EVOLUTION OF THERMOTOLERANCE IN SEASONAL ENVIRONMENTS: THE EFFECTS OF ANNUAL TEMPERATURE VARIATION AND LIFE-HISTORY TIMING IN *WYEOMYIA SMITHII*

Gregory J. Ragland[1,2,3] and **Joel G. Kingsolver**[1]

[1]*Department of Biology, CB-3280, University of North Carolina, Chapel Hill North Carolina 27599*

[2]*Department of Entomology and Nematology, PO Box 110620, University of Florida, Gainesville, Florida 32611*

[3]*E-mail: gragland@ufl.edu*

In organisms with complex life cycles, the adaptive value of thermotolerance depends on life-history timing and seasonal temperature profiles. We illustrate this concept by examining variation in annual thermal environments and thermal acclimation among four geographic populations of the pitcher plant mosquito. Only diapausing larvae experience winter, whereas both postdiapause and nondiapause adults occur only during the growing season. Thus, adults experience transient cold stress primarily during the spring. We show that adult cold tolerance (chill coma recovery) is enhanced in spring-like conditions via thermal acclimation but is unaffected by diapause state. Moreover, adult mosquitoes from northern populations were more cold tolerant than those from southern populations largely because acclimation responses were steeper in the north. In contrast to cold tolerance, there was no significant acclimation of heat tolerance (heat knockdown), and no significant differences in heat tolerance between northern and southern populations. Field temperature data show that because of evolved differences in diapause timing, adult exposure to cold stress is remarkably consistent across geography. This suggests that geographic variation in cold tolerance may not be the result of direct selection on adults. Our results illustrate the importance of the interplay between phenological and thermal adaptation for understanding variation along climatic gradients.

KEY WORDS: Acclimation, cline, cold tolerance, diapause, geography, heat tolerance, phenology.

Tolerance of stressful (deleterious) temperatures is a major adaptation to thermally variable environments. All organisms are physiologically limited to a defined temperature range (Huey and Kingsolver 1993; Hoffmann et al. 2003), and in seasonal environments with high annual temperature variation stressful temperatures at the limits of that range are frequently encountered at some point during the life cycle (Stevens 1989). The general expectation is that populations from colder habitats are more cold tolerant, whereas populations from warmer habitats are more heat tolerant. This expectation is often confirmed, suggesting geographic variation in temperature-mediated selection (Van Berkum 1988; David

et al. 2003; Castaneda et al. 2004; Sorensen et al. 2005; Winne and Keck 2005; Collinge et al. 2006).

Rearing temperature often substantially influences temperature stress tolerance (Levins 1969; Huey et al. 1999; Bubliy and Loeschcke 2005), and these acclimation responses can evolve (Hoffmann and Watson 1993; Kingsolver and Huey 1998). Consider a typical temperate environment with well-defined summers and winters. In this seasonally fluctuating environment, stressful cold temperatures occur primarily when average temperatures are relatively low. Therefore, increased cold tolerance expressed at low developmental temperatures would be expected to confer a

selective advantage. In contrast, selection rarely acts directly on cold tolerance expressed at higher developmental temperatures because stressful cold temperatures rarely occur during the warmest portion of the year. Thus, selection may favor individuals with strong cold acclimation responses in addition to individuals that are cold tolerant across immediate environmental or long-term developmental temperatures. Because a relationship between pre-exposure temperature and thermotolerance is widespread, acclimation responses are arguably the most appropriate phenotype for assessing local adaptation to temperature extremes (David et al. 2004).

Empirical evidence suggests that thermotolerance frequently varies with geography, but there is limited evidence for intraspecific geographic variation in acclimation responses for thermotolerance. Most studies with *Drosophila* have failed to find any evidence for intraspecific variation in acclimation, often across large geographic distances (Yamamoto and Ohba 1982; Kimura 1988; Hoffmann and Watson 1993; Gibert and Huey 2001; Bubliy et al. 2002; Hoffmann et al. 2005), although Ayrinhac et al. (2004) present a notable exception. Klok and Chown (2003) found no intraspecific variation in thermotolerance acclimation across elevation in sub-Antarctic weevils, whereas Hawes et al. (2007) inferred latitudinal variation for supercooling point acclimation in the Antarctic mite *Halozetes belgicae* based on the results of multiple studies. Thus, there is some evidence for geographic variation in thermotolerance acclimation, but studies outside of the *Drosophila* literature are rare.

Both spatial and temporal heterogeneity in the thermal habitat of a given population may complicate geographic trends in selection on thermotolerance and acclimation responses. Temperature varies with topography, vegetative cover, soil depth, and other environmental factors, and a number of studies demonstrate how selection on thermotolerance can vary with microhabitat (Kingsolver 1979; Leather et al. 1993; Feder et al. 1997; Sinclair 2001; Irwin and Lee 2003). Likewise, both predictable (e.g., diurnal, seasonal) and unpredictable (e.g., storm front) temporal fluctuations in temperature exert variable selection on thermotolerance (Tauber et al. 1986; Kostal and Simek 2000; Zani et al. 2005b; Danks 2006). Seasonal variation is particularly important because thermal conditions may vary greatly among life-history stages or events.

