross-product terms were not included because of limited sample size.

A potential statistical artefact can arise in the estimation of selection on tolerance. Often there will be a strong positive correlation between mean and variance in fitness across paternal half-sibling families – i.e. families with low mean fitness have low variance in fitness, and those with high mean fitness have high variance in fitness. In our dataset this correlation was 0.81 ($P < 0.001$) and remained high even after log-transformation ($r = 0.80$, $P < 0.0001$). Therefore, those families with low mean fitness are constrained by their low variance in fitness to have flatter slopes in the linear regressions estimating tolerance, and will be estimated as highly tolerant. In these situations, it is difficult to determine if a pattern of natural selection against tolerance is indeed real – that is, because these families are tolerant and tolerance has a high fitness cost. Alternatively, selection against tolerance could be an artefact of low vigour families, with low fitness across a range of environments, being mistakenly estimated as tolerant simply because their low variance in fitness constrains the slope of fitness on damage. To address this problem, we repeated the selection analyses leaving out the 25 or 50 families (out of 75) with the lowest fitness variance. This technique reduces bias by excluding the families whose fitness variance is most likely to constrain their estimated slopes (tolerance); however, this approach also limits statistical power by reducing sample sizes. Results of these analyses using exactly the same selection gradient model as the full analysis indicated that there was still highly significant selection against tolerance (see Results). Thus, we provide strong evidence that the selection against tolerance reported below is not an artefact, because the highest fitness variance families that remained in the analysis with only 25 families are not constrained to any particular slope (tolerance).

We also employed this 'subset method' to test for artefacts in the measures of the cost of tolerance (see Results). In addition, we pursued an alternative approach to evaluate whether mean-variance correlations were affecting our conclusions: we standardized the fitness of each family to a mean of zero and variance of one (Z -scores) prior to estimating tolerance, thus eliminating any differences between families caused by differences in mean fitness or fitness variance. The Z -score estimates of tolerance were highly correlated with our original estimates ($r = 0.67$, $P < 0.001$), and we still obtained significant directional selection against tolerance ($P = 0.03$) in the same selection gradient model as was used for the other analyses. None of our alternative selection gradient analyses produced qualitatively different results, i.e. all factors that were significant in the main analysis stayed significant and none that were not significant became significant.

RESULTS

In our study population, 92% of the seeds we had planted germinated in the field and nearly 26% of the emergent seedlings were completely defoliated (most killed) by the frost (mean \pm SE per cent frost damage: 49 ± 1). Less than 1% of the germinants (16 plants) emerged after the frost event. Surrounding native vegetation, such as new growing shoots of *Rhus typhina*, also experienced heavy frost damage. Later in the season, post-frost insect herbivory on plants was substantial, with a mean \pm SE of 24.0 ± 0.7 per cent leaf damage.

We found heritabilities significantly different from zero for only a few traits: germination time, leaf number, trichome density and early flower number (Table 1). The heritability of frost tolerance was marginally significant ($P = 0.06$) using the more powerful test of differences among sires in the full-sibling slopes, and this remained nearly as significant ($P = 0.08$) when initial seed weight was included as a covariate. In an ANCOVA for fitness, with sire, dam nested within sire [dam(sire)], frost damage, frost damage \times dam(sire) and frost damage \times sire interactions, we failed to detect a significant frost damage \times sire interaction [$P = 0.29$, testing the frost damage \times sire interaction over the frost damage \times dam(sire) mean square]. Nevertheless, the tests for variation among sires in the full-sibling slopes, as well as our analyses of the genetic correlations between frost tolerance and other traits, costs of frost tolerance and natural selection on tolerance, all suggest that frost tolerance was genetically variable in our study population.

With the exception of initial seed mass, the other traits had heritabilities near zero, but most of these traits had substantial amounts of additive variance as measured by the coefficient of additive variance (CV_A ; Table 1) (Houle 1992). The low heritabilities and lack of statistical significance result from the very large error variances, which

reflect the large amount of environmental variance (V_E) in the field, especially when the error variances are much larger than the dam variance components, as they are here (Falconer & Mackay 1996). In addition, for three traits (initial seed mass, frost resistance and lifetime fruit mass) the dam variance component was about an order of magnitude larger than the sire variance component, indicating the strong influence of non-additive genetic and/or maternal effects on the phenotypic variance of these traits. Not surprisingly, maternal effects were strongest for seed mass, the only trait for which the dam variance component was larger than the error variance component. This is solid evidence for maternal effects rather than non-additive genetic variance because there is less dominance variance in the dam variance component than in the error variance component (Falconer & Mackay 1996, p. 167).

Costs of resistance and tolerance

Frost resistance and tolerance showed different patterns of costs. Frost resistance (one minus proportion leaf damage) showed no significant correlation with fitness in the absence of damage ($n = 75$, $r = -0.13$, $P = 0.25$). In contrast, frost tolerance (slope of the regression of fitness on proportion frost damage, Fig. 1) showed a highly significant negative correlation with fitness in the absence of damage ($n = 75$, $r = -0.97$, $P < 0.0001$; Fig. 2). This result indicates that plant genotypes tolerant of frost damage (i.e. less affected in terms of fitness than less tolerant genotypes) pay a cost that is realized in the absence of frost damage. Of the estimated covariance between fitness in the absence of damage and frost tolerance (-162.95), a substantial portion (approximately 39%) was because of the statistical artefact introduced by estimating these parameters from the same regression for each paternal half-sib family. Nevertheless, the corrected covariance (-99.79) is significantly different from zero (99% CI: -102.7 to -86.9), indicating that more

Table 1 Descriptive statistics, variance components (VC) and heritabilities

Trait	<i>n</i>	Mean	SEM	CV_A	Sire VC	Dam VC	Error VC	b^2
Initial seed mass (mg)	2014	4.95	0.04	16.96	0.18	2.39	1.12	0.19
Germination time (days)	1857	6.62	0.06	14.93	0.24	0.86	4.91	0.16*
Leaf number	1162	3.46	0.03	13.58	0.06	0.03	0.75	0.26***
Frost resistance	1857	0.51	0.01	11.49	0.0008	0.01	0.17	0.02
Frost tolerance	1857	-0.357	0.02	46.32	0.007	-	0.06	0.41(*)
Herbivore resistance	1229	0.76	0.01	3.84	0.0002	0	0.06	0.01
Trichome density	1162	75.75	1.39	26.55	101.10	165.69	1981.21	0.18*
Leaf toughness	1162	68.36	0.45	4.01	1.87	3.79	233.50	0.03
Early flower number	1159	15.32	0.77	65.48	25.16	11.13	649.19	0.15*
Lifetime fruit mass	2014	18.59	0.63	21.28	3.91	47.76	723.23	0.02

CV_A is the coefficient of additive genetic variation or evolvability (Houle 1992).

Significance levels for heritabilities are from log-likelihood tests of the sire variance component. * $P < 0.05$; *** $P < 0.001$; (*) $P = 0.06$.

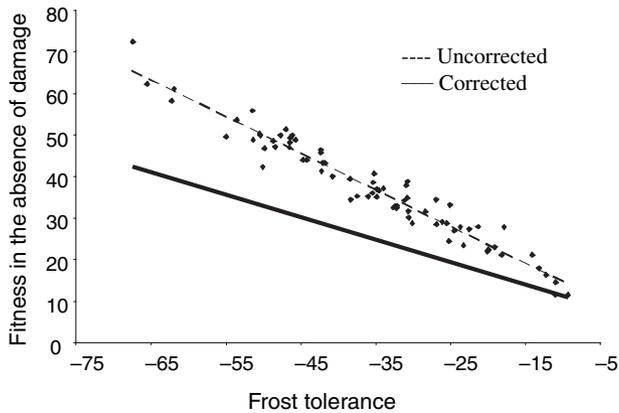


Figure 2 Fitness costs of tolerance to frost damage for the 75 paternal half-sib families. Each data point represents the estimate of the slope (tolerance) and y -intercept (fitness in the absence of damage) from a regression of fitness on damage for that paternal half-sib family. Because the two plotted variables are estimated with the same linear regression, the covariance between them was corrected for this source of bias (see Materials and methods). For heuristic purposes, we portray the corrected covariance between these two parameters as a regression line (solid line), calculated with the same intercept and variance in the independent variable as one would obtain from a simple regression with uncorrected data (dashed line). As such, the covariance between x and y is the only term that differs between the regression lines.

tolerant paternal half-sib families have lower fitness in the absence of frost damage – i.e. that tolerance is costly (Fig. 2).

Just as with the estimates of selection on tolerance (see Materials and methods), there is a possible artefact in this cost-estimate because of families with low mean and variance in fitness being constrained to low slope and intercept in the regression of fitness on proportion frost damage. We attempted to address this possibility in the same ways as for selection on tolerance: (1) by estimating costs with only the 50 sire families with the highest variance in fitness, (2) by estimating costs with only the 25 families with the highest variance in fitness, and (3) with all of the data, but with tolerance estimated for each family after fitness had been transformed to a mean of zero and variance of 1 (Z -scores). Results of these analyses were equivocal – we found evidence of significant costs of tolerance using the 50 families with the highest variance in fitness (corrected covariance = -17.80 , 99% CI: -20.51 to -9.01). However, we failed to detect significant costs in analyses using the top 25 families or with tolerance estimated from Z -scores (top 25: corrected covariance = 20.41 , 99% CI: 14.1 – 37.1 ; Z -score estimates: corrected covariance = 0.0072 ; 99% CI: 0.004 – 0.03). As such, although our data provide some evidence for a fitness cost of tolerance, we cannot exclude the possibility that

our finding of a cost is a result of the correlation between the mean and variance of fitness.

Genetic selection on resistance and tolerance

In our genetic selection analysis, we found strong directional selection for larger initial seed mass (Table 2). The simple genetic correlation between fitness (lifetime fruit mass) and initial seed mass was also positive and highly significant, in agreement with the directional selection gradient. Therefore, in addition to the fact that strong maternal effects on seed mass have been found in this and other studies (Mazer 1987), our genetic selection analysis predicts that this trait could also evolve to larger values in nature.

We found directional selection for increased plant resistance to frost and herbivory, and for decreased tolerance to frost (Table 2). The marginally significant quadratic term for frost tolerance was because of a single family: when this one family is excluded from the analyses, the quadratic term is not significant ($P = 0.46$), although the rest of the model remains nearly identical. Results of selection analyses removing the 25 or 50 families (out of 75) with the lowest fitness variance indicated that there was still highly significant selection against tolerance ($P < 0.001$ for both), suggesting that selection against tolerance was not an artefact of the correlation between mean and variance in fitness. These findings of natural selection against tolerance suggest that the costs of tolerance described previously are indeed biologically significant, and that the costs of tolerance outweighed the benefits in this experiment.

We detected highly significant negative correlational selection on frost resistance and tolerance after removing the effects of the directional selection on these traits (Table 2, Fig. 2). Selection thus favoured combinations of traits, indicating that frost resistance and tolerance were alternatives: the combinations of high frost resistance and low tolerance and low resistance and high tolerance resulted in higher fitness than when both resistance and tolerance were high or both were low. Note that the directional selection on these traits indicates that plants with high resistance and low tolerance had higher fitness than plants with low resistance and high tolerance.

Although we measured several traits as life-history correlates or possible mechanisms of resistance and tolerance, we found little selection on these traits (Table 2) and few genetic correlations among them (Table 3). Indirect selection favoured later germinating seeds and larger plants, both of which were correlated with initial seed mass.

DISCUSSION

We detected directional selection for increased plant resistance to frost and herbivory, yet decreased tolerance

Table 2 Additive genetic selection analysis (Rausher 1992) and additive genetic correlations between the traits and fitness

Trait	β	SE	P	γ	SE	P	r_A	P
Initial seed mass	0.06	0.02	0.002	-0.22	0.18	0.22	0.45	0.0001
Germination time	-0.01	0.01	0.31	0.01	0.05	0.84	-0.29	0.01
Leaf number	0.01	0.02	0.56	0.06	0.18	0.74	0.35	0.002
Frost resistance	1.84	0.43	< 0.0001	-49.6	57.8	0.39	0.37	0.001
Frost tolerance	-0.002	0.0002	< 0.0001	0.0001	0.00003	0.03	-0.73	< 0.0001
Herbivore resistance	2.40	0.99	0.02	341.0	407.1	0.41	0.36	0.001
Trichome density	-0.0002	0.0006	0.76	0.0001	0.0001	0.64	-0.05	0.70
Leaf toughness	0.01	0.007	0.13	-0.01	0.02	0.56	0.22	0.06
Early flower number	-0.002	0.001	0.08	0.001	0.0006	0.17	0.16	0.17
Frost resist \times tolerance				-0.11	0.03	0.0002		

The β and γ columns are the linear and quadratic selection gradients, respectively, calculated using best linear unbiased prediction (BLUP) estimates of breeding values. The linear coefficients were determined from the first-order model only ($R^2 = 0.76$, $P < 0.0001$), while the second-order coefficients were determined from the full model with the linear, squared and cross-product terms ($R^2 = 0.85$, $P < 0.0001$). The r_A column represents the additive genetic correlations between the trait and fitness, estimated as Pearson product-moment correlations between BLUP breeding values. $n = 75$. Significant selection gradients and correlation coefficients are shown in boldface.

Table 3 Additive genetic correlations (r_A) among traits calculated as Pearson product-moment correlations among best linear unbiased prediction (BLUP) estimates of breeding values ($n = 75$)

	Initial seed mass	Germination time	Leaf number	Frost resistance	Frost tolerance	Herbivore resistance	Trichome density	Leaf toughness
Germination time	-0.40***							
Leaf number	0.30**	-0.30**						
Frost resistance	0.05	0.00	0.45***					
Frost tolerance	-0.32**	0.19(*)	-0.13	-0.01				
Herbivore resistance	-0.06	-0.03	0.14	0.26*	-0.19(*)			
Trichome density	-0.24*	0.14	-0.05	0.20(*)	0.03	-0.04		
Leaf toughness	0.06	-0.06	0.06	0.11	-0.10	0.04	0.10	
Flower number	0.21(*)	-0.12	0.31**	0.34**	-0.17	0.01	0.09	-0.03

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; (*) $P < 0.10$.

to frost (Table 2). The directional selection for decreased tolerance is perhaps surprising at first glance. However, this result is consistent with our (somewhat equivocal) evidence for a cost of tolerance (Fig. 2). The selection for increased frost resistance is intuitive in light of the lack of significant costs of resistance. Thus, the qualitative pattern from these main effects is that selection is favouring one, but not the other defence strategy against frost damage. Our evidence for selection against tolerance is from a selection gradient analysis (Table 2), which removes the indirect selection caused by correlations with other traits in the analysis. Therefore, this selection against tolerance cannot be caused by correlations with the other traits included in the analysis such as seed mass and germination time.

Direct and indirect selection on seed mass (Stanton 1984; Mazer 1987; Gomez 2004; Verhoeven *et al.* 2004) and germination time (Gonzalez-Astorga & Nunez-Farfan 2000; Donohue 2002; Griffith *et al.* 2004) have been

shown to be of evolutionary importance in other studies. For example, selection for larger seeds may lead to increased competitive ability and resistance to abiotic stress, but may also result in higher levels of seed predation (Gomez 2004; Verhoeven *et al.* 2004). Geographic variation in climatic patterns has resulted in population differentiation in seed germination times as extreme as winter vs. summer annual life-histories in naturalized populations of *Arabidopsis thaliana* (Donohue 2002; Griffith *et al.* 2004). We found strong genetic selection on seed mass in our study, but no significant direct selection on germination time (Table 2), so we would predict adaptive evolutionary change in seed mass but not germination time in the near future.

Of the genetic correlations we detected (Table 3), perhaps the most interesting is the correlation between resistance to frost and resistance to herbivory. This suggests the possibility that there are shared underlying traits causing

resistance to both frost and herbivory; however, none of the traits we measured are likely to be one of these shared traits, as none were correlated with both frost and herbivore resistance (Table 3). The strong positive correlation between frost resistance and leaf number is likely caused only by the fact that leaf number was measured 11 days after the frost, so that those plants that were least damaged by the frost had the most leaves. In addition, there was a marginal correlation between trichome density and frost resistance, suggesting that trichomes may serve a protective function. Frost tolerance was negatively correlated with initial seed mass. As indicated above, however, this correlation is accounted for in the selection gradient analysis, so that the selection against tolerance cannot be due to indirect selection through seed mass.

Resistance and tolerance as alternative strategies

Our result of finding negative correlational selection on frost resistance and tolerance further indicates that resistance and tolerance may be alternative strategies. Selection appeared to act the most strongly against combinations of high frost tolerance coupled with high frost resistance and low frost tolerance combined with low frost resistance (Fig. 3). These data provide thus provide convincing support for the prediction that tolerance and resistance are redundant strategies against environmental stresses (Van Der Meijden *et al.* 1988; Simms & Triplett 1994; Fineblum & Rausher 1995).

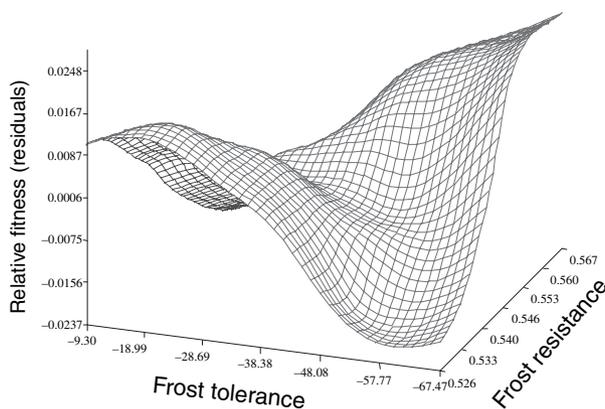


Figure 3 Fitness surface depicting the correlational selection on resistance and tolerance to frost damage. Relative fitness is estimated as the residuals from a regression that contained all terms in the full quadratic model (Table 3) except the cross-product between resistance and tolerance. Thus, the surface only shows the correlational pattern of selection and does not include the directional selection on these traits. The surface was generated in PROC G3GRID (SAS 1999) using a spline interpolation with a smoothing coefficient of 0.3.

The negative correlational selection for frost resistance and tolerance has yet to result in the evolution of a negative additive genetic correlation between these traits (Table 3). It is possible that such relationships are only favoured by intense selection episodes that are relatively infrequent; the frequency of selection has been shown theoretically to be important in the evolution of complex traits (Gomulkiewicz & Kirkpatrick 1992). Nonetheless, our study measures selection on frost resistance and tolerance, and it is unknown how common this correlational selection may be in nature. Climate change is expected to increase the frequency and intensity of frosts in some habitats (Inouye 2000), and thus intense selection events, such as that observed here, could become increasingly important.

Evolution of resistance and tolerance

Theory on the alternate evolution of resistance vs. tolerance has largely emerged from the literature on plant–herbivore interactions (Van Der Meijden *et al.* 1988; Simms & Triplett 1994; Fineblum & Rausher 1995; Strauss & Agrawal 1999; Tiffin 2000; Weinig *et al.* 2003). For *A. thaliana*, Mauricio *et al.* (1997) reported natural selection on two resistance traits and tolerance to herbivory, but failed to detect negative correlational selection on resistance and tolerance. In similar fashion, working with the Landsberg erecta × Columbia recombinant inbred lines of *A. thaliana*, Weinig *et al.* (2003) also failed to detect negative correlational selection on resistance and tolerance to rabbit herbivory. Tiffin & Rausher (1999) did not detect direct selection on tolerance to herbivory in *Ipomoea purpurea*, although tolerance was costly and correlational selection on tolerance and resistance was present. The pattern of correlational selection described by Tiffin & Rausher (1999) was complex: both positive and negative correlational selection gradients were detected, and negative correlational selection was only found for tolerance to one type of damage and resistance to another. Thus, the theoretical prediction that selection should favour either resistance or tolerance to the same type of damage, but not both, has only received modest support in plant–herbivore studies (see also Pilsen 2000).

Despite the fact that periodically strong selection events are difficult to predict and study, we serendipitously were able to estimate selection because of a late-season frost. This event imposed strong natural selection to increase frost resistance and decrease frost tolerance, likely because of the undetectable cost of resistance and a high cost of tolerance. Selection also favoured a negative correlation between these traits, consistent with theoretical predictions based on the redundancy of the two strategies. Periodically intense selection events, such as this catastrophic frost, are likely an important component of the ecology and evolution of natural populations (Bumpus 1899; Grant & Grant 1993;

Spiller & Agrawal 2003) and are becoming increasingly common because of large environmental perturbations caused by human activities (Inouye 2000; Reznick & Ghalambor 2001).

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