

**THE EFFECT OF LEAF SHAPE ON THE THERMOREGULATION  
 AND FROST TOLERANCE OF AN ANNUAL VINE,  
*IPOMOEA HEDERACEA* (CONVOLVULACEAE)<sup>1</sup>**

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- *Premise of study:* Leaf shape is predicted to have important ecophysiological consequences; for example, theory predicts that lobed leaves should track air temperature more closely than their entire-margined counterparts. Hence, leaf-lobing may be advantageous during cold nights (~0°C) when there is the risk of damage by radiation frost (a phenomenon whereby leaves fall below air temperature because of an imbalance between radiational heat loss and convective heat gain).
- *Methods:* Here, we test whether radiation frost can lead to differential damage between leaf shapes by examining a leaf-shape polymorphism in *Ipomoea hederacea*, where leaves are either lobed or heart-shaped depending on a single Mendelian locus. We logged leaf temperature during midautumn, and measured chlorophyll fluorescence and survival as proxies of performance. Furthermore, we tested if the leaf-shape locus confers freezing tolerance using freezing assays on leaf tissue from different leaf shapes.
- *Key results:* We found that lobed leaves consistently remain warmer than heart-shaped leaves during the night, but that no pattern emerged during the day, and that temperature differences between leaf shapes were typically small. Furthermore, we found that leaf types did not differ in frost tolerance, but that a 1°C decrease leads to a transition from moderate to complete damage.
- *Conclusions:* Our results demonstrate that *Ipomoea hederacea* leaf shapes do experience different nighttime temperatures, and that only minor temperature differences can lead to disparate levels of freezing damage, suggesting that the differential thermoregulation could result in different levels of frost damage.

**Key words:** boundary layer; cline; energy budgets; freezing tolerance; *Ipomoea hederacea*; leaf shape; thermoregulation; radiational cooling.

Leaf shape is one of the most diverse traits among plant species, and there is a long history of investigation of the physiological, ecological, and evolutionary consequences of this variation (reviewed by Nicotra et al., 2011). Given that leaves are the primary photosynthetic surface of most plants, it is generally accepted that variation in leaf shape is likely to have evolutionary and ecological consequences. A great deal of literature focuses on the influence of leaf shape on thermoregulation under hot vs. cool or arid vs. mesic conditions (or some combination thereof); for example, highly dissected or complex leaves are thought to be more effective at maintaining cooler leaf surfaces, and avoiding excessive water loss (Givnish and Vermeij, 1976; Givnish, 1988). Comparatively fewer studies examine the impact that leaf shape may have on regulating temperature under cold, nighttime conditions, despite the well-known potential

for shape to affect convective/radiational cooling under cold conditions (Leuning and Cremer, 1988; Jordan and Smith, 1995; Lambers et al., 2006). Here, we test whether a leaf-shape polymorphism in Ivyleaf morning glory, *Ipomoea hederacea* (L.) Jacquin (Convolvulaceae), can differentially influence the thermoregulation of leaves during midautumn nights when there is an elevated risk of cold-damage as a result of radiational cooling.

Plant energy-budget theory predicts that an increase in leaf dissection will facilitate convective heat exchange by reducing the effective boundary layer (thin layer of stagnant air surrounding the leaf that impedes on the inward and outward movement of heat; Gurevitch, 1988; Schuepp, 1993; Nobel, 2005; Lambers et al., 2006). Support for the notion that leaf dissection increases convection comes in a variety of analyses; past studies have employed computer simulation analyses (Gurevitch, 1988; Roth-Nebelsick, 2001), leaf cut-outs or replica models (Vogel, 1970; Grace et al., 1980; Gottschlich and Smith, 1982; Stokes et al., 2006), and metal-coated leaves (Gurevitch and Schuepp, 1990). The boundary layer of a leaf (*BL*) can be modeled by:

$$BL = 4\sqrt{\frac{D}{V}} \quad (\text{Eq. 1})$$

where *D* is the length of the leaf, and *V* is airspeed (Nobel, 2005). Each lobe on a dissected leaf behaves like a single

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smaller leaf, and hence  $D$  is relatively small compared to an entire-margined leaf of comparable overall size. Convective heat exchange ( $H_C$ ) is expressed as:

$$H_C = -2K^{Air} \frac{(T_{Leaf} - T_{Air})}{BL} \quad (\text{Eq. 2})$$

where  $T$  is temperature and  $K^{Air}$  is the thermal conductivity of air (Nobel, 2005). Eq. 2 leads to two predictions: First, leaf dissection should increase heat- and/or drought-avoidance, because an improved  $H_C$  will enable leaves to dissipate heat across its  $BL$  swiftly, preventing the leaf from readily overheating and potentially resorting to transpirational cooling (Givnish, 1988; Nobel, 2005; Lambers et al., 2006).

Second, based on the same boundary layer and heat exchange principles, leaf dissection may function to maintain warmer leaves in cold conditions, especially during clear nights. Under these conditions, leaves would radiate infrared radiation ( $IR$ ) or long-wave heat toward the cold sky/atmosphere, potentially dropping below air temperature (see e.g., Sage and Sage, 2002); a phenomenon termed radiation cooling (Jordan and Smith, 1995).  $H_C$  moving into the leaf will constantly counter-balance the loss of  $IR$  heat, especially if the leaf falls below air temperature. However, the  $BL$  will impede on  $H_C$ , making it less efficient than  $IR$  loss. Thus, by increasing  $H_C$ —by reducing  $BL$ —dissected leaves could potentially remain warmer relative to entire margined leaves. When air

temperatures are cold enough, the difference in  $H_C$  among different shaped leaves could render some leaves susceptible to frost damage, while others may remain warm enough to avoid damage.

In contrast to predictions based on dissection reducing the boundary layer and hence  $H_C$ , an alternative set of predictions could be made based on differences in leaf perimeter between entire and lobed/dissected leaves. In particular, data from several species of varying leaf shape and type suggest that frost damage occurs primarily near leaf margins, due to reduced water content and boundary layers in these areas of the leaf (Smith et al., 2004). Because lobed and dissected leaves have greater edges and perimeters for a fixed area, these data would lead to the prediction that lobed leaves should be more susceptible to frost damage. Ultimately, the net balance of whether lobing/dissection leads to increased or decreased susceptibility to radiation frost because of the relative magnitude of its effects on radiational cooling or frost damage is an empirical issue.

The potential for differences in radiational cooling—and therefore differential frost—among leaf-shape variants has rarely been addressed. Ivy leaf morning glory, *Ipomoea hederacea*, expresses a leaf-shape polymorphism (lobed and heart-shaped) that is determined by simple Mendelian inheritance (Elmore, 1986; Bright, 1998), making it a good candidate to study leaf-shape effects on radiational cooling. The lobing allele ( $L$ ) is partially dominant to the heart allele ( $l$ ) (Elmore, 1986; Bright, 1998), such that heterozygotes ( $Ll$ ) show a less lobed phenotype (Fig. 1). Individuals undergo a developmental transition

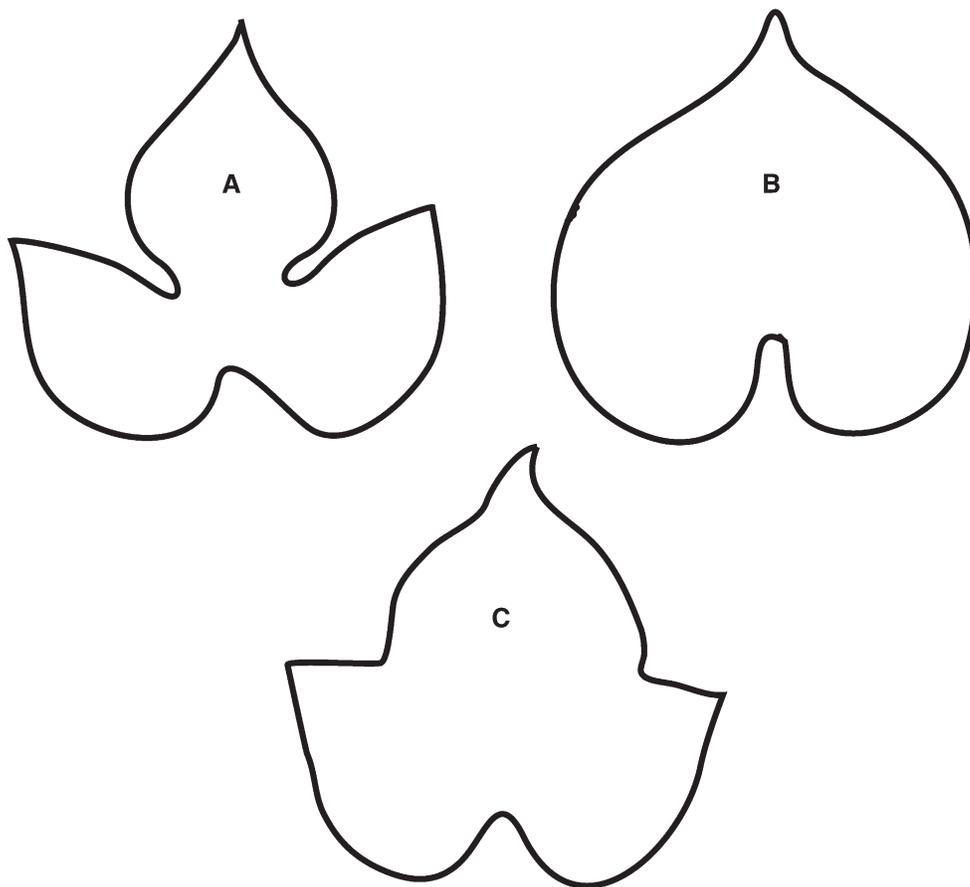


Fig. 1. Outlines of the typical shape of a leaf taken from a (A) homozygous lobed, (B) homozygous heart, and (C) heterozygous individual of *Ipomoea hederacea*.

such that all leaf-shape genotypes begin with heart-shaped leaves, and those with at least one *L* allele will express lobes by the fifth true leaf (Campitelli et al., 2008). Additionally, this polymorphism follows a putatively adaptive latitudinal cline in the eastern United States; the lobed phenotype dominates the northern part of the range, while the southern end of the range is composed of many polymorphic populations, but the heart phenotype is more abundant (Bright, 1998; Bright and Rausher, 2008; see Fig. 1 of Campitelli and Stinchcombe, 2013). The direction of the leaf-shape cline is consistent with lobed leaves in northern habitats having an advantage due to the effects of radiational cooling, and inconsistent with the hypothesis that frost damage should be greater on lobed genotypes because of their increased perimeter to area ratios. However, differences in leaf temperature and freezing tolerance between leaf shapes of this species have not previously been examined.

Here, we test whether the leaf-shape polymorphism in *Ipomoea hederacea* impacts thermoregulation during periods of radiational cooling (i.e., during cloudless nights), and whether these impacts are in the direction that could produce the observed cline in leaf shape. Specifically, we sought to answer the following questions: (1) Do the leaf-shape genotypes show consistent differences in temperature, and does this have an effect on the performance of the leaves? And (2) What temperature does *I. hederacea* leaf tissue experience freezing damage, and does this differ among leaf-shape genotypes?

## MATERIALS AND METHODS

**Natural History**—Ivyleaf morning glory, *Ipomoea hederacea*, is an annual weedy vine distributed throughout the eastern United States. It is commonly found in open fields, especially where the soil has been recently disturbed (e.g., roadside ditches, the edges of crop fields, abandoned fields), and thus frequently experiences full exposure to the sky. It germinates between May and August (depending on geographical location) and persists until the first lethal frost. Flowering is initiated approximately 6–8 wk postgermination, and often continues until a lethal frost. The production of small fruits (1 per flower) begins shortly after the initiation of flowering, and also continues until a lethal frost. Frost damage during fruit maturation has significant negative effects on seed viability (Simonsen and Stinchcombe, 2010).

**Crossing and Planting Design**—We generated crossed lines for the following experiments by crossing two inbred lines originating from two different populations in North Carolina, USA, each of which was either homozygous lobed or heart. Inbred lines had been self-fertilized in the greenhouse for 7 generations prior to being crossed. Because lobing is dominant, we used the lobed parent as the pollen donor and heart parent as the pollen recipient such that any heterozygote lobed progeny in the  $F_1$  population indicated a successful cross.  $F_2$  and  $F_3$  lines were subsequently generated by self-fertilization and single-seed descent from the  $F_1$  generation; the parents of experimental plants were grown in a common greenhouse environment to equalize maternal environmental effects. The advantage of using individuals from a cross in the following experiments was 2-fold: (1) All of our comparisons between leaf shapes were within a species (as opposed to interspecific comparisons that might be confounded with phylogenetic history), and (2) the Mendelian basis of the leaf-shape polymorphism suggests that only tightly linked genes that cosegregate with leaf shape should differ between the genotypes because recombination will break up other genetic associations, increasing the likelihood that any differences are due to leaf shape per se. The variation we observe in  $F_2$  and  $F_3$  progeny will be limited to the effects of the leaf-shape locus, and any other loci that were polymorphic between the original parents. While using only two parental genotypes for our cross will limit the amount of genetic variation segregating for quantitative traits, our ability to make conclusions about the effects of leaf shape will be unaffected, because shape is determined by a single Mendelian locus that is polymorphic in the  $F_2$  and  $F_3$  populations.

All seeds were scarified with a razor blade, and then planted into either Single Ray Leach Cone-tainers (Stuewe and Sons, Tangent, Oregon, USA;

*Rooftop temperature monitoring experiment*, described in the paragraph below) or 100 cm<sup>2</sup> square pots (Kord Products, Brampton, Canada; *Freezing tolerance assay experiment*, described below under Freezing Tolerance Assay), that were filled with a mixture of Pro-Mix (Premier Tech., Rivière-du-Loup, Canada) and triple-mix (Fairgreen Sod Co., Markham, Canada) in a 3:1 ratio, and 3–4 mo slow release Osmocote fertilizer (14-14-14 N-P-K; Scotts Co., Mississauga, Canada); 300 mL of fertilizer pellets were added to 75 L of soil mixture. Pots were bottom-watered three times weekly and Cone-tainers were both mist-sprayed twice daily and bottom-watered indefinitely to establish a solid water column. Once the plants achieved their first true leaves, they were bottom-watered three times per week. Greenhouse conditions were set to a 16-hour day photoperiod with a temperature regime of 26°C days/22°C nights.

**Rooftop Temperature Monitoring Experiment**—We first sought to determine if there is a directional pattern to the temperature ranking between lobed and heart-shaped leaves during cold evenings, and whether this could be associated with leaf performance and survival of the plants. On 26 September 2009, we planted 80  $F_2$  seeds in a greenhouse set to the conditions above, and following several weeks of growth (20 October 2009) when plants had completed their developmental transition to adult leaf shapes, we transferred these plants into a greenhouse kept at 12°C during the day, and 10°C at night for seven days to acclimate them in preparation for outdoor conditions. Prior to being acclimated, plants were actively growing but had not begun flowering or showing signs of senescence, and were ~4 wk old (plants in the field live for ~4–6 mo).

On 29 October 2009, we transferred plants into a 1.5 m by 1.5 m garden bed approximately 0.5 m deep, located on the rooftop of the Earth Sciences Centre (University of Toronto, Ontario, Canada; 43.66128°N, 79.40023°W); the three leaf-shape genotypes were randomly assigned positions within this garden. We then filled the garden bed with damp soil, completely surrounding the Cone-tainer pots to buffer the soil in each cone from freezing; frozen soil would potentially facilitate ice propagation in through the roots and lead to damage that is unrelated to thermoregulation by the leaves. To record leaf temperature, we attached copper-constantan (type-E) thermocouples (Omega, Laval, Canada) to the center of the bottom surface of a single leaf from 24 randomly selected plants (8 of each genotype) ensuring that the thermocouple tip was kept away from the midvein. The offset of each thermocouple was initially calibrated using an ice-bath at 0°C. Temperature was logged every 10 min using OM-CP-QUADTEMP thermocouple dataloggers (Omega). The experiment was terminated on the morning of 18 November 2009 (20 nights and 19 d) because of a lethal frost.

Directionality was determined by first calculating the mean temperature of each leaf shape for each time point in our time series (2826 time points). We then scored the fraction of measurements where the mean temperature of heart leaves was colder than lobed leaves. We estimated these fractions separately for nights and days: For 29 October to 31 October 2009 we used a sunset time of 6 pm and a sunrise of 8 am. Due to daylight savings time in Toronto, we used a sunset time of 5 pm and a sunrise time of 7 am for the remaining days (1 November to 18 November 2009). These cutoffs were chosen based on sunrise/sunset data from Environment Canada (<http://www.weatheroffice.gc.ca/>). Accordingly, our data set was comprised of 14-hour nights (84 time points per night, except on the night of 31 October/1 November when daylight savings brought this to 74), and 10-hour days (60 time points per day).

We evaluated leaf performance by measuring the maximum quantum yield ( $F_v/F_m$ ) of a single marked leaf on each of the 80 plants on cloudless nights (the effects of radiational cooling are diminished during overcast conditions because of a lack of exposure to an open sky) using a Pocket PEA chlorophyll fluorimeter (Hansatech Instruments Ltd., Amesbury, Massachusetts, USA).  $F_v/F_m$  can be used as a proxy of photosynthetic efficiency (Genty et al., 1989; Lambers et al., 2006).  $F_v/F_m$  values of healthy terrestrial plants are ~0.8, and decreases from this are reliable indicators of plant stress (Cavender-Bares and Bazzaz, 2004). In particular, leaf damage due to cold temperatures leads to a reduction of  $F_v/F_m$  (Strand and Öquist, 1985; Rizza et al., 2001; Bykova and Sage, 2012), allowing comparisons of  $F_v/F_m$  to be used as an indicator of cold-injury. Because  $F_v/F_m$  must be measured on dark-acclimated material (Maxwell and Johnson, 2000), we recorded it during the night, just prior to sunrise (5 am – 7 am EST). This time is also typically (although not always) the coldest time of the night, which is optimal for detecting differences in photosynthetic inhibition between leaf-shape genotypes. In addition, we scored the proportion of each genotype that survived each day (survival). We scored plants as dead if all of its leaves had completely wilted as a result of frost damage, and we verified these assessments by returning each putatively dead plant to optimal conditions in the greenhouse; we found that no plants showed any sign of regrowth. We recognize that plants may not have died strictly from freezing damage to leaves,

however, this assessment allowed us to test for associations between tolerance to cold temperatures and leaf-shape genotype.

**Freezing Tolerance Assays**—It is generally accepted that the principal cause of freezing damage in plant tissue is by disruption of the cellular membrane (Steponkus, 1984). There are various hypotheses as to how the plasma membrane may incur damage, and these are extensively reviewed in Steponkus (1984). We performed assays of freezing tolerance by measuring the amount of ion leakage through cellular membranes (first developed by Dexter et al., 1930), a method that does not require prior knowledge of the freezing injury mechanism. We used the following general protocol for each experiment, and then below describe the rationale and details that are unique to each assay.

We grew plants in the greenhouse to a size of > 15 leaves (individuals had not begun flowering), and then we removed two circular leaf discs (18 mm diameter) from the 5th–10th leaf for each plant using a copper cork-borer, rinsed them briefly with deionized distilled water (ddH<sub>2</sub>O), dab dried, and transferred leaf discs to a 20 mL glass scintillation vial. We then exposed leaf tissue to one of several target cold temperatures by using a temperature controlled chamber (S-1.2C-B Thermotron, Holland, Michigan, USA). Temperature profiles for each assay were as follows: 22°C to 4°C at a rate of 7.5°C h<sup>-1</sup>, hold at 4°C for 1 h, 4°C to 0°C at 7.5°C h<sup>-1</sup>, hold at 0°C for 1 h, 0°C to target temperature (where target refers to the temperature that is being tested) at 2°C h<sup>-1</sup>, hold at target temperature for 5 h, target to 0°C at 2°C h<sup>-1</sup>, hold at 0°C for 1 h, 0°C to 22°C at 7.5°C h<sup>-1</sup>. We used a stepwise design for our temperature profile to provide the leaf tissue enough time to adjust its physiology to the ambient conditions in the chamber, and thereby reduce the risk of tissue damage resulting from a rapid decline or increase in temperature. Once the chamber achieved the target temperature, we added ice crystals as nucleators to each vial to initiate the formation of ice, and avoid super cooling in the tissue (Zhen and Ungerer, 2008).

Following the temperature treatment, we added 15 mL ddH<sub>2</sub>O to each vial, and allowed 24 h for ions to leak and equilibrate with the surrounding water. We then measured the conductivity of the water with an Ultrameter II electrical conductivity meter (Myron L Co., Carlsbad, California, USA) to obtain our treatment conductivity ( $C_{Treat}$ ). Next, we boiled the glass vials for 30 min to induce complete damage of the leaf tissue, and subsequently allowed another 24 h to leak all remaining ions, before recording a second conductivity measurement ( $C_{Total}$ ). We calculated relative conductivity ( $RC$ ) as follows:

$$RC = \frac{C_{Treat}}{C_{Total}} \quad (\text{Eq. 3})$$

Using  $RC$  allowed us to control for natural variation in the amount of cellular contents of different plants, enabling us to compare the degree of freezing damage across samples.  $RC$  ranges from zero to one such that larger values indicate more ion leakage, and hence greater tissue damage.

**Freezing Tolerance of Non-Acclimated Tissue**—We investigated freezing tolerance in nonacclimated tissue for two reasons. First, it allowed us to determine whether there are intrinsic physiological differences in freezing tolerance among leaf-shape genotypes while eliminating differential acclimation between genotypes as a source of variation. Second, testing nonacclimated tissue also simulates early season cold snaps which might occur after plants have developed their adult leaf shape. We used 86 F<sub>2</sub> individuals (25 lobes: 45 heterozygotes: 16 hearts) that were grown in a greenhouse (conditions listed under Freezing Tolerance Assays) and tested three target temperatures (−2°C, −3°C and −4°C), as well as a control temperature (22°C) to determine baseline relative conductivity; preliminary measurements showed no damage above −2°C.

**Freezing Tolerance of Cold-Hardened Tissue**—Several studies have shown that cold-hardened plants show enhanced freezing tolerance relative to nonacclimated plants (e.g., European accessions of *Arabidopsis* from northern latitudes show a greater increase in freezing tolerance relative to accessions from southern latitudes following cold-acclimation; Zhen and Ungerer, 2008). We tested for cold acclimation, and differences in acclimation between leaf-shape genotypes, to simulate late-season conditions when all plants are exposed to chilling temperatures, and plants are typically maturing fruit. We performed our freezing tolerance assay on 73 F<sub>3</sub> individuals (34 lobes: 20 heterozygotes: 19 hearts). We grew plants to a size of >15 leaves in the greenhouse, a then carried out a two-phase acclimation treatment: (Phase 1) one week at 15°C day/10°C night with a 14 h photoperiod, and (Phase 2) a second week at 10°C day/5°C night employing the same light regime. Following each phase we carried out a

freezing tolerance assay using −4°C as our target temperature; our assay on nonacclimated material (described previously at Freezing Tolerance of Non-Acclimated Tissue) showed −4°C as lethal, indicating that differences in acclimation among genotypes would be manifest at −4°C or colder.

**Statistical Analyses**—For our temperature monitoring experiment, we examined if the fraction of time spent colder significantly deviated from 50% when comparing the two homozygous genotypes (i.e., heart vs. lobed). We split our analysis into nights and days, and performed a separate  $\chi^2$  test on each category to determine which genotype was colder more often (e.g., if heart leaves spent > 50% of the time colder than lobed leaves on night one, we scored this as heart colder). We carried out this analysis using the FREQ procedure in SAS 9.2 (SAS, Cary, North Carolina, USA). In addition to splitting the data between day and night, we also analyzed which leaf shape was warmer (or colder) during periods when the average of all leaf temperatures (i.e., both leaf shapes combined) were above and below air temperature. Because these results were similar in statistical significance and pattern to analyses of days and nights, we only report on days and nights separately.

We used permutation tests to evaluate the statistical significance of the temperature difference between lobed and heart individuals, considering nighttime and daytime measurements separately. The high-dimensionality and low sample size of our data (several thousand observations of temperature on 16 individuals) precluded traditional repeated measures ANOVA and MANOVA. In brief, we first estimated the mean difference in leaf temperature for heart and lobed individuals (i.e.,  $\bar{x}_{H} - \bar{x}_{L}$ ) for every time point. The distribution of  $\bar{x}_{H} - \bar{x}_{L}$  characterizes temperature differences between heart and lobed leaves throughout the nights of experiment; if lobed leaves are typically warmer than heart-shaped leaves, the mean of this distribution ( $\bar{x}_{difference}$ ) will be < 0. We tested the statistical significance of  $\bar{x}_{difference}$  by randomly permuting genotype labels (and leaving the rest of the data unaltered) 9999 times and estimating where the observed  $\bar{x}_{difference}$  fell in the permuted null distribution. Importantly, because all temperature measurements for a single individual are randomly assigned a new genotype label, this approach accounts for the nonindependence created by multiple measurements per individual in generating the null distribution of  $\bar{x}_{difference}$ . We implemented permutation testing using PopTools (Hood, 2010).

We compared photosynthetic efficiency of leaves ( $F_V/F_M$ ; *Rooftop temperature monitoring experiment*) and frost damage (*Freezing tolerance assays*) between leaf-shape genotypes over the duration of the experiment using repeated-measures ANOVA by employing the MIXED procedure in SAS (Littell et al., 2006), which uses restricted maximum likelihood. Fixed effects in the model included plant id, leaf-shape genotype, and trial number (i.e., each day or target temperature is a single trial for our *Rooftop temperature experiment* and *Freezing tolerance assays* respectively), and we included plant identification (id) as a repeated effect to account for repeated measurements of the same individual. For our  $F_V/F_M$  data, we report results from an unstructured covariance structure, and for our freezing tolerance assays, we report results from an autoregressive heterogeneous covariance structure, because these provided the best fit variance-covariance structures based on Akaike and Schwarz information criteria. Hypothesis tests and interpretations were robust to the choice of error covariance structure for all repeated measures models.

We analyzed the proportion of each genotype that survived using the LIFETEST procedure in SAS, which compares the time to an event (in this case, mortality) among leaf-shape genotypes.

## RESULTS

**Rooftop Temperature Monitoring Experiment**—Lobed leaves maintain warmer temperatures throughout the majority of each night; during all 20 nights, heart-shaped leaves registered as colder greater than 50% of the time ( $\chi^2 = 20$ ,  $df = 1$ ,  $P < 0.0001$ ; Fig. 2A). When we combine all nights, heart leaves register as colder than lobed leaves 1584 out of a possible 1674 time points (95%), suggesting that lobed leaves may attain convective heat more efficiently at night. In contrast, leaf shape was not an accurate predictor of temperature ranking during the day; 12.5 out of 19 d, heart leaves registered as cooler greater than 50% of the time ( $\chi^2 = 1.89$ ,  $df = 1$ ,  $P = 0.17$ ; Fig. 2B). Combining all 19 d, heart leaves registered as colder 770 out of a possible 1152 measurements (66%), suggesting that when

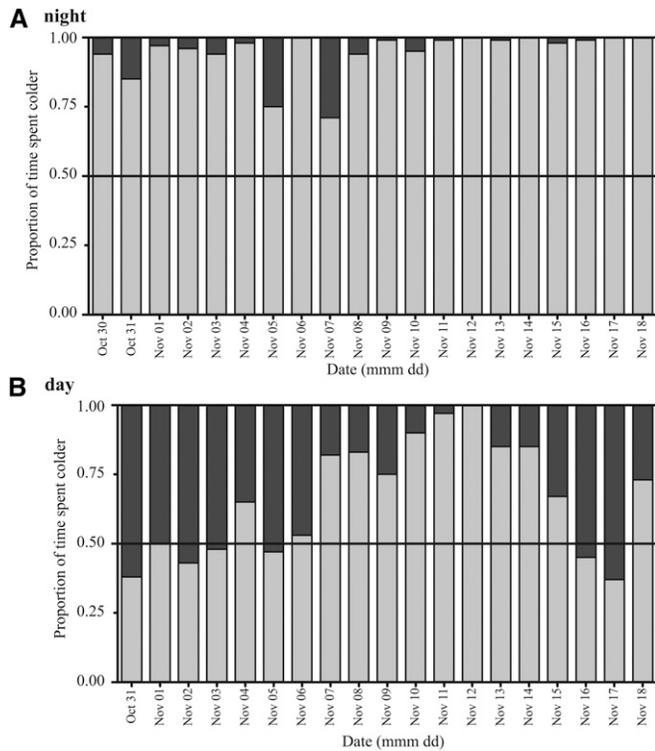


Fig. 2. The proportion of time that on average heart-shaped leaves (light gray bars) or lobed leaves (dark gray bars) are colder for each (A) night or (B) day, during our *Rooftop temperature monitoring experiment*. Each bar represents a single night or day.

exposed to sunlight, other physiological, morphological, or environmental factors are influencing leaf temperature, potentially reducing the role that leaf shape plays in governing thermoregulation. When heterozygotes are included, they appear to behave like heart-shaped individuals (Appendix S1; see Supplemental Data with the online version of this article), suggesting the lobes are not deep enough on heterozygotes to generate a similar leaf shape effect as homozygous lobed genotypes. Alternatively, a locus affecting leaf temperature that is tightly linked with the leaf-shape locus, but with opposite patterns of dominance, could also explain these results.

The magnitudes of temperature differences presented above are often narrow, i.e., the average temperature difference between leaf-shape genotypes (heart minus lobe) for each night and day are typically within 1°C (night, Fig. 3A; and day, Fig. 3B). The 95% confidence limits for the empirical null distribution, estimated from permuting genotype labels, range from -0.25 to 0.25 nighttime measurements and -0.5 to 0.51 for daytime measurements. Across all nighttime observations  $\bar{x}_{\text{difference}} = -0.16$  ( $P = 0.104$ ), while for daytime measurements  $\bar{x}_{\text{difference}} = 0.17$  ( $P = 0.26$ ). We provide a full time series of leaf temperature measurements, as well as air temperature, in Appendix S2.

Chlorophyll fluorescence ( $F_V/F_M$ ) did not significantly differ among genotypes (Table 1, Appendix S3a).  $F_V/F_M$  did significantly vary from day to day as a result of ambient temperature fluctuations (Table 1). We note that  $F_V/F_M$  was typically quite low (always < 0.6) potentially indicating chronic photo-inhibition; however, we did not detect any photo-bleaching, or signs of senescence, and frost damage only occurred on the final day of the experiment—all suggesting that leaves were recovering

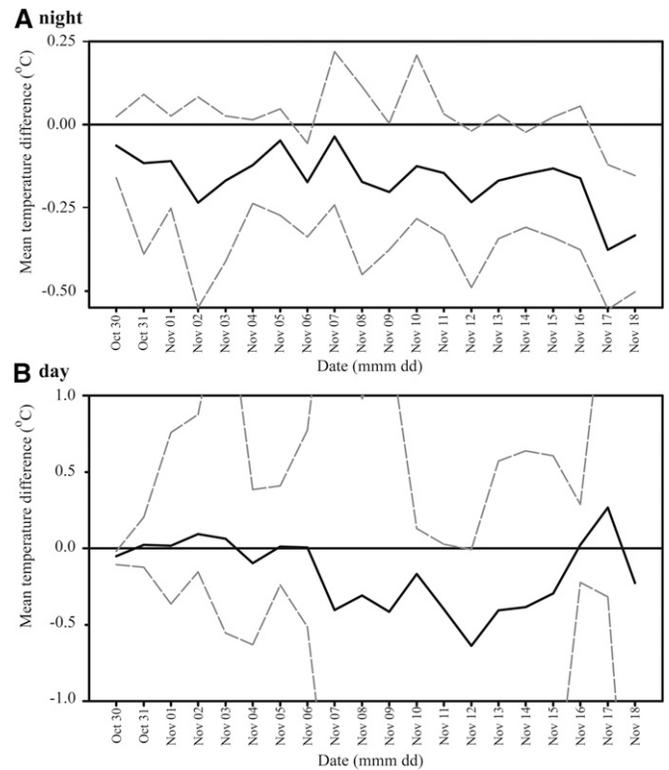


Fig. 3. The difference in temperature (mean of heart leaves minus mean of lobed leaves; solid black line) for each (A) night and each (B) day during our *Rooftop temperature monitoring experiment*. Below the zero line indicates nights (or days) that hearts were on average colder, while above the zero line indicates nights (or days) that lobes were on average colder. The gray dashed lines show the maximum positive and negative differences between leaf temperatures.

from low  $F_V/F_M$  and functioning normally during the days. In addition, even if we restrict the data on  $F_V/F_M$  to the first day of measurement (which corresponds to 4 d of rooftop growth) when  $F_V/F_M$  values are greatest, we still see no differences between leaf-shape genotypes (data not shown). Similar to our photosynthetic efficiency analysis, the proportion of plants that

TABLE 1. ANOVAs and survival statistics for our *Rooftop temperature monitoring experiment* (rooftop  $F_V/F_M$  and rooftop survival refer to results on chlorophyll fluorescence and proportion of surviving individuals respectively), and *Freezing tolerance assays* (nonacclimated and cold-hardened refer to results on nonacclimated and acclimated leaf tissue respectively).

Experiment	Source	Effect	P
Rooftop $F_V/F_M$	Genotype	$F_{2,76} = 1.69$	0.19
	<b>Day</b>	<b><math>F_{7,76} = 327.25</math></b>	<b>&lt; 0.001</b>
	Genotype × Day	$F_{14,76} = 0.75$	0.72
Rooftop survival	Genotype	$\chi^2 = 0.98$	0.61
	Genotype	$F_{2,83} = 0.49$	0.61
Nonacclimated	<b>Treatment</b>	<b><math>F_{3,246} = 718.42</math></b>	<b>&lt; 0.001</b>
	Genotype × Treatment	$F_{6,246} = 0.67$	0.67
	Genotype	$F_{2,70} = 0.19$	0.83
Cold-hardened	<b>Phase</b>	<b><math>F_{2,140} = 156.83</math></b>	<b>&lt; 0.001</b>
	Genotype × Phase	$F_{4,140} = 2.04$	0.093

Notes: Boldface indicates significance. The  $\chi^2$  test for rooftop survival used two degrees of freedom.

remained alive following each night was not significantly different among genotypes (Table 1, Appendix S3b).

**Freezing Tolerance Assays**—Two important findings emerged from our assay of freezing tolerance on nonacclimated material: (1) A decrease in temperature of 1°C (treatments of –2°C to –3°C) results in a considerable increase in cellular leakage suggesting that *Ipomoea hederacea* is quite sensitive to even small changes in temperature, and (2) the three leaf-shape genotypes do not differ in their ability to tolerate cold temperatures (Fig. 4A; Table 1).

Cold-hardening did not significantly increase the freezing tolerance of individuals, nor did it generate significant differential freezing tolerance among genotypes (Fig. 4B). There was a trend for lobed leaves to incur the least damage after *phase 1*, but the most damage after *phase 2* of cold-hardening, leading to a trend in the interaction term genotype  $\times$  phase (Table 1). However, the biological relevance of this interaction is unclear, because all leaf tissue experienced extremely high levels of freezing damage (i.e., > 70% relative conductivity which is likely to be lethal) in both phases of the assay (Fig. 4B).

## DISCUSSION

Three main results emerged from our study of thermoregulation of *Ipomoea hederacea* leaf-shape genotypes: First, we confirmed the prediction that lobed individuals maintain warmer leaves during clear and cold nights relative to heart-shaped leaves. Second, although this pattern is upheld consistently throughout each night of the experiment, the extent of the temperature difference between these two homozygous genotypes is typically small. As a result, the performance measures we recorded were not significantly different among genotypes. Third, *I. hederacea* leaves are sensitive to temperatures below –2°C (–4°C when acclimated), and the temperature difference between leaf tissue that incurred moderate damage and highly damaged tissue is a single degree Celsius. We first elaborate on these results below, and then, in light of our findings, we discuss the hypothesis that radiational cooling may contribute to the latitudinal cline for leaf shape in *I. hederacea*.

**Differential Radiational Cooling, Leaf Performance, and Frost Tolerance**—Our rooftop experiment provides evidence that lobed leaves are generally more efficient at maintaining warmer temperatures than heart-shaped leaves during midautumn nights (Fig. 2A), and that this pattern is quite stable, occurring 95% of the logged measurements. Hence, we find support for the prediction that lobed leaves remain warmer at night, potentially as a result of boundary layer differences. We failed to detect any evidence that lobing, by increasing perimeter to area ratios, leads to increased frost damage. However, the extent of the temperature disparity between lobes and hearts was quite small, often within 1°C (Fig. 3A), and only approached marginal significance. Furthermore, we did not detect differential survival among the three leaf-shape genotypes throughout the duration of the experiment.

It is possible that the temperature patterns we detected may result from physiological or phenotypic traits (e.g., trichome density, stomatal density/conductance, leaf thickness)—that are known to influence thermoregulation—and are potentially genetically linked with leaf shape. Preliminary work has demonstrated that *Ipomoea hederacea* exhibits a great deal of variation

in stomatal density—potentially leading to transpirational differences—between heart and lobed lines, but there are no mean differences between these lines for both trichome density or leaf thickness/toughness (K. L. Ostevik and J. R. Stinchcombe, unpublished data). Although stomata are thought to mostly close at night when photosynthesis is not occurring (Lambers et al., 2006), there is increasing evidence that nocturnal conductance may occur (Caird et al., 2007), albeit at a greatly reduced level; as such, transpiration rates should not be a major factor at night. For any of these traits to contribute to temperature differences between leaf-shape genotypes, loci influencing them would have to be physically linked to the leaf-shape locus. We note that the preliminary data on stomatal density and trichomes were taken on inbred lines (including the parents of our crossed lines here), and further exploration of such physiological traits in an F<sub>2</sub> population is required.

While the directionality of temperature differences was consistent at night, the magnitude of the disparity between leaf morphs was small. A possible explanation for the small difference is that variation between the microclimate of each plant in our experimental garden was large enough to reduce the potential of detecting temperature differences. Our experiment was designed to explore the overall behavior of leaf shapes within a population rather than focus on individual leaves, because we were ultimately interested in whether radiational cooling could differentially impose selection on leaf shape within a population. An alternative experimental setup would have been to organize our plants in triplicate (one of each genotype), and carry out our measurements treating each triplicate as a replicate. Such a design would minimize microclimatic variation within a triplicate (i.e., by standardizing the height of leaves from the ground, direction leaves are facing, size of leaves etc.), while potentially maximizing the temperature differences between leaf shapes. However, it is unlikely that individuals would be arranged as such in nature, and hence a triplicate design might not reveal biologically important effects likely to produce natural selection in the field. Instead, our experimental design reflects a more natural situation, that is likely more conservative with respect to detecting differences, and so the consistency of our results likely represent a real effect.

Data from other plant species are at times consistent with the hypothesis that an increase in leaf lobing (dissection) may be an adaptation for cooler climates. Typically the pattern of leaf lobing correlating with cooler temperatures is supported if the plants in question experience ample exposure to the sky, and hence possess a higher risk of radiation frost (Jordan and Smith, 1995). For example, Royer et al. (2009) demonstrated that *Acer rubrum* L. seeds originating from northern populations yield trees that express leaves with greater dissection compared to seeds derived from southern populations in North America, and that these traits were negatively correlated with mean annual temperature. Jordan and Smith (1995) compared leaves of *Taraxacum officinale* F.H. Wigg. and *Erigeron peregrinus* (Banks ex Pursh) Greene along two sky exposure gradients, with the goal of evaluating the relationship between leaf size and susceptibility to radiation frost: (1) An elevation gradient with higher elevation populations experiencing a reduced *IR* input, and (2) a microsite exposure gradient with the amount of canopy coverage determining *IR* input. In both scenarios they discovered that leaf size decreased with a reduction in *IR* input, supporting the idea that a reduced boundary layer may be advantageous when there is an increased risk of radiation frost conditions.

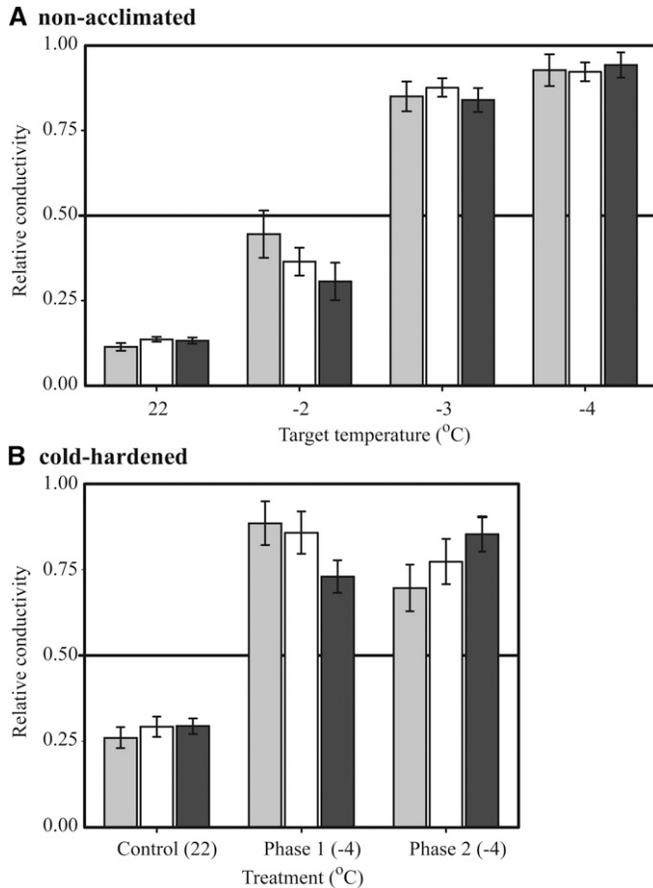


Fig. 4. Relative conductivity (mean  $\pm$  SE) of (A) nonacclimated and (B) cold-hardened leaf tissue from our *Freezing tolerance assays*. Light gray bars represent heart-shaped individuals, white bars represent heterozygotes, and dark gray bars represent lobed individuals. Phase 1 and 2 refer to the two stages of acclimation we used prior to scoring relative conductivity. Note that in both phases in (B), leaf tissue was treated at  $-4^{\circ}\text{C}$ .

Leaf dissection, lobing, or smaller size, however, does not always correlate with an increased risk of radiation frost. For example, Gurevitch (1988) demonstrated that low-altitude individuals of *Achillea millefolium* L. have highly dissected leaves relative to high-altitude individuals of the same species. Gurevitch (1988) interpreted these findings in the context of when the plant is photosynthetically active during the daytime; she concluded that low-altitude plants express highly dissected leaves that can couple more closely to air temperature, enabling these individuals to avoid overheating. The alternate ecological patterns associated with leaf shape (dissection associated with heat dissipation or coupling with air temperatures) highlights an important point, i.e., leaf shape is a complex phenotypic trait that is likely evolving under multiple selective forces (Nicotra et al., 2011), and that similarly shaped leaves are sometimes predicted by functional arguments to perform well under contrasting environments.

Although radiation frosts only occur at night, we also explored daytime leaf temperature to determine if there were any consistent patterns. We found no evidence that the different leaf shapes had significantly different daytime temperature (Figs. 2B and 3B), which was unexpected given the extensive literature suggesting that lobed leaves should remain relatively cooler

under sunny conditions (Givnish and Vermeij, 1976; Givnish, 1988). In fact, although not significant, our data are typically in the opposite direction of the predicted pattern, suggesting that other factors contribute to daytime thermoregulation. It is likely that other factors play a major role in determining leaf temperature during the daytime (e.g., transpiration), that diminish the importance of leaf shape.

Photosynthetic efficiency, as measured by leaf fluorescence ( $F_v/F_m$ ), typically tracks temperature fluctuations closely (see e.g., Groom and Baker, 1992; Bilger et al., 1995; reviewed by Maxwell and Johnson, 2000). Accordingly, the absence of significant differences among leaf-shape genotypes in  $F_v/F_m$  was unsurprising. Daytime measurements of  $F_v/F_m$  would be useful for testing whether there were any slight or lingering effects of the low nighttime temperature on leaves, and we plan to incorporate these into future studies.

Nonacclimated leaf tissue suffered near complete freezing damage at  $-4^{\circ}\text{C}$ , and was not different among the three leaf-shape genotypes (Fig. 4A). Additionally, *Ipomoea hederacea* showed no noticeable acclimation response because cold-hardened leaf tissue, irrespective of leaf-shape genotype, also experienced near-complete freezing damage at  $-4^{\circ}\text{C}$  (Fig. 4B). The lack of acclimation-potential is somewhat surprising given that *I. hederacea* occupies a range that extends into Pennsylvania, USA, and thus is regularly exposed to cold acclimating temperatures. Given the lack of acclimation response, this suggests that the morphological effects of leaf shape on radiational cooling might be pronounced.

**Clinal Variation in Leaf Shape**—One of our motivations for exploring the thermoregulatory and frost-tolerant properties of *Ipomoea hederacea* under cold conditions is derived from the prevalence of lobed individuals occupying the northern end of its range (Bright, 1998; Campitelli and Stinchcombe, 2013). While we were not able to detect significant performance differences among leaf-shape genotypes in our experiments, *I. hederacea* leaf tissue is highly sensitive to subzero temperatures—as revealed by our freezing tolerance assays—with complete damage being induced with only a  $1^{\circ}\text{C}$  decrease (Fig. 4A). Hence, even minor differences in leaf temperature could potentially lead to significantly disparate levels of freezing injury. We thus cautiously hypothesize that, on rare occasions air temperature may stabilize on a critical value that causes differential damage among leaf phenotypes, resulting from the interaction of radiational cooling and convective heat transfer. Such events could translate to fitness differences if this were to happen in the early stages of the lifecycle when the plants are particularly vulnerable (e.g., Army and Upper, 1973; Agrawal et al., 2004), or in later stages when they still require photosynthetic energy to mature their fruits/seeds. Whether these rare events occur frequently enough over generations to provide lobed individuals a fitness advantage in northern populations—that experience these conditions more frequently—remains an open empirical question. Future studies that manipulate the degree of radiational cooling (see Aston and Paton, 1973, for potential methodology) and assess whether the two leaf shapes experience differential damage at a critical temperature in a controlled setting would be beneficial. Such an experiment, combined with an exploration of historical climate data on the frequency with which the critical temperature occurs across *I. hederacea* populations, would allow population genetic modeling to determine if these events have the potential to lead to a fitness advantage for lobed genotypes in northern populations.

**Conclusions**—The purpose of our study was to determine if radiational cooling could lead to consistent temperature differences between the distinct leaf shapes of *Ipomoea hederacea*, and whether any differences in thermoregulatory ability could result in consequences for fitness components or physiological performance. Our study is the first, to our knowledge, to demonstrate that different leaf shapes within a species can exhibit consistent differences in temperature at night as a result of radiational cooling, although we were not able to detect significant differences in physiological performance or fitness components. Further exploration into the functional importance of radiational cooling by manipulating the degree of exposure of plants to an open sky—by covering some individuals while leaving others uncovered at nighttime—and tracking leaf temperature and photosynthetic efficiency in larger experimental field settings, will help to determine the significance of the risk of radiation frost as a selective agent contributing to the evolution of leaf shape.

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