In organisms with complex life cycles such as insects, particular life-history stages or physiological states (e.g., diapause) are often timed to coincide with specific seasonal time windows. Seasonal timing may impact environmental conditions experienced by particular stages or physiological states (Bradshaw et al. 2004; Ragland and Kingsolver 2007). Thus, selection on acclimation and thermotolerance may depend on life stage or physiological state when temperatures fluctuate seasonally. Thermotoler-

ance does often vary across the life cycle (Lee and Denlinger 1985; Kostal 1994; Kostal and Simek 1995; Krebs 1999; Tsutsayeva and Sevryukova 2001; Zani et al. 2005a; Terblanche et al. 2007), and this variation is often well explained by field thermal ecology (Evans and Brust 1972; Lee and Denlinger 1985). In studies of geographic variation in thermotolerance, however, the selective forces that drive local adaptation are often inferred from average temperatures or geographic proxies such as altitude or latitude (e.g., Krebs and Loeschcke 1995; Gibert and Huey 2001; Hallas et al. 2002; Hoffmann et al. 2002; David et al. 2003; Castaneda et al. 2004). Without knowledge of life-history timing and annual temperature variation, it is unclear which life-history stages are under direct selection for temperature tolerance.

To explore the relationship between seasonal timing, thermal environment, and temperature tolerance, we measured adult heat and cold tolerance across rearing temperatures in four geographic populations of the pitcher plant mosquito, *Wyeomyia smithii*. Local adaptation of life-history timing has been studied extensively in this temperate mosquito (e.g., Bradshaw and Lounibos 1977; Bradshaw and Holzapfel 1986; Bradshaw et al. 2000, 2004), facilitating geographic comparisons of the thermal environment of specific life stages or physiological states. Of particular significance is the timing of hibernal diapause. *Wyeomyia smithii* overwinters in larval diapause (a dormant, metabolically depressed state), and diapause in higher latitude populations is timed such that the growing season is relatively shorter than that of lower latitude populations (Bradshaw and Lounibos 1977).

Bradshaw et al. (2004) showed that higher latitude populations attain higher fitness than lower latitude populations when exposed to stressful winter conditions in the diapausing stage, whereas the reverse is true in stressful summer conditions experienced during active growth and reproduction. Here we ask whether variation in temperature stress tolerance of adults accompanies this geographic variation in fitness. Because diapause occurs during the larval stage in *W. smithii*, the adult stage only encounters the thermal environment associated with the warmer months of the year. By focusing on a single stage with a known seasonal window we can examine direct associations between environmental temperature and acclimation responses.

We address four specific questions about selection on and evolution of thermotolerance and acclimation responses of adult *W. smithii*. First, is there geographic variation in tolerance and acclimation to cold? Second, does diapause state alter cold tolerance? Third, is there geographic variation in tolerance and acclimation to heat? Finally, how does phenological (life-history timing) adaptation impact patterns of selection on cold and heat tolerance via alteration of the frequency of stressful cold or hot temperatures experienced by adult mosquitoes?

Methods

STUDY ORGANISM AND SAMPLING

Adult female pitcher plant mosquitoes obligately oviposit into the water-filled leaves of the purple pitcher plant (*Sarracenia purpurea*), where the larvae develop until eclosion. Photoperiodic cues induce and terminate a diapause state occurring in the larval stage. Long-day conditions promote direct development, whereas short-day conditions induce diapause (Bradshaw and Lounibos 1972).

Geographic populations of *W. smithii* are found in eastern North America, ranging from northern Florida to the Great Lakes region inland and Newfoundland along the coast (Armbruster et al. 1998). Adults are weak flyers and the host plants are patchily distributed, so gene flow between populations is minimal or absent (Istock and Weisburg 1989). Populations arrayed along a latitudinal cline differ in critical photoperiod (CP), the photoperiod at which 50% of a sample initiates or terminates diapause (Bradshaw and Lounibos 1977). CP increases (and the length of the growing season concomitantly decreases) with increasing altitude and latitude (Bradshaw and Lounibos 1977). Phylogeographic analyses suggest that geographic populations fall into two distinct, intraspecific clades: a northern clade occurring north of North Carolina or at high elevations in North Carolina, and a southern clade from North Carolina to Florida (Armbruster et al. 1998; W. E. Bradshaw, unpubl. data).

We collected ∼1000 larvae from 50 or more individual pitcher plants in the spring and fall of 2004 from each of four geographic populations (Table 1). Two of these populations are from the southern clade (FL and NC Coast), two from the northern (NC Mtn., MAS), and all have diverged in diapause timing as determined by CP (Bradshaw and Lounibos 1977). Moreover, there is a clear distinction between northern and southern clade populations in the frequencies at which they experience hot summer and cold winter temperatures (Ragland and Kingsolver 2007). Sampled larvae were brought to the laboratory and reared to adulthood as in Hard et al. (1992) under long-day conditions (LD 16:8) and fluctuating (sinusoidal) temperatures ranging from 13°C to 29°C. Adults were allowed to swarm in five gallon cages provided biweekly with a fresh pitcher leaf and a honey-water-soaked sponge

Table 1. Geographic locations of the sampled *W. smithii* populations.

Geographic Location	Lat./Lon. (deg)	Altitude (m)
Florida gulf coast (FL)	30°N 85°W	10
North Carolina coast (NC Coast)	34°N 78°W	20
North Carolina mountains (NC Mtn.)	35°N 83°W	900
Central Massachusetts (MAS)	42°N 72°W	265

for nutrition. Eggs were collected every three days until all adults in a cage had died. Freshly hatched larvae were then reared under diapause-inducing conditions (LD 8:16, 19° C) to synchronize all individuals in the same developmental stage. Diapausing larvae were then returned to long-day conditions to initiate the next generation. We maintained laboratory colonies at >500 individuals per population per generation, and we initiated new generations with a constant proportion of eggs from each egg collection date. Armbruster et al. (1997) calculate that even with a smaller colony size (250) propagated for 20 generations the expected decline in additive genetic variance is only 5.8%. Moreover, variation in body size and development time within and among three of the four populations was similar in the F_2 and F_3 generations (Ragland and Kingsolver, 2008; G. Ragland, unpubl. data).

EXPERIMENTAL DESIGN

We assayed cold and heat tolerance of adults using three experiments: tolerance and acclimation to cold in two rearing treatments with fluctuating temperatures in nondiapause conditions (Experiment I); cold tolerance in postdiapause conditions (Experiment II); and tolerance and acclimation to heat in two fluctuating temperature rearing environments in nondiapause conditions (Experiment III).

Experiment I. Tolerance and acclimation to cold: Chill coma recovery

Chill coma recovery assays are commonly used to assess cold tolerance in arthropods (David et al. 1998, 2003; Gibert and Huey 2001; Gibert et al. 2001; Castaneda et al. 2005; Zeilstra and Fischer 2005). In the assay, individuals are exposed to a stressful cold temperature (often 4°C or lower) for an extended period of time, inducing a comatose state. After this exposure, individuals are returned to a more benign temperature and measured for recovery time, or the latency to regain locomotory capacity (David et al. 1998). This assay often correlates well with other measures of cold tolerance in *Drosophila* (Anderson et al. 2005), although cold tolerance responses in insects are complex, and variation in chill coma recovery does not always capture all of the variation in other metrics of tolerance (Lee and Denlinger 1990; Rako and Hoffmann 2006). However, tropical *Drosophila* species exhibit consistently longer chill coma recovery times than temperate species, and similar latitudinal trends are often observed intraspecifically as well (Hallas et al. 2002; Hoffmann et al. 2002; Castaneda et al. 2005). This evidence suggests that the chill coma assay is associated with general resistance to cold stress (Gibert et al. 2001).

To assess geographic variation in both plastic responses to rearing temperature and average cold tolerance, we measured chill coma recovery of adults from each of the four geographic populations (MAS, NC Coast, NC Mtn., and FL) reared from egg hatch to adulthood in two fluctuating environments designed to

mimic natural diurnal temperatures measured in pitcher plants in the field during a relatively warm ("Warm" treatment, $\bar{T} = 22°C$, range $= 16.5°C$) and relatively cool ("Cool" treatment, $\bar{T} = 18°C$, range $= 16.5°C$) spring day (Fig. 1). Daily minima at or below 0°C are much more likely to occur during the spring than during the summer, so warmer rearing temperatures are less realistic. We avoided using colder temperatures to avoid potentially high mortality, as survival to pupation is relatively low (<50%) when larvae are reared at 16°C constant (Ragland and Kingsolver, 2008).

From the F_4 laboratory generation we initiated cohorts of newly hatched (within 24 h) larvae by haphazardly selecting and transferring 25 individuals to a 150×25 mm culture dish filled with 170 mL distilled water. Eight cohorts per population were assigned to each temperature treatment. Each dish was initially provided with 1.00 mL standard food suspension (0.05 g/mL 4:1 guinea pig chow to freeze-dried brine shrimp), and larvae were transferred to fresh dishes once per week until all larvae had pupated. Fresh food suspension was provided at each transfer in a temporal pattern designed to mimic food levels in a pitcher plant leaf (Bradshaw and Holzapfel 1986): 1.75, 2.5, and 3.0 mL were added for the first three weeks, and 2.5 mL every week thereafter. Pilot studies suggested that this feeding regime maintained ad libitum conditions (G. Ragland, unpubl. data).

Commencing on the day that the first pupa appeared, pupae were removed from dishes, sexed, and transferred to individual wells in 24-well culture plates. From these pupae we selected a subset from each dish to obtain a uniform sample across the entire range of pupation dates in a single dish. Selected pupae were transferred to 50 mL centrifuge tubes with 15 mL distilled water for eclosion, and two days after eclosion each adult was

Figure 1. Diurnal temperature profiles for the rearing treatments in the chill coma recovery (black lines) and heat knockdown (gray lines) experiments. Diurnal temperatures fluctuated around means ($\bar{T}$) of 20 and 27°C for the heat knockdown experiment and around means of 18 and 22°C for the chill coma experiment.

assayed for chill coma recovery. All assays were carried out from 1300 to 1400 h to avoid any confounding diurnal effects. The assay itself consisted of (1) a 20-min exposure to 24°C, during which individuals were transferred to stoppered glass test tubes, (2) immersion in melting ice (0°C) for 1.5 h, and (3) return to 24°C. Immediately after returning the comatose adults to 24°C, we recorded the time required for a righting response (comatose individuals lie on their backs). Mortality was low in both rearing environments (<8%; similar for all populations). Sample sizes for the 18 and 22°C mean treatments were 83 and 77 (FL), 75 and 54 (NC Coast), 79 and 73 (NC Mtn.), and 67 and 51 (MAS).

Experiment II. Chill coma recovery and diapause status
Thermal tolerance in insects can depend on life-history stage (Krebs and Loeschcke 1995), and pupal and adult *W. smithii* are substantially less tolerant of extreme temperatures than the egg and larval stages (Zani et al. 2005a). Similarly, the physiological state of a given life-history stage may influence thermotolerance. In *W. smithii* the postdiapause generation (individuals undergoing larval diapause before developing to adulthood) that develops in the spring is more likely to experience stressful cold temperatures than nondiapause generations. Any carry-over physiological effects of diapause could potentially alter the physiology of post-diapause compared to nondiapause development (e.g., Tauber and Tauber 1987). Indeed, reproductive success is much reduced in postdiapause compared to nondiapause generations in *W. smithii* (Bradshaw et al. 1998). Because Experiment I was conducted during nondiapause development, we also measured cold tolerance of postdiapause individuals from two populations. From the F_3 laboratory generation we haphazardly selected diapausing larvae from the FL and MAS laboratory colonies to initiate five cohorts per population. These larvae were maintained for three months at 8:16 LD and 18°C constant, with 30 larvae per dish. After three months, the cohorts of diapausing larvae were transferred to the "cool" treatment and reared concurrently with the nondiapausing larvae (long-day conditions caused the larvae to break diapause and develop to adulthood). Chill coma assays were performed as described above.

Experiment III. Tolerance and acclimation to heat: Knockdown proportion
Knockdown temperature is often used to measure heat tolerance in arthropods (Huey et al. 1992; Berrigan and Hoffmann 1998; Hoffmann et al. 2002; Bubliy and Loeschcke 2005; Hoffman 2005; Sorensen et al. 2005). This trait is typically measured by exposing individuals or groups to increasing temperatures and recording the temperature at which an individual becomes comatose (Huey et al. 1992). Heat knockdown temperature responds readily to selection in *Drosophila* and has been shown to correlate with additional heat tolerance phenotypes (Huey et al. 1992; Bubliy and
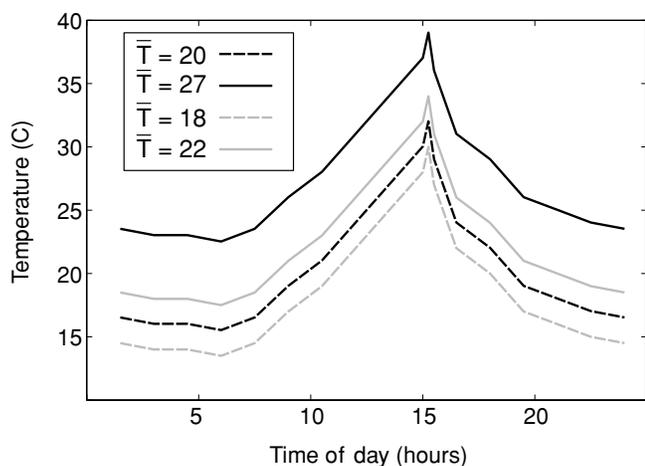
Loeschcke 2005). However, traits such as critical thermal maximum and upper lethal temperature are often uncorrelated with heat knockdown (Hoffmann et al. 1997, 2003; Folk et al. 2007). These results caution that heat knockdown, although clearly related to heat tolerance, may be governed by different physiological mechanisms than other traits contributing to heat tolerance.

Geographic and genetic variation in survival of stressful high temperatures has previously been assessed in *W. smithii* (Armbruster et al. 1999). We employed a modification of a knockdown assay to examine an additional, potentially uncorrelated heat tolerance phenotype. Rather than measuring knockdown temperature, we measured the proportion of a population sample that maintained locomotory ability after a fixed exposure to a fixed temperature. This assay is a variant of the static method defined by Lutterschmidt and Hutchison (1997), wherein a single stressful temperature (rather than ramping temperatures) is employed. Because a low knockdown proportion indicates high heat tolerance, we report (1-knockdown proportion) as the measure of heat tolerance.

To measure both acclimation and tolerance as in the previous experiment, we reared eight cohorts of larvae from each population under two fluctuating temperature regimes designed to mimic a typical cool ("Cool" treatment, $\bar{T} = 20°C$, range $= 16.5°C$) and warm ("Warm" treatment, $\bar{T} = 27°C$, range $= 16.5°C$) summer day in the field (Fig. 1). From the F_5 generation we selected newly hatched larvae, reared cohorts, and selected pupae as described above. Two days after eclosion, adults were transferred to 15-mL centrifuge tubes and allowed to equilibrate at 24°C for 15 min. The centrifuge tubes were then immersed in a temperature-controlled water bath held at $42.5 \pm 0.1°C$ for 5 min. This temperature and duration of exposure were predetermined to induce heat coma in 20–60% of adults sampled from each population. After the 5-min exposure, tubes were returned to 24°C and individuals were scored as either able or unable to fly. Under benign environmental conditions *W. smithii* will fly upwards when disturbed. Gently rapping mosquito-containing centrifuge tubes on the laboratory bench elicited a very clear-cut response: the individual either remained at the bottom of the tube or flew toward the top. Sample sizes for the 20 and 27°C mean treatments were 84 and 104 (FL), 113 and 84 (NC Coast), 54 and 76 (NC Mtn.), and 90 and 94 (MAS).

STATISTICAL ANALYSES

For the analysis of nondiapause development (Experiment I), fixed effects of population, temperature, and sex on chill coma recovery time were analyzed in a likelihood framework using a linear fixed effects model implemented in Proc Mixed, SAS ver. 9.1 (SAS Institute 2004, Cary, NC). Chill coma recovery times were natural log transformed prior to analysis to improve normality and homoscedasticity. Initially we analyzed the data in a mixed model including the random effect of cohort nested within temperature by population, but because this random effect did not significantly improve the fit of the model (assessed by the Akaike Information Criterion, or AIC), it is not included here. We calculated an AIC score for the model that included all main effects, then calculated AIC scores for more complex models containing all possible combinations of interactions between population, temperature, and sex. The results we present include only the best of these models selected by the (lowest) AIC (Johnson and Omland 2004). In addition to the best model, we also present *F*-statistics associated with each model term. In cases in which *F*-tests indicated a nonsignificant ($P > 0.05$) main effect, comparison to the AIC score for a model excluding that main effect always indicated nonsignificance. From the linear model we also estimated linear contrasts to compare the average value across temperatures and the slope of the relationship between rearing temperature and chill coma among populations (Ragland and Kingsolver 2007). Rather than calculating all pairwise contrasts, we compared the mean slope of the two northern clade populations to the mean slope of the two southern clade populations.

The effects of physiological state (non- vs. postdiapause development: Experiment II) on chill coma recovery were also analyzed in a fixed effects linear model (SAS Proc Mixed) including terms for population, sex, temperature, and physiological state. Cohort effects were again excluded from the model as they did not lower the AIC. The best-fitting model was selected as above, and only the best model is presented in the results.

Because heat knockdown data (Experiment III) were binary, we applied a logistic fixed effects model including population, temperature, and sex as effects (SAS Proc Glimmix). Model selection and linear contrasts were carried out as above.

WEATHER DATA

Data from temperature loggers placed in individual pitcher leaves in the field show that temperatures in pitcher leaves are highly correlated with local air temperature (Kingsolver 1979) and weather station data (fig. 2 in Bradshaw et al. 2000). To confirm correlations with weather station data we collected temperature data from one northern and one southern clade population. We placed temperature loggers in four and five pitcher plant leaves (ranging from heavily shaded to full sun) at the NC coast and NC Mtn. sites from mid August to late October of 2003 and 2004, respectively. We estimated linear, first-order regressions of individual leaf temperatures on proximal weather station data for each site. During the winter a cover of snow will insulate pitcher plants from subzero (°C) temperatures (Evans and Brust 1972; Bradshaw et al. 2004) but snow is not a confounding factor during the growing season.

To assess the frequencies of cold and hot temperatures in different geographic populations, we retrieved 50 years of daily

minimum and maximum air temperature data for weather stations less than 5 km from each sampled site (US National Climatic Data Center, http://www.ncdc.noaa.gov/oa/ncdc.html). Years for which any of the stations reported missing values for any month of the year were excluded, and the resulting 35-year subset was analyzed. For each site we calculated the frequency distribution of maximum and minimum temperatures including data for the entire year binned into 1°C intervals. In addition, we calculated the frequency distribution of maximum and minimum temperatures for only the growing season of each site, defined as the days of the year with daylength longer than the CP of that population (Ragland and Kingsolver 2007). We included civil twilight in estimates of daylength because *W. smithii* likely includes twilight cues in photoperiod perception (Bradshaw and Phillips 1980), although excluding civil twilight did not qualitatively change the results. Within each class of temperature measures (max. and min.) we performed pairwise comparisons between whole-year and growing season distributions within each population using two-sample Kolmogorov–Smirnov tests. We also performed pairwise comparisons among populations within each temperature measure by time window (whole-year/growing season) combination.

Results

COLD TOLERANCE, ACCLIMATION, AND DIAPAUSE STATUS: CHILL COMA RECOVERY

Population of origin, sex, and temperature significantly affected chill coma recovery time (Table 2A). Recovery times were faster at the lower rearing temperature for all populations (Fig. 2A), and averaged across temperatures, northern clade populations recov-

Table 2. Results of linear mixed model (I,II) and logistic model (III) ANOVA for chill coma recovery (I,II) and heat knockdown proportion (III). I, II and III correspond to experiments I, II, and III described in the methods section.

Trait	Effect	df	*F* value	*P* value
I. Chill coma rec.	Pop	3	42.42	<0.001
	Temp	1	152.16	<0.001
	Sex	1	30.10	<0.001
	Pop × Temp	3	8.25	<0.001
II. Chill coma rec.				
	Pop	1	107.49	<0.001
	Sex	1	5.44	0.020
	PS*	1	1.77	0.18
III. Heat knockdown	Pop	3	5.58	0.001
	Temp	2	2.19	0.140
	Sex	1	0.19	0.661

*Physiological State: non- or postdiapause.
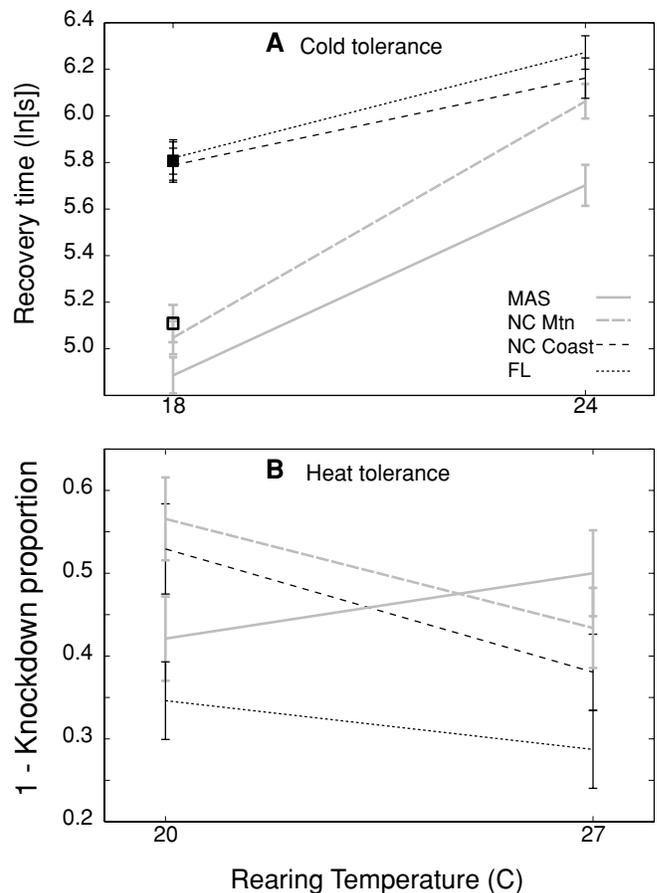
Figure 2. Least-squared means $\pm$ SE for log-transformed chill coma recovery time (A) and 1-heat knockdown proportion (heat tolerance; (B). Black and gray lines represent southern (FL, short dashed; NC coast, long dashed) and northern (MAS, solid; NC Mtn., dashed) clade populations, respectively. In panel (A), the lines connect means for nondiapause development, whereas the closed square and open square symbols depict the means of postdiapause development for FL and MAS, respectively.

ered significantly faster than southern clade populations ($F_{1,545} = 118.07$, $P < 0.001$). Females recovered significantly faster than males ($F_{1,545} = 30.10$, $P < 0.001$), but there were no significant interactions between sex and population or temperature. There was a significant interaction between population and temperature, and linear contrasts demonstrate that the slope of the recovery time versus temperature relationship was steeper for northern clade populations than for southern clade populations ($F_{1,545} = 21.72$, $P < 0.001$). With only two rearing temperatures we cannot exclude the possibility that these acclimation responses are actually nonlinear and differ in more complex ways than simply linear slopes. However, combining these data with measures of chill coma (using an identical assay) at 20 and 27°C constant rearing temperature for three of the four populations (excluding MAS) does suggest a monotonic acclimation response between

18 and 27°C (G. Ragland, unpubl. data). Whether or not the underlying functional relationship is truly linear, our data indicate that the northern clade acclimation response is stronger than that of the southern clade over the range of rearing temperatures included here.

Physiological state (post- vs. nondiapause: Experiment II) had no effect on chill coma recovery time in the cool treatment (Table 2B). The square symbols in Figure 2A show mean (± 1 SE) recovery time of postdiapause FL and MAS adults relative to nondiapause adults: recovery times for each population were indistinguishable based on physiological state.

TOLERANCE AND ACCLIMATION TO HEAT: HEAT KNOCKDOWN

Population of origin significantly affected heat tolerance (1-knockdown proportion), but sex and rearing temperature did not (Table 2C). In three of the four populations a higher proportion was knocked down at lower rearing temperatures, but the trend was nonsignificant (Fig. 2B). Southern clade populations did not exhibit consistently greater heat tolerance than did northern populations. Compared to the average value of the northern clade populations, one southern clade population (NC Coast) had a statistically indistinguishable average heat tolerance ($F_{1,693} = 1.29$, $P = 0.257$) whereas the other had lower heat tolerance ($F_{1,693} = 16.34$, $P < 0.001$).

LOCAL TEMPERATURES VERSUS STATION DATA

Table 3 presents the range and average values for intercepts, regression coefficients, and coefficients of determination estimated from regressions of individual leaf maxima and minima on station data. All regressions were highly significant ($P < 0.0001$) with high r^2 values. The variation in regression parameters suggests that there is substantial temperature variation among leaves, a result noted in several other studies (Kingsolver 1979; Bradshaw et al. 2000, 2004). On average, however, the regressions predict the higher leaf maxima (30–40°C) to be 0.2 to 1.2°C higher than weather station data at the NC coast site and about 1.4 to 4.1°C cooler at the NC Mtn. site. Leaf minima are predicted on average to be about 1–2°C warmer than weather station data at both sites. Air temperatures (experienced by adult mosquitoes) tend to be less

extreme than the warmest and coldest leaves at a site (Kingsolver 1979). The variation in shade provided by taller vegetation that affects leaf temperature (Kingsolver 1979; Bradshaw et al. 2000, 2004) will also create variation in air temperature, but our leaf data suggest that average air temperatures at a site do not deviate substantially from those measured at proximal weather stations.

PHENOLOGICAL ADAPTATION AND THE FREQUENCY OF COLD AND HOT CONDITIONS

All pairwise differences in maximum and minimum temperature distributions between the growing season and the entire year and among populations were highly statistically significant ($P < 0.0001$). Moreover, frequency data in particular temperature ranges reveal well-defined effects of phenology and population of origin. When considered over the entire year, the two northern clade populations (NC Mtn. and MAS) clearly experience higher frequencies of cold daily minimum temperatures than the two northern clade populations (NC Coast and FL) (Fig. 3A). A cover of snow will minimize exposure of frozen pitcher leaves to temperatures below zero (Bradshaw et al. 2004), so actual temperatures in a pitcher plant are often much closer to zero than below-zero measurements of air temperature would suggest. This effect truncates the low end of the minimum temperature distribution (primarily for northern clade populations) for the entire year. Accounting for the insulating effects of snow does not change the observation, however, that northern clade populations experience temperatures at or below zero much more frequently than southern clade populations during the entire year (Fig. 3A). Conversely, the two southern clade populations (NC Coast and FL) experience higher frequencies of hot daily maximum temperatures than the two northern clade populations (NC Mtn. and MAS) (Fig. 3B).

Accounting for differences in the length of the growing season (due to differences in CP among populations) drastically changes the frequency distributions of minimum temperatures (Fig. 3C). After correcting for the timing of diapause at each site, none of the populations experience daily minimum air temperatures below about −7°C during the growing season (Fig. 3C). Because of evolved differences in diapause timing, all populations experience more similar frequencies of minimum temperatures at or below zero during the growing season, although the MAS

Table 3. Summary of regression analysis of individual leaf minimum (min) and maximum (max) temperature on proximal weather station data. Ranges and averages (in parentheses) are provided for estimates of regression parameters (intercept and regression coefficient) and coefficients of determination (r^2).

		Intercept	Regression Coefficient	r^2
NC Mtn.	max	4.9–8.5 (6.7)	0.61–0.89 (0.73)	0.67–0.77 (0.70)
	min	−1.2–3.1 (1.6)	0.69–1.0 (0.87)	0.76–0.85 (0.81)
NC coast	max	−4.6–0.98 (−2.8)	0.91–1.3 (1.1)	0.80–0.87 (0.84)
	min	−0.27–1.6 (0.80)	0.89–0.93 (0.91)	0.95–0.97 (0.96)
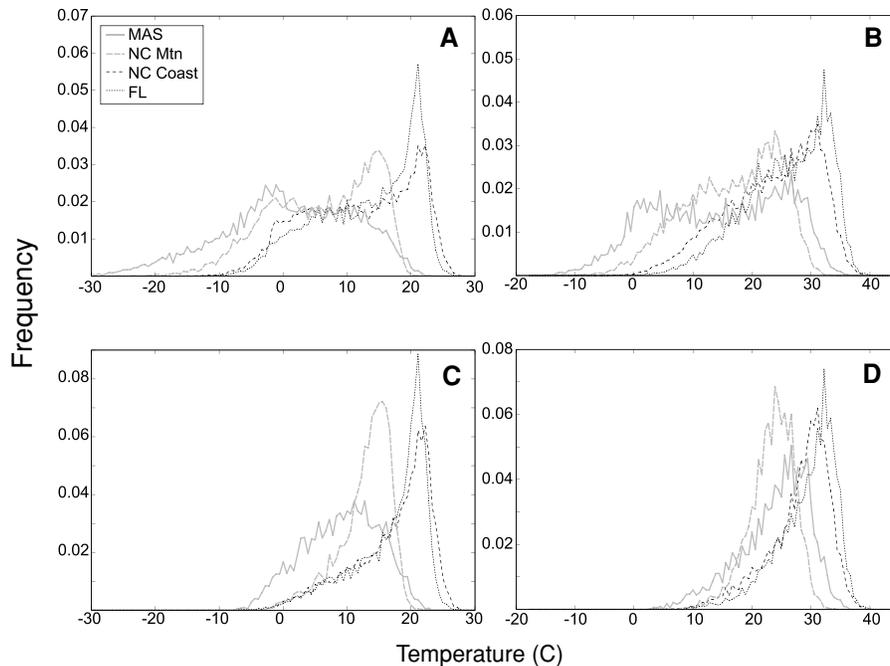
Figure 3. Frequency distributions of daily minimum (A,C) and maximum (B,D) temperatures for each sampled geographic location as estimated from the 35-year weather dataset for the entire year (A,B) and for the phenologically defined growing season only (C,D).

population still experiences a higher frequency of cold minimum temperatures (Fig. 3C).

In contrast, accounting for differences in diapause timing does not drastically change the high-temperature end of these distributions (Fig. 3D). As a result, the two southern clade populations experience higher frequencies of hot daily maximum temperatures than the two northern clade populations during the growing season.

Discussion

GEOGRAPHIC VARIATION IN ADULT COLD AND HEAT TOLERANCE

Latitudinal trends in chill coma recovery times have been reported for a variety of interspecific comparisons in arthropods. For example, Gibert et al. (2001) found that of 84 *Drosophila* species, 26 temperate species recovered significantly faster than 48 tropical species (the distributions of recovery times do not overlap). A similar pattern has been observed for intraspecific variation within a handful of species, including *Drosophila melanogaster* (Hoffmann et al. 2002), *Drosophila serrata* (Hallas et al. 2002), and the isopod *Porcellio laeris* (Castaneda et al. 2005), where populations at higher latitudes exhibit faster recovery times. These studies suggest that chill coma recovery is associated with an important adaptation for cold tolerance. Our results for geographic variation in cold tolerance in *W. smithii* paint a similar picture. Averaged across rearing temperatures, adult *W. smithii* from north-

ern clade populations had substantially faster chill coma recovery times than those from southern clade populations. Enhanced adult cold tolerance as measured by chill coma recovery therefore accompanies the relatively greater fitness achieved by northern populations during times of winter cold stress (Bradshaw et al. 2004; Zani et al. 2005b). Because we have only one metric of cold tolerance, it is possible that geographic patterns could differ for other tolerance measures such as critical thermal minimum. In fact, more detailed mechanistic studies incorporating multiple tolerance metrics could be useful for revealing variation in specific physiological responses to cold stress. However, the correspondence between chill coma recovery and composite fitness metrics is compelling evidence that northern clade populations are relatively more tolerant of stressful cold temperatures.

The same general geographic pattern holds for both nondiapause (Experiment I) and postdiapause (Experiment II) individuals (Fig. 2A): diapause status of the larvae had no detectable effect on cold tolerance in resulting adults. This is of interest because diapausing larvae of *W. smithii* overwinter, so postdiapause adults are more likely to experience cold temperatures during early spring. In contrast, adults resulting from nondiapausing larvae are less likely to experience cold temperatures as they occur during the summer or early fall—adults that occur in cold fall conditions are unlikely to generate offspring that reach the larval diapause stage before onset of winter (Bradshaw et al. 2004). Although diapausing larvae are more tolerant of subzero temperatures than nondiapause larvae (Evans and Brust 1972), our results suggest

that this enhanced cold tolerance does not carry over into post-diapause adults. By comparison, diapause occurs during the adult stage in many *Drosophila*. In *D. melanogaster*, chill coma recovery is correlated with survival of stressful cold temperatures (Anderson et al. 2005), and overwintering survival is a plausible selective factor (Gibert et al. 2001). Diapause occurs in the larval stage in *W. smithii*, so cold winter temperatures could only affect adult cold tolerance via correlated selection on diapausing larvae. Whether cold tolerance is genetically correlated across physiological states (diapause) or life stages is generally unknown, although Krebs et al. (1998) showed that expression of Hsp70, a common (high and low) temperature stress-induced protein, is positively genetically correlated across life-history stages in *D. melanogaster*. Upregulation of Hsp70 is common in overwintering insects and has been shown to strongly associate with cold tolerance (Rinehart et al. 2007).

In contrast to our results for cold tolerance, northern and southern clade *W. smithii* adults did not differ significantly in heat tolerance (1-knockdown proportion). Although there was significant overall population variation in heat tolerance, this was due to lower heat tolerance in one of the southern clade populations (FL). This nontrend agrees well with previous results showing that survival of lethal high temperatures (Armbruster et al. 1999) and population replacement rate (R_0) under heat stress (Zani et al. 2005b) are unrelated to geography. An additional study shows that when stressed to the brink of extinction in high temperature environments for multiple generations, southern populations do achieve higher yearlong replacement rates than northern populations (Bradshaw et al. 2004). Therefore, it is possible that an alternative measure of heat tolerance might better correlate with geographic differences in summer heat. But, the cumulative body of evidence suggests that geographic populations clearly diverge more in cold tolerance than in heat tolerance (Bradshaw et al. 2004; Zani et al. 2005b).

THERMAL ACCLIMATION IN COLD AND HEAT TOLERANCE

Temperatures experienced during development or immediately prior to temperature stress often strongly influence thermotolerance (Huey et al. 1999; Hoffmann et al. 2003; Bradshaw et al. 2004), and acclimation responses may evolve in addition to thermotolerance per se. Thermal acclimation has been widely reported in many animals (Kingsolver and Huey 1998), although rigorous tests of the beneficial acclimation hypothesis are more uncommon and provide mixed results (Deere and Chown 2006). Our results show that cooler rearing temperatures decreased chill recovery times (and increased cold tolerance) in adult *W. smithii*, just as cold temperature acclimation improves cold tolerance in diapausing larvae (Evans and Brust 1972). A similar pattern demonstrating a strong relationship between rearing temperature and chill

coma recovery has been shown in *Drosophila* (Gibert and Huey 2001; Hoffmann et al. 2005), although acclimation responses may vary depending on the tolerance measure, the severity of the temperature stress, and the duration of the acclimation period (Rako and Hoffmann 2006). The life stage that experiences the acclimation temperature may also influence cold tolerance (Terblanche and Chown 2006). Here we applied the same ecologically realistic rearing environment to the larval and adult stages, but it is certainly possible that short-term and long-term thermal environments are of unequal importance. In contrast to chill coma recovery, heat tolerance was unrelated to rearing temperature. Studies of *Drosophila* suggest that higher rearing temperatures often confer increased heat tolerance (Levins 1969; Bradshaw et al. 2004; Hoffmann et al. 2005), but this does not appear to be the case in *W. smithii*, even over a substantial (7°C) change in mean rearing temperature.

An important result from our studies is the presence of significant geographic variation in the strength of thermal acclimation in cold tolerance (Fig. 2A). Northern clade populations of *W. smithii* exhibited steeