Ecology and Evolution

Testing potential selective agents acting on leaf shape in *Ipomoea hederacea*: predictions based on an adaptive leaf shape cline

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Abstract

Leaf shape is a highly variable phenotype, and is likely influenced by many sources of selection. *Ipomoea hederacea* exhibits an adaptive latitudinal cline in leaf shape, which is controlled by a single Mendelian locus: lobed individuals dominate the north with entire-shaped individuals mostly in the south. We test if the following candidate selective agents, suggested by the literature, are responsible for the cline: differential insect herbivory, genetic correlations with other clinal traits like flowering time and growth rate, and thermoregulatory differences. We planted 1680 F_3 individuals, segregating for leaf shape, in the north of *I. hederacea*'s range, where we expected lobed genotypes to have higher fitness. Individuals were assigned to insect removal or control treatments, and we scored herbivory, flowering time, growth rate, leaf temperature, and fitness (seed number). Herbivory, flowering, and growth rate had significant fitness effects, but none differed between leaf shapes. Lobed leaves were consistently warmer at night, but no performance advantage was detected. Finally, we detected no overall fitness differences between leaf shape genotypes, whether we controlled for other traits under selection or not. Our data suggest these candidate selective agents may not be important contributors to the cline, and alternative approaches to understanding the mechanisms maintaining the leaf shape cline in *I. hederacea* may be necessary.

Introduction

Isolating the agents of selection acting on ecologically important traits is a central goal in evolutionary ecology (Wade and Kalisz 1990; MacColl 2011). Identifying the causes of selection often poses a significant challenge because the connection between a phenotype and fitness is typically obscured by the many ecological sources of natural selection that could be occurring (MacColl 2011). One method to establish the possible agents of selection is to identify environmental variables (abiotic or biotic) that correlate with the trait of interest, and perform experiments that directly manipulate the putative agent of selection, demonstrating the fitness consequences of variation in that trait (e.g., Simms and Rausher 1989; Wade and Kalisz 1990). Manipulation of selective agents in experimental studies has been particularly useful for understanding the forces leading to temporal or geographical patterns of traits (see for e.g., Kettlewell 1956;

Cook et al. 2012). Here, we examine whether four different candidate selective agents – all suggested by previous studies – are operating, separately or in combination, in a way that maintains an adaptive latitudinal cline in leaf shape in ivyleaf morning glory, *Ipomoea hederacea*.

Ipomoea hederacea exhibits a leaf shape polymorphism whereby individuals either express lobed or entire-shaped leaves depending on their genotype at a single Mendelian locus (Elmore 1986; Bright 1998). Although the molecular genetic basis of leaf shape is unknown, crosses between entire-shaped and lobed individuals consistently show lobing to behave as a single Mendelian locus (Elmore 1986; Bright 1998; B. E. Campitelli, pers. obs.), with heterozygotes showing intermediate lobing (Fig. 1A); we refer to each leaf shape genotype as entire-shaped, heterozygote, or lobed. Bright (1998) first documented a latitudinal cline for leaf shape in the eastern United States (US), where the southern range contains a mixture of monomorphic entire-shaped populations and polymor-
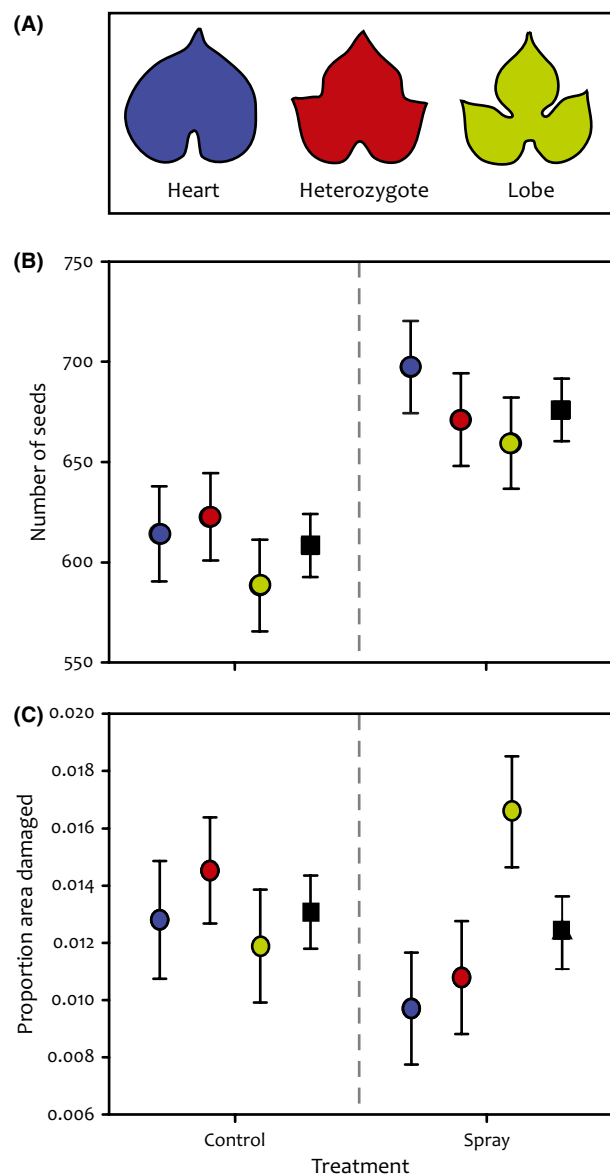
Figure 1. (A) Example illustrations of the three leaf shapes of *Ipomoea hederacea*. The colors of the leaves represent the color scheme that is employed throughout this paper, (B) Mean (lsmeans ± 1 SE) seed production, and (C) mean (lsmeans ± 1 SE) leaf area damaged in the control and insect removal (spray) treatments. Circles represent the three leaf shapes separately, and the black squares represent the overall mean for all plants.

phic populations, and northern populations are predominantly lobed. Bright and Rausher (2008) further showed that natural selection acts on leaf shape in *I. hederacea* in experimental populations just south of the putative cline center in North Carolina; they detected directional selection consistent with the cline in 1 year, and balancing selection the following year. Bright (1998) also showed that white rust fungus imposes selection on leaf shape;

however, the direction of selection opposed the clinal pattern (i.e., entire-shaped plants were most affected by the fungus). Previously, we examined whether the leaf shape cline could be nonadaptive in origin by comparing patterns of clinal variation at the leaf shape locus to a set of putatively neutral loci; we found strong evidence that the cline was maintained by selection and that the leaf shape locus was a genomic outlier (Campitelli and Stinchcombe 2013). Our analysis further showed that the center of the cline – where most of the transition in leaf shape occurs – is near the North Carolina/Virginia (US) border, extending westward. In the current study, we evaluate the contribution of a series of hypothesized selective agents and potential mechanisms that may be responsible for the predominance of lobed genotypes in Northern populations.

Insect herbivory

Several studies have demonstrated that insect herbivores can potentially impose selection on leaf shape (reviewed by Brown et al. 1991): for example, Rausher (1978) showed that female *Battus philenor* butterflies search for specific leaf shapes to oviposit on, potentially leading to the divergence in leaf shape in its two host species. Insect herbivores impose differential leaf damage to the two homozygous leaf shape genotypes of *I. hederacea,* suggesting that insects could be an important selective agent on leaf shape variation in *I. hederacea* (Campitelli et al. 2008). Latitudinal gradients in insect herbivory have been reported (e.g., Adams and Zhang 2009; Moles et al. 2011); however, for insect herbivores to be a selective agent contributing to the leaf shape cline, a prerequisite is that they impose differential selection on leaf shape. Our first hypothesis is that insect herbivores impose selection on leaf shape, such that lobed genotypes are favored north of the clinal boundary.

The leaf shape cline is a correlated response to selection on other traits

Another possibility is that the leaf shape cline is not due to direct selection on leaf shape per se, but instead that it reflects a correlated response to selection on other clinally varying traits. For example, it is widely accepted that flowering phenology can have major fitness consequences (reviewed by Elzinga et al. 2007; Harder and Johnson 2009; Munguía-Rosas et al. 2011), and two lines of evidence suggest that flowering time is under clinally varying selection in *I. hederacea*, presumably because of differences in season length. First, Simonsen and Stinchcombe (2010) detected strong directional selection favoring earlier flowering genotypes in an experiment carried out north of *I. hederacea*'s current range. Second, two other

independent experiments have detected significant latitudinal clines for flowering time such that northern populations tended to flower earlier (Klingaman and Oliver 1996; A. J. Stock, B. E. Campitelli, and J. R. Stinchcombe, unpubl. data). Because of the evidence of clinal variation in flowering time (over the same geographic range as the leaf shape cline) and the importance of flowering time for fitness generally and in this species, it is necessary to determine if the leaf shape cline is due to the ecological and physiological consequences of leaf shape directly, or because of correlations between leaf shape and other clinally varying traits. If flowering time is genetically correlated with leaf shape – through either linkage disequilibrium or pleiotropy – leaf shape may be evolving as a correlated response to selection on flowering phenology (Lynch and Walsh 1998). Accordingly, our second hypothesis is that the leaf shape cline is an indirect result of selection on phenology by season length.

Higher growth rates are typically correlated with an increased reproductive capacity in herbaceous plants (e.g., Aarssen and Taylor 1992). Simonsen and Stinchcombe (2010) observed marginally significant positive selection for larger midseason plant size, suggesting that growth rate may also be important for fitness in *I. hederacea*. In addition, they detected correlations between flowering time and growth rate, further suggesting that growth rate may influence seed output through its effects on flowering phenology. Our third hypothesis is that individuals with different leaf shape genotypes may express differential growth rates, as a result of a potential genetic correlation, and thus the leaf shape cline is a correlated response to selection on growth rate (again, presumably due to changes in season length across the range).

Thermoregulatory differences between leaf shape genotypes

The striking leaf shape variation in *I. hederacea* suggests it may be important for its ecophysiology. There is abundant theoretical (Gurevitch 1988; Roth-Nebelsick 2001) and empirical (Vogel 1970; Grace et al. 1980; Gottschlich and Smith 1982; Gurevitch and Schuepp 1990; Stokes et al. 2006) evidence that the shape of a leaf significantly affects its thermoregulatory properties. Increases in leaf dissection (e.g., lobing compared to entire-shaped) facilitate the movement of convective heat (H_C) between a leaf and the surrounding air (H_C moves down temperature gradients), through a reduction in the boundary layer (thin layer of air that impedes the movement of heat: Gurevitch 1988; Schuepp 1993; Nobel 2005; Lambers et al. 2006). Thus, lobed leaves are predicted to exchange H_C more efficiently, and so under hot and sunny conditions they should remain cooler by more rapidly dissipating H_C. However, at night

when radiational cooling (a well-documented phenomenon that is unaffected by leaf shape and boundary layer dynamics: Leuning and Cremer 1988; Jordan and Smith 1995) causes leaves to cool below air temperature (e.g., Jordan and Smith 1995; Sage and Sage 2002), lobed leaves should remain relatively warmer by absorbing H_C from the surrounding environment more readily. We have shown elsewhere that the nighttime temperature predictions hold true in *I. hederacea* (Campitelli 2013), but were unable to measure fitness in that experiment. Our final hypothesis is that the different leaf shapes will exhibit different thermoregulation patterns, specifically at night, in a manner consistent with the cline.

To test if any of these hypotheses are potential factors contributing to clinal variation in leaf shape, we carried out a common garden field experiment in the northern part of *I. hederacea's* range, where we could make the clear prediction that lobed individuals should have a higher fitness. We then sought to determine if leaf shape was under selection by insect herbivores, was correlated with other clinally varying traits, or had thermoregulatory consequences, and whether any of these mechanisms would lead to lobed genotypes having a fitness advantage in Northern populations. Specifically, we addressed the following questions: (1) Do lobed individuals have higher overall fitness? (2) Do lobed leaves receive less insect damage, and does leaf damage correlate with fitness? (3) Are flowering time and growth rate influenced by leaf shape genotype in a manner that confers higher fitness to lobed individuals? (4) Are lobed leaves able to thermoregulate more efficiently thus enabling them to avoid the detrimental effects of heat stress or cold damage?

Material and Methods

Natural history

Ivyleaf morning glory, *Ipomoea hederacea* (L.) Jaquin (Convolvulaceae), is an annual vine commonly found in recently disturbed habitats (e.g., roadside ditches and crop fields) throughout the eastern US; it extends from the deep south (Florida to Texas) up through the great lakes region. It germinates from May to August, and persists until a hard frost. Flowering commences approximately 8 weeks postgermination, and continues until a season-ending frost. It produces hermaphroditic flowers visited by two species of *Bombus* bees (Ennos 1981); selfing rates range between 20% and 93% depending on both location and year (Ennos 1981; Hull-Sanders et al. 2005). Primary seed dispersal is due to gravity, though some long-distance secondary dispersal is likely achieved through agricultural activity, as in other *Ipomoea* species that share a common habitat and range (Epperson and Clegg 1986).

In the eastern US, *I. hederacea* is frequently attacked by many natural enemies (Bright 1998; Stinchcombe and Rausher 2001); herbivore damage by mammalian (e.g., white tailed deer, *Odocoileus virginianus*) and insect (e.g., grasshoppers, flea hoppers, and lepidopteran larvae) folivores, as well as insect frugivores (e.g., corn earworm, *Helicoverpa zea*) is common, and fungal pathogens (e.g., white rust [*Albugo ipomoeae-panduranae*] and orange rust [*Coleosporium ipomoea*]) often infect leaves. In our experiment we detected no fungal attack, and we excluded deer by erecting 2.5 m fences around the plot.

Crossing design and experimental setup

We generated recombinant families by crossing two inbred lines (a homozygous entire and homozygous lobe) that originated from North Carolina, USA, and had experienced seven generations of selfing prior to the cross; we used the lobed parent as the pollen donor such that lobed progeny signified a successful cross. After two generations of selfing and single seed descent, we obtained an F_3 population that we used in our experiment. The advantage of employing crossed individuals is that recombination will break up any genetic associations of traits of interest and the leaf shape locus (except tightly linked loci), such that any differences we detect between leaf shape genotypes are more likely to be a direct result of the leaf shape locus.

Our experiment consisted of eight seeds from each of 210 F_3 families for a total of 1680 plants. Because we knew the leaf shape genotype of the F_2 population, we used the rules of Mendelian inheritance to select 210 families that would yield an approximate 1:1:1 ratio of lobed, heterozygote, and entire-shaped individuals, respectively; to achieve this we used 35 entire, 140 heterozygote, and 35 lobed families. Using eight seeds per line, the predicted yield is 560 individuals per-leaf shape genotype (lobed and entire = [35 homozygote families × 8 seeds family^{-1}] + 0.25[140 heterozygote families × 8 seeds family^{-1}]; heterozygotes = 0.5[140 heterozygote families × 8 seeds family^{-1}]). The final ratio of leaf shape genotypes that survived transplant was 565 lobed: 513 heterozygote: 509 entire, which did not significantly deviate from the expected 1:1:1 ratio ($\chi^2 = 3.69$, df = 2, $P = 0.16$). Leaf shape genotype is identifiable based on phenotype (Fig. 1A; Bright and Rausher 2008; Campitelli and Stinchcombe 2013).

On 6 and 7 June 2010, we planted scarified seeds into flats containing Fafard 3 Mix (Conrad Fafard Inc., MA). We used two groups of seeds: (1) The original 1680 used to start the experiment, and (2) an additional two seeds per family (420 seeds) to be used as replacements for any seedlings that died immediately after transplantation. All flats were mist watered twice daily and allowed to germinate in a greenhouse. On 17 June, all original seedlings were transplanted at 1.5 m spacing in a randomized block design consisting of four spatial blocks (420 plants block^{-1}, 2 plants family^{-1} block^{-1}), into an open field at the Blandy Experimental Farm, University of Virginia (US; 39.058109°N, −78.057625°W, 173 m altitude). Blandy is located in a predominantly lobed region: five nearby populations within 10 km have lobed frequencies of ~75–80% (Campitelli and Stinchcombe 2013), suggesting that lobed genotypes should have a fitness advantage at our field site. Flats containing replacements were also moved into the field so as to experience the same climatic conditions, but were kept in their flats until needed. Prior to transplant, natural vegetation in the field was mowed, and we plowed rows using a hand-push Troy-Bilt® tiller (native vegetation was mowed bimonthly thereafter). We watered seedlings twice daily for 2 weeks posttransplant to facilitate establishment, and then discontinued watering. We provided plants 1.5 m stakes to prevent tangling. On 6 July, we replaced all unsuccessful transplants with an individual from the same family (49 entire + 44 heterozygote + 58 lobe = 151 plants; not statistically different from the expected 1:1:1 ratio; $\chi^2 = 2.08$, df = 2, $P = 0.35$). A terminal frost on the night of 23rd October ended the experiment.

We assigned plants randomly to two treatments: half were allocated to an insect removal treatment by spraying them with a mixture of the insecticides carbaryl (Sevin®; Garden Tech, KY) and *Bt* (Bug B Gon Max®; Ortho, OH) with the remaining half assigned to a control treatment (water spraying). The insecticides were prepared following the manufacturers' instructions, and all applications (spray and control) were administered at 2-week intervals beginning 12th July; both insecticides have been shown to have no direct influence on fitness in *I. hederacea* (Stinchcombe and Rausher 2001). We randomized treatments such that all families had a single representative in each treatment in each block.

Traits and variables measured

We were primarily interested in four potential mechanisms of selection on leaf shape (or the leaf shape locus), and if they conferred differential fitness to individuals with different leaf shapes: defense against insect herbivores, flowering phenology, growth rate, and thermoregulation.

Insect herbivory

To score insect herbivory, we estimated the proportion of total leaf area removed by herbivory. Near the end of the

season (5–14 October) we took digital images of three selected leaves on each plant (one from the bottom, mid, and top) for digital image analysis in ImageJ v1.46 (http://rsbweb.nih.gov/ij/index.html). We used the mean damage of all three leaves from a plant as the damage score for that plant.

Flowering time and relative growth rate

We scored flowering time as the number of days between sow date in the greenhouse and date of first flower. To estimate relative growth rates, we counted the number of leaves on each plant at four time points (day 1, 3 weeks, 7 weeks, and 12 weeks), and then used the following equation (Blackman 1919; Hunt et al. 2002):

$$RGR = \frac{\ln(M_2) - \ln(M_1)}{t_2 - t_1} \qquad (1)$$

where M_1 and M_2 are leaf counts at time one (t_1) and time two (t_2), respectively, and where time is the number of days from planting. We calculated three growth rates: RGR_{early}, RGR_{mid}, and RGR_{late} using data from leaf counts at day 1, 3 weeks, and 7 weeks, to estimate t_1 and M_1, and data from leaf counts at 3, 7, and 12 weeks to estimate t_2 and M_2. We assumed M to be 1 for day 1 measurements because $\ln(0)$ is undefined. We expect temporally adjacent growth rates to be correlated because they share a data point in common.

Temperature and leaf performance

We recorded leaf thermoregulation by selecting a single leaf from 22 randomly selected plants (controlling for leaf size, orientation, and height above ground) by attaching copper-constantan (type-E) thermocouples (calibrated to 0°C in an ice bath) to the bottom surface of leaves, and logging temperature every 10 min using OM-CP-QUAD-TEMP dataloggers (Omega, QC, Canada). To ensure comparable measurements between leaf shape genotypes, we attached thermocouples to the middle of the leaf, adjacent to but not touching the midvein. Because of a limited number of thermocouples, we restricted our temperature recordings to 11 lobed and 11 entire shaped individuals, and recorded air temperature by placing the remaining thermocouples in a probe housing designed to reduce the impact of sunlight. As described below, we compare leaf temperatures to each other, rather than to air temperature.

To determine if differential thermoregulation affected leaf performance, we measured chlorophyll fluorescence (F_v/F_m; or maximum quantum yield) of two leaves (positioned at the base and top of the plant) from 120 randomly chosen plants (10 per leaf shape genotype per block), using a Pocket PEA chlorophyll fluorimeter (Hansatech Instruments Ltd., MA). F_v/F_m is a proxy for photosynthetic efficiency and is sensitive to temperature fluctuations, and so is often used as an indicator of leaf performance under varying temperatures (Genty et al. 1989); hence it is useful for performance comparisons between leaf shapes that are predicted to thermoregulate differently. F_v/F_m is recorded on dark-acclimated leaves (Maxwell and Johnson 2000), so we performed measurements just prior to sunrise (5 AM–7 AM EST); this tends to be the coldest time of the night, which is optimal for detecting photosynthetic inhibition. Measurements began on 13th September, and took place every clear night that fell below 5°C thereafter; overcast conditions inhibit radiational cooling because of the requirement for exposure to open sky, and so we did not measure F_v/F_m on such nights. We simultaneously recorded an instantaneous temperature measurement of the upper surface of these same leaves using an OS531 handheld infrared thermometer (Omega, QC, Canada) to examine direct impacts of temperature on F_v/F_m. We restricted our temperature monitoring, F_v/F_m, and instantaneous temperature measurements to original plants in the control treatment. Due to logistical constraints, we were unable to measure other aspects of leaf-level ecophysiology (e.g., stomatal conductance).

Fitness

To estimate lifetime fitness, we collected mature seeds from all plants on a daily basis immediately after seed capsules began to mature.

Data analysis

To assess if any traits significantly differed between leaf shape genotypes, and if they had significant fitness effects, we employed a series of mixed model ANOVAs. For each response variable (e.g., insect damage, flowering time, relative growth rate, seed output), we included the fixed effects of block, treatment (control and spray), leaf shape genotype (entire, heterozygote, lobed), and a leaf shape × treatment interaction; denominator degrees of freedom for hypothesis tests for fixed effects were determined by the Kenward–Roger approximation. We estimated variance components for two random effects: family and the family × treatment interaction. We note that in these models, planting cohort (original or replacement) had significant effects on several traits; reanalyzing the data separately by cohort did not change the patterns or interpretations of the data. Hence, we chose to include planting cohort as a fixed effect to maximize sample size

and power. All mixed model ANOVAs were carried out in SAS 9.2 (Proc Mixed, SAS Institute, NC), assuming normally distributed residuals. Residuals were always unimodal, but often deviated significantly from normality. Accordingly, we verified all hypothesis tests with permutation tests, following Heath et al. (2010); because permutation and parametric-based hypothesis tests were identical in pattern and significance, we only present the latter.

Insect herbivory, flowering time, and relative growth rate

To assess whether any quantitative traits (e.g., damage, flowering time, RGR_{early}, RGR_{mid}, and RGR_{late}) differed between leaf shape genotypes, we used each trait as the response variable in individual models, and the fixed effects listed above as independent variables. For insect damage as a response variable, we used a logit transformation, following recommendations by Warton and Hui (2011). We explored the potential for genetic variation in traits by removing either family or family × treatment from the model, and comparing the model fit (−2 log likelihood) to the full model using a chi-squared test with one degree freedom; we halved the P-value from the chi-squared test because it is one tailed (Littell et al. 2006).

To analyze the effects of each trait measured on fitness, we used seed output as our response variable, and included all the measured traits as covariates. We also explored additional models including quadratic effects of quantitative traits on fitness, models excluding quantitative traits as covariates, and models excluding heterozygote genotypes, which are rare in natural populations (Campitelli and Stinchcombe 2013).

Temperature and chlorophyll fluorescence

For the temperature monitoring data, we first assessed the directionality of leaf temperature between genotypes by estimating the mean temperature of each leaf shape (here, lobed, and entire only) for each of the 10,988 time points in our time series. We then examined if the fraction of time spent colder significantly deviated from 50% when comparing the two leaf shapes. We split our analysis into nights and days, and performed a separate chi-squared test on each category (Proc Freq, SAS) to determine which genotype was colder more often. Nights and days were determined by using sunrise and sunset data for Washington, DC, (www.noaa.gov); all points between sunset and sunrise were assigned to nights, and vice-versa for days.

The structure of our datalogger dataset (many measurements on 22 individuals) prevented us from using repeated measures ANOVA to evaluate the statistical significance of temperature differences between lobed and entire shaped leaves. Instead, we first estimated the mean temperature difference between entire- and lobed-shaped leaves ($\bar{x}_{entire} - \bar{x}_{lobe}$) for all time points; if lobes are consistently warmer at night, the mean of this distribution ($\bar{x}_{difference}$) will be negative. We assessed the statistical significance of $\bar{x}_{difference}$ by comparing it to an empirical null distribution of temperature differences generated by randomly permuting leaf shape labels 9999 times using PopTools (Hood 2010); a significant difference would be confirmed if $\bar{x}_{difference}$ fell outside the 95% confidence limits of the null distribution. By randomly reassigning leaf shape in each permuted sample to all temperature measurements of an individual, our permutation tests account for the nonindependence of repeated measures on an individual. We performed this analysis separately for nighttimes and daytimes (as above). We performed similar analyses to test for differences in the variance in leaf temperature between leaf shape genotypes, but found no significant differences or even marginal trends; as such, we only present results for hypothesis tests for $\bar{x}_{difference}$.

We compared F_v/F_m and instantaneous temperature measurements between leaf shape genotypes (here, all three leaf shapes) over the duration of the experiment using repeated-measures ANOVA using Proc Mixed (SAS). We accounted for repeated measurements on the same plants by setting each plant as a repeated effect. We report results from a compound symmetry variance–covariance structure, because it provided the best fit.

Results

Insect herbivory

Insects have a significant effect on fitness: when insect herbivores are removed there is on average an 11% increase in seed output (Fig. 1B; Table 1). However, we failed to detect significant fitness differences between leaf shape genotypes in either treatment (Fig. 1B; Leaf shape and Leaf shape × treatment terms, Table 1), indicating that insect herbivores are not imposing differential selection on leaf shape in either treatment. Although we detected increased fitness in the spray treatment, we did not find evidence of differential insect damage to leaves between treatments (Fig. 1C; Table 2), suggesting that reduced leaf damage is not the cause of increased fitness in the spray treatment. There was a significant treatment × leaf shape genotype effect on damage driven by an increased level of damage to lobed plants in the spray treatment (Table 2); the biological interpretation of this interaction is unclear because the higher damage occurred in the spray treatment where insect herbivores should

Table 1. Mixed model ANOVA statistics showing the effects of all fixed variables (block, treatment, planting cohort, leaf shape), measured traits (damage, flowering time, RGR_{early}, RGR_{mid}, RGR_{late}), and random variables (family and family × treatment) on lifetime fitness (number of seeds).

Source	Effect size	P-value
Block	$F_{3,1014} = 4.94$	**0.0021**
Treatment	$F_{1,182} = 18.96$	**<0.0001**
Planting Cohort	$F_{1,1207} = 1.23$	0.2681
Leaf shape	$F_{2,819} = 0.94$	0.3916
Damage	$F_{1,1203} = 0.09$	0.7590
Flowering time	$F_{1,618} = 477.13$	**<0.0001**
RGR_{early}	$F_{1,1235} = 463.31$	**<0.0001**
RGR_{mid}	$F_{1,1236} = 465.74$	**<0.0001**
RGR_{late}	$F_{1,1229} = 40.66$	**<0.0001**
Leaf shape × treatment	$F_{2,737} = 0.4$	0.6695
Family	$\chi^2 = 1.7$	0.0961
Family × treatment	$\chi^2 = 0.3$	0.2919

Subscripts indicate numerator degrees of freedom, and denominator degrees of freedom estimated by the Kenward–Roger approximation. Boldface indicates a significant effect.

have been removed. We note that leaf damage was always less than 2% (Fig. 1C).

We also used the average leaf area from our photographed leaves, and the final leaf count from our growth rate data to estimate total leaf surface area (mean leaf area × number leaves), and tested for differences in total photosynthetic area between leaf shape genotypes. We found significant differences in mean area per leaf between genotypes ($F_{2,1177} = 7.31$, $P = 0.0007$), with entire-shaped and heterozygote leaves being significantly larger than lobed leaves, but not different from each other. We failed to detect any significant differences among genotypes in total leaf number ($F_{2,878} = 0.72$, $P = 0.49$), and a marginal trend in total leaf area per plant ($F_{2,1016} = 2.39$, $P = 0.09$), suggesting that variation

in total leaf number eliminated the per-leaf size advantage of entire-shaped genotypes. When comparing only the two homozygote classes, we found that per-leaf area was larger for entire-shaped genotypes ($F_{1,692} = 12.86$, $P = 0.0004$), no differences in total leaf number ($F_{1,385} = 1.24$, $P = 0.27$), and significantly greater total leaf area per plant for entire-shaped genotypes ($F_{1,505} = 4.76$, $P = 0.0296$). Total leaf surface area, however, did not affect fitness (all three leaf shape genotypes, $F_{1,1231} = 0.36$, $P = 0.55$; homozygote leaf shape classes only, $F_{1,816} = 0.01$, $P = 0.93$).

Flowering time and relative growth rate

We found strong directional selection favoring earlier flowering plants (Fig. 2A; Table 1), and significant genetic variation for flowering time (indicated by the family term in Table 2). Figure 2A shows a family means regression of seed set on flowering time; these family means were estimated for each treatment separately because of a significant family × treatment interaction for flowering time (control $R^2 = 0.52$ and spray $R^2 = 0.45$, both $P < 0.0001$). We found the same result for phenotypic data (Table S2). Flowering time did not differ between leaf shape genotypes, as all three leaf shapes display flowering times that span the full flowering distribution (Fig. 2B; Table 2). Since we employed crossed individuals, this suggests that there is no genetic relationship between the leaf shape locus and flowering time through pleiotropy or tight linkage.

All three relative growth rates were significant predictors of fitness (Table 1), as indicated by phenotypic correlations between growth rate and seed output (Fig. 3A–C). Individuals that grew fast early and midway through the season (RGR_{early} and RGR_{mid}; Fig. 3A and B) and those that slowed their growth later in the season had a higher fitness (Fig. 3C; Table 1). Although there is some curvature in the

Table 2. Mixed model ANOVAs examining the effects of fixed variables (block, treatment, planting cohort, leaf shape, leaf shape × treatment) and random variables (family, family × treatment) on each measured trait (insect herbivory, flowering time, RGR_{early}, RGR_{mid}, and RGR_{late}).

Source	Damage[1]	Flowering	RGR_{early}	RGR_{mid}	RGR_{late}
Block	$F_{3,997} = 1.45**$	$F_{3,1170} = 34.9**$	$F_{3,1196} = 58.9**$	$F_{3,1563} = 155.5**$	$F_{3,1315} = 69.2**$
Treatment	$F_{1,368} = 0.28$	$F_{1,206} = 2.2$	$F_{1,209} = 0.08$	$F_{1,1563} = 8.0*$	$F_{1,1309} = 0.03$
Planting Cohort	$F_{1,1260} = 0.55$	$F_{1,1381} = 102.9**$	$F_{1,1539} = 601.1**$	$F_{1,1563} = 10.3*$	$F_{1,1458} = 0.04$
Leaf shape	$F_{2,1002} = 0.68$	$F_{2,1520} = 1.9$	$F_{2,900} = 0.2$	$F_{2,1563} = 0.3$	$F_{2,1037} = 1.7$
Shape × treatment	$F_{2,1003} = 4.39*$	$F_{2,838} = 1.8$	$F_{2,852} = 0.3$	$F_{2,1563} = 0.4$	$F_{2,1419} = 2.5$
Family	$\chi^2 = 0.0$	$\chi^2 = 157.9**$	$\chi^2 = 0.3$	$\chi^2 = 0.0$	$\chi^2 = 8.7*$
Family × treatment	$\chi^2 = 0.0$	$\chi^2 = 4.5*$	$\chi^2 = 0.8$	$\chi^2 = 0.0$	$\chi^2 = 0.0$

Subscripts indicate numerator degrees of freedom, and denominator degrees of freedom estimated by the Kenward–Roger approximation.
[1]For insect damage as a response variable, we applied a logit transformation of log[(observed damage + minimum damage)/1−(observed damage + minimum damage)].
Boldface indicates a significant effect; *P < 0.05, **P < 0.001.
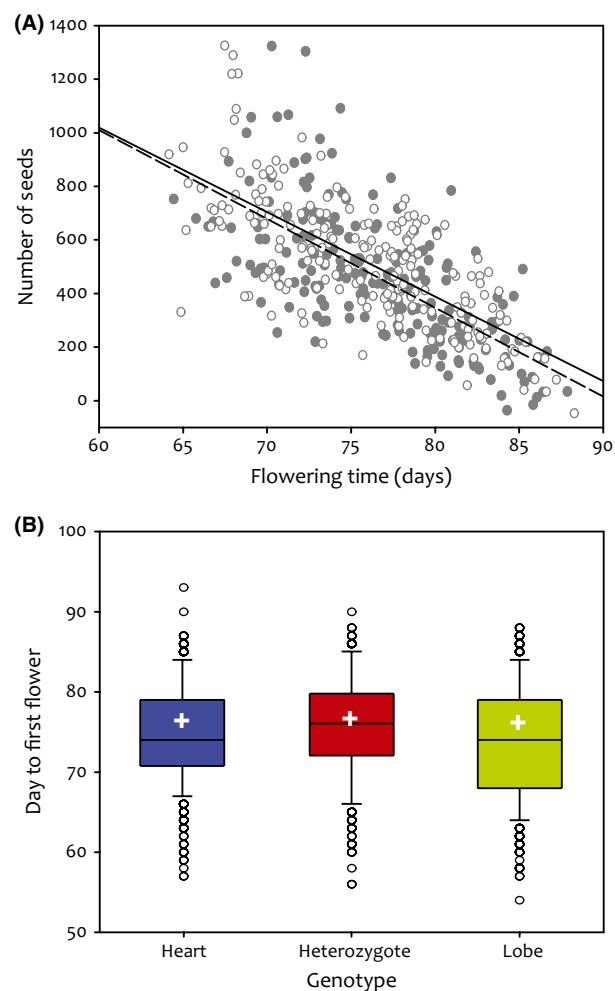
(A)

(B)

Figure 2. (A) Family means (lsmeans) showing the effect of flowering time on seed production. The control (filled circles and solid line; $R^2 = 0.52$, $P < 0.0001$) and spray treatment (open circles and dashed line; $R^2 = 0.45$, $P < 0.0001$) are shown separately due to a significant family × treatment effect on flowering time. (B) Boxplots showing the distribution of flowering time for each of the three leaf shape genotypes. White crosses represent the mean (lsmeans), and the black line in each box shows the median flowering time.

relationship between growth rates and fitness (Fig. 3), there is no evidence of an intermediate optima/minima indicative of true stabilizing/disruptive selection (sensu Mitchell-olds and Shaw 1987), and as such we only present linear fits of directional selection.

None of the three growth rate measures were different between leaf shapes (Fig. 3D; Table 2). We detected significant genetic variation for RGR_{late} (family term in Table 2), and a stronger negative relationship between family means of RGR_{late} and seed output ($R^2 = 0.17$, $P < 0.0001$). Plants in the control treatment had a slightly larger RGR_{mid} (Table 2), suggesting that herbivory may have induced faster growth during this portion of the

growing season. Although RGR_{early} and RGR_{mid}, and RGR_{mid} and RGR_{late} are serially correlated (because of a common data point), the statistical model including all of these terms accounts for these correlations, which were moderate ($0.05 < |r_p| < 0.36$, Table S2; $0.03 < |r_g| < 0.34$, Table S3). Full phenotypic and genotypic correlations among the measured traits (fitness, damage, flowering time, $RGRs$) are presented in Tables S2 and S3, respectively.

Temperature and chlorophyll fluorescence

Our temperature monitoring confirmed that lobed leaves are consistently warmer than entire-shaped leaves throughout the majority of nights; entire-shaped leaves spent >50% of the time colder on 73 out of 77 nights ($\chi^2 = 62.8$, df = 1, $P < 0.0001$; Fig. 4A). Although clear temperature directionality was apparent, the magnitude of the mean difference in temperature between the two leaf shapes was always within 1°C, with the maximum difference occurring in early September (Fig. 4B). In contrast to nighttime, no apparent temperature pattern emerged during the daytime; entire-shaped leaves spent >50% of the time colder on 35 out of 76 days ($\chi^2 = 0.47$, df = 1, $P = 0.49$; Fig. S1). Permutation tests revealed that the 95% confidence limits of the null distribution ranged from −0.3 to 0.3°C for both nighttimes and daytimes (determined separately). For all measurements the mean difference ($\bar{x}_{entire} - \bar{x}_{lobe}$) for nighttimes was −0.22°C ($P = 0.08$) and for daytimes was 0.10°C ($P = 0.25$).

Both instantaneous temperature measurements and F_v/F_m did not significantly differ between leaf shapes on the 11 clear nights we measured these parameters (Fig. S2; Table S1). We detected a position effect on both instantaneous temperature and F_v/F_m (Table S1); leaves at the top of the plant were generally warmer, and as a result had higher photosynthetic efficiency. We found no evidence of leaf shape genotype × position interactions, suggesting that position effects did not differ between genotypes.

Total and direct effects of leaf shape on fitness

After accounting for insect herbivory, flowering time, and all three growth rates, we found that leaf shape had no overall fitness effect, suggesting that selection on these quantitative traits is not masking fitness differences between leaf shapes (Fig. S3; Table 1). Including quadratic terms for the quantitative traits did not reveal fitness differences between leaf shapes ($F_{2,835} = 0.94$, $P = 0.39$). Within the subgroup of 22 individuals that we detected consistent nighttime temperature differences (Fig. 4; Table 2), we also detected no fitness difference between
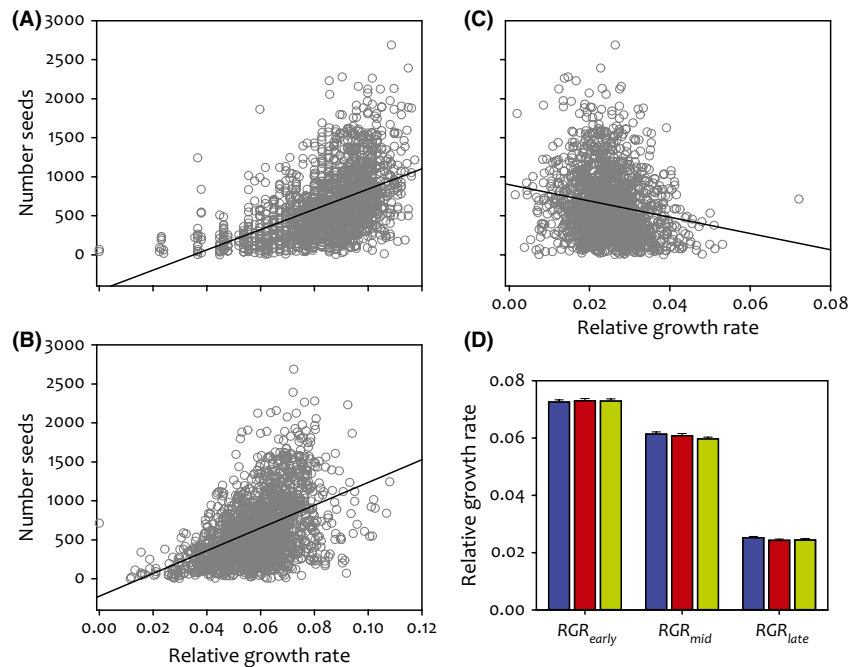
Figure 3. The phenotypic relationships between seed production and (A) early-season relative growth rate (RGR_{early}; $R^2 = 0.26$, $P < 0.0001$), (B) midseason relative growth rate (RGR_{mid}; $R^2 = 0.24$, $P < 0.0001$), and (C) late-season relative growth rate (RGR_{late}; $R^2 = 0.05$, $P < 0.0001$). (D) Mean relative growth rates (lsmeans ± 1 SE) for entire (blue), heterozygote (red), and lobed (green) leaf shape genotypes.

lobed and entire-shaped individuals ($F = 0.011$, df = 1, $P = 0.91$), suggesting that the temperature pattern we detected did not influence seed output. We also reran our analysis of selection on leaf shape removing all other measured traits from the model (to estimate total selection, without removing the effects of potentially correlated traits), and likewise did not detect net selection on leaf shape (Table S5).

Because heterozygotes are rare in natural populations (Campitelli and Stinchcombe 2013) suggesting they may not be central to the predominance of lobed genotypes in northern latitudes, we reran our selection analysis on the two homozygote leaf shape genotypes. Selection analysis with only homozygotes revealed similar patterns, and did not change any of the interpretations from the full analysis (Table S4).

Discussion

We predicted that *I. hederacea* individuals with lobed leaves would be more fit relative to their entire-shaped counterparts in an experimental population in the northern part of its current US range, where lobed individuals have been found in much higher frequency (Bright 1998; Campitelli and Stinchcombe 2013). Specifically, past experiments and data from the literature lead to the predictions that lobed individuals would sustain less insect

damage, flower earlier in the growing season, grow faster, or thermoregulate in a manner that enabled them to achieve a higher performance or fitness (or some combination of these factors). While we found that several of these ecological variables imposed natural selection on *I. hederacea*, none of them show strong evidence of acting differentially on leaf shape, with the potential exception of temperature. Below we discuss our results in the context of past studies and literature, suggest hypotheses for the lack of selection on leaf shape, and discuss the implications of our findings for understanding leaf shape variation in *I. hederacea*.

Selection on *I. hederacea* and the leaf shape polymorphism

Insect herbivory

We found that insect herbivory reduced the fitness of *I. hederacea*, which is a common finding (e.g., Simms and Rausher 1989; reviewed by Marquis 1992): plants in the control treatment exhibited an average reduction in fitness of 11% (Fig. 1B; Table 1). These fitness effects could not be attributed to leaf chewing (folivorous) insects because the overall amount of leaf damage incurred by plants in each treatment was not significantly different (Fig. 1C; Table 2). In addition, there was an interaction between leaf
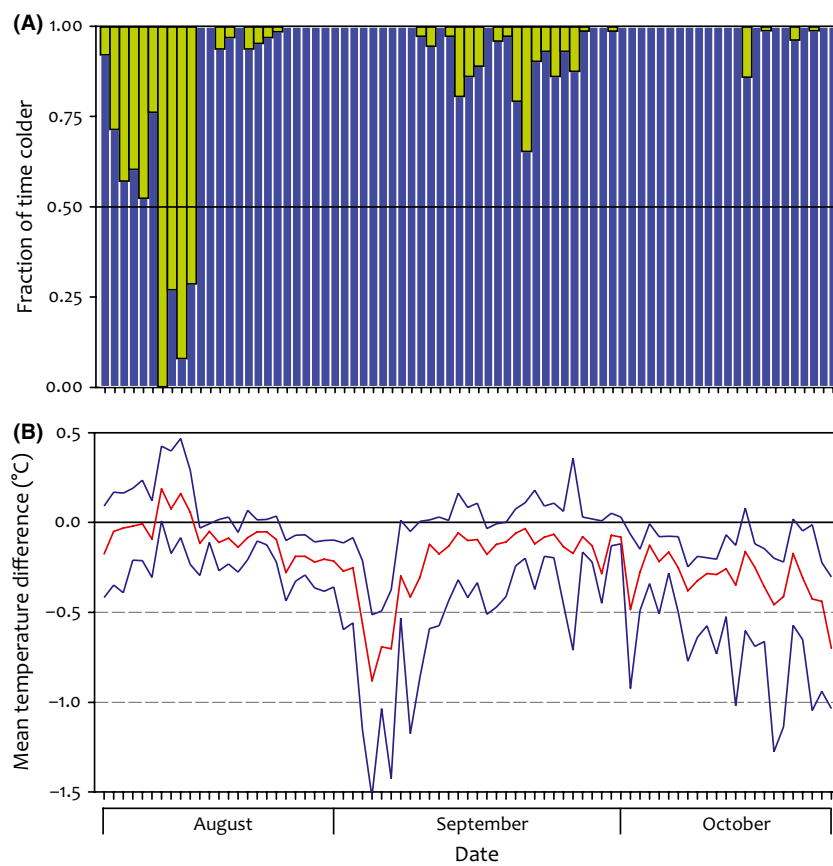
Figure 4. (A) The proportion of time that on average entire-shaped leaves (blue bars) or lobed leaves (green 0062ars) are colder during the night. Each stacked bar represents a single night. (B) The maximum, average, and minimum value of temperature differences (entire-shaped leaves minus mean of lobed leaves) for each night. When the red-colored line is below the *x*-axis, it indicates nights that entires were on average colder, while points above zero indicate nights that lobes were on average colder.

shape and treatment on damage driven by a significant increase in leaf damage to lobed leaves in the spray treatment (Fig. 1C; Table 2); however, this damage difference did not translate into a fitness difference between leaf shapes in the spray treatment, supporting the interpretation that leaf chewing herbivores are not responsible for the higher fitness of plants in the spray treatment. As we only measured insect folivory, it is possible that other types of insect herbivores (e.g., granivores or mucivores) – presumably also sensitive to insecticides – contributed to the fitness differences between treatments. We observed no mucivory (e.g., aphids or the like), and a low level of granivory by corn earworm larvae (*Helicoverpa zea*; Lepidoptera), but were unable to quantify their abundance or damage levels. Corn earworm larvae are a common and important enemy of *Ipomoea* species; they burrow their heads into seed pods and consume its contents, imposing a direct fitness cost (Simms and Rausher 1989). Therefore, it is possible that the reduction in fitness to control plants may have been a result of granivory by *H. zea*, at least in

part. Overall, leaf damage and fitness did not differ significantly between leaf shapes within either treatment (Fig. 1B and C), suggesting that insect herbivores do not impose differential selection on leaf shape in *I. hederacea*, and therefore are unlikely to be involved in the maintenance of the leaf shape cline.

Flowering time

We found similar flowering time patterns to past work (Simonsen and Stinchcombe 2010): there was significant genetic variation in flowering time, and strong directional selection favoring earlier flowering (Fig. 2A; Tables 1 and 2). We also expected to detect selection for earlier flowering because our experiment was conducted in the northern part of *I. hederacea*'s range, where terminal frosts likely occur earlier than where the seeds for the parents of our cross-originated (NC). Two previous studies (Klingaman and Oliver 1996; A. J. Stock, B. E. Campitelli, and J.R. Stinchcombe, unpubl. data) demonstrated latitudinal clines

for flowering time with northern populations flowering earlier, suggesting that earlier flowering is adaptive in northern populations. Earlier flowering should be selectively favored under such conditions because it enables individuals to reproduce and mature seed prior to being killed by earlier autumn frosts. Despite strong selection for earlier flowering phenology, flowering time did not differ between leaf shapes, suggesting that the leaf shape locus is not genetically associated with flowering time. Therefore, the selective mechanisms that are generating a flowering time cline are not indirectly influencing leaf shape, and hence are likely not contributing to the leaf shape cline.

Relative growth rate

Individuals who rapidly accumulated biomass early and midway through the season tended to have a higher reproductive output (Fig. 3A and B; Table 1). In addition, selection favored individuals that slowed their growth later in the season (Fig. 3C; Table 1), suggesting there are benefits to reallocating resources, presumably to flower and seed production. Growth patterns of this type are common in many annual plant species that have only a single opportunity to reproduce (e.g., Reed and Holland 1919; Harper and Ogden 1970). Preliminary work showed a latitudinal cline for midseason growth rate (A. J. Stock, B. E. Campitelli, and J. R. Stinchcombe, unpubl. data) suggesting that selection is acting on this trait across the range, and past studies have revealed quantitative genetic variation in growth rates within populations (Simonsen and Stinchcombe 2010). However, we find no evidence that growth rates vary between leaf shapes at any point during the season (Fig. 3D, Table 2), indicating that differences in relative growth rate between leaf shape genotypes are unlikely to explain the leaf shape cline.

Thermoregulation and leaf performance

We found that during the vast majority of nights, lobed leaves remained consistently warmer than entire-shaped leaves (Fig. 4A); however, the mean difference in temperature between lobed and entire-shaped leaves was only marginally significant relative to a null distribution (Fig. 4B). No patterns of temperature directionality or differences between leaf shapes emerged during the daytime (Fig. S1). Our findings in this study are similar to previous work (Campitelli 2013), suggesting that the temperature profiles we have observed represent a real effect of leaf shape on thermoregulation. Similar to our previous study, we found that these persistent patterns were not enough to generate differential performance or fitness between leaf shapes, likely because of their small magnitude. We have previously tested whether other physiological traits (i.e., specific leaf

area, trichome density, vein density and stomatal density) also known to potentially affect thermoregulation differently between leaf shapes, and failed to detect differences between genotypes (Campitelli & Stinchcombe, unpublished data). We note that evapotranspiration through stomates – which will further cool leaves – can occur at night, but is typically greatly reduced by stomatal closure (5–15% of daytime levels: reviewed by Caird et al. 2007). The effects of evapotranspiration on nighttime leaf temperature relative to convective heat exchange and radiational heat loss – either buffering or amplifying predicted boundary layer effects – needs further consideration. Regardless of the proximate physiological mechanisms (boundary layer dissection and convective heat exchange or evapotranspiration), their net, cumulative effects appear to have minor consequences for leaf temperature and fitness. Further experimentation that manipulates the potential for radiational cooling may be an effective method to determine the biological relevance of the thermoregulatory patterns we have observed.

Leaf shape

None of the variables we measured showed significant differences between leaf shapes (with the exception of thermoregulation, though we detected no fitness effect). We also failed to detect significant fitness differences between leaf shapes either when statistically controlling for the effects of natural selection on other quantitative traits (Fig. S3, Table 1), suggesting that leaf shape fitness differences are not being masked by strong selection on other traits; analyses of total selection, not controlling for other traits, likewise failed to detect effects of leaf shape on fitness. Combined, our results suggest that leaf shape is either a selectively neutral trait in *I. hederacea*, and that the leaf shape cline may have arisen stochastically, or that we have not detected the selective agent. The hypothesis of selective neutrality is not supported by past studies: (1) Bright and Rausher (2008) detected natural selection acting on leaf shape in *I. hederacea* in four separate field experiments carried out in two consecutive years. (2) The leaf shape locus exhibits a steep cline and is a highly significant outlier locus relative to the rest of the genome, while there is complete nonclinality at putatively neutral molecular markers (Campitelli and Stinchcombe 2013), suggesting the leaf shape locus is experiencing (or has experienced) divergent selection.

Why were there no fitness effects of leaf shape?

One potential explanation is that we inadvertently excluded a selective agent that otherwise would have

generated fitness differences between leaf shapes. For example, because of the destructive potential of deer (they are capable of consuming entire experiments in their totality in a few days), we chose to exclude deer herbivory. Although Bright (1998), Bright and Rausher (2008) and Stinchcombe and Rausher (2001) employed fences, *I. hederacea* individuals within their experiments experienced significant deer damage. In addition, Bright (1998) detected a significantly greater amount of deer damage on entire-shaped individuals, and in that particular experiment lobed plants had a higher fitness; however, deer herbivory was not manipulated experimentally. Stinchcombe and Rausher (2001) found selection for greater resistance to deer herbivory, though they did not detect a leaf shape effect. Therefore, it is possible that the exclusion of deer unintentionally removed a significant selective agent on leaf shape in *I. hederacea*, and so further experimentation manipulating deer accessibility may provide valuable insight. Although Bright (1998) also discovered significantly different levels of fungal damage on leaf shapes, we did not detect any fungal infection in our experiment.

A second possible explanation is that early season variables impose the strongest selection on leaf shape, and hence we failed to capture the primary selective agent by transplanting in the early summer. For example, it has been shown that early season frosts can inflict major fitness costs on plants (e.g., Agrawal et al. 2004), and given our thermoregulation results (Fig. 4), perhaps lobed individuals would more effectively avoid damage during early season cold snaps. However, Bright and Rausher (2008), who detected selection on leaf shape, planted their experiments at similar (early July 1997) or later (31 July 1996) times. Furthermore, they conducted their experiments ~350 km south of our site in North Carolina, US, where early season episodic cold snaps are even less frequent, suggesting that selection on leaf shape in their experiments was unlikely a result of early season cold temperatures. Bright and Rausher (2008) also planted scarified seeds directly into the field (as opposed to transplanting seedlings), and so differential selection on leaf shape may occur at the early developmental stages in the field rather than early in the season. Nonetheless, in their experiments, an equal number of each leaf shape germinated and survived to reproduction (see Table 1 in Bright and Rausher 2008), and so the fitness differences they detected are likely not due to selection at the seedling stage. Additionally, preliminary germination and freezing trials in the lab have failed to detect differences between genotypes in the proportion of seeds germinating or their mean time to germination following a range of temperature treatments (R. Kim, B. E. Campitelli, and J. R. Stinchcombe, unpubl. data).

A third possibility is that 2010 was a relatively benign year with respect to selection on leaf shape in *I. hederacea*

in Virginia. In other words, perhaps temporally fluctuating selection governs the leaf shape cline in *I. hederacea*, and our experiment occurred during a year of relaxed selection. There are many instances of episodic or fluctuating selection from detailed multiyear studies: For example, in the classic case of Darwin's medium ground finch (*Geospiza fortis*) in the Galápagos, a period of strong selection (brought on by a drought in 1977) lead to a significant shift in beak depth (reviewed by Boag and Grant 1984). Episodic selection on beak depth was subsequently documented several times since 1977 with longer intermittent periods of little or no selection (Gibbs and Grant 1987), demonstrating temporally variable selection pressures. In a more extreme example, Dominguez and Dirzo (1995) found strong directional selection – in opposite directions – on flowering time in *Erythroxylum havanense* over two consecutive years. In addition, even Bright and Rausher (2008) detected a shift in selection on leaf shape in *I. hederacea* from 1996 to 1997 in North Carolina (US); while Stinchcombe and Rausher (2001), who also did their experiment in NC, did not detect any selection on leaf shape in 1998. What is apparent from many multiyear studies is that the direction and magnitude of selection often oscillates (reviewed by Bell 2010), especially for single-locus traits; hence, it is entirely possible that we did not detect selection on leaf shape because of relatively weak selection in that year.

Future prospects of leaf shape in *I. hederacea*

Identifying selective agents of ecologically important traits remains a significant challenge in evolutionary biology. Even detailed long-term investigations of known fitness-related traits do not always turn up a selective agent. For example, Fisher and Ford (1947) initiated an 8-year survey of natural populations of the scarlet tiger moth (*Panaxia dominula*), that revealed significant changes in allele frequencies at a single-locus wing spot polymorphism. They ruled out genetic drift as the primary driver of these changes, suggesting natural selection. Despite 60 years of study on this polymorphism in the same populations, the selective agent(s) still have not been fully resolved (Cook and Jones 1996; O'Hara 2005). Given that leaf shape is likely to experience selection from multiple sources (reviewed by Nicotra et al. 2011), determining the environmental variables that are imposing selection on, and governing the geographical distribution of this trait in *I. hederacea*, will likely remain a difficult but important task.

Despite being unable to isolate selective agents acting on leaf shape in *I. hederacea*, this study contributes to our understanding of leaf shape evolution by providing evidence that several candidate selective agents may not

be important contributors to the cline. First, our crossing design allowed us to eliminate any potential associations of leaf shape with documented flowering time and growth rate clines, despite finding strong selection on both of these traits. Second, although we find consistent thermoregulatory patterns (Fig. 4), we also find no leaf-level ecophysiological performance differences suggesting that differential thermoregulation is likely not contributing to the clinal variation. Finally, we can conclude that insect herbivores are unlikely to contribute to the leaf shape cline; although other sources of herbivory (e.g., deer) may contribute. However, if temporally fluctuating selection is the major selective force maintaining the leaf shape cline, these four candidate selective mechanisms may need to be reconsidered. Therefore, future work should involve a combination of population surveys and multiyear experimental evolution (e.g., Scarcelli and Kover 2009) to determine if episodic selection is maintaining the observed leaf shape cline, and to continue to hone in on the selective agents acting on this trait in *I. hederacea*.

Acknowledgments

Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Repeated measure ANOVAs of the effects of fixed variables (block, leaf shape, position of leaf, time, leaf shape × time, position of leaf × time) and random variables (family) on two ecophysiological traits (chlorophyll fluorescence and instantaneous temperature), measured on 11 clear and cold nights.

Table S2. Phenotypic correlation coefficients (Pearson) between all traits in the control treatment (above diagonal), and spray treatment (below the diagonal).

Table S3. Family means (genetic) correlation coefficients (Pearson) between all traits in the control treatment (above the diagonal), and the spray treatment (below the diagonal).

Table S4. Mixed model ANOVA statistics showing the effects of all fixed variables (block, treatment, type, leaf shape), and measured traits (damage, flowering time, RGR_{early}, RGR_{mid}, RGR_{late}) on lifetime fitness (number of seeds) when only considering the two homozygote leaf shape classes (lobed and entire-shaped).

Table S5. Net fitness effects of leaf shape without controlling for the effects of other quantitative traits.

Figure S1. (A) The proportion of time that on average entire-shaped leaves (blue bars) or lobed leaves (green bars) are colder during the day. Each stacked bar represents a single day. (B) The difference in temperature (mean of entire leaves minus mean of lobed leaves ± SE; note, we only show SE toward zero) for each day. Points below the zero line indicate days that entires were on average colder, while points above zero indicate days that lobes were on average colder. In b, filled circles show nights where error bars do not cross zero, and blue solid lines represent the upper and lower maximum temperature differences.

Figure S2. Mean (lsmeans ± 1 SE) chlorophyll fluorescence (F_v/F_m; circles and solid lines) and instantaneous temperature measurements (squares and dashed lines) for entire (blue), heterozygote (red), and lobed (green) leaf shape genotypes for 11 clear and cold nights in the autumn of 2010.

Figure S3. Mean (lsmeans ± SE) seed production of individuals with each leaf shape after statistically removing the effects of insect damage, flowering time, all three growth rates, and our fixed effects described in the main text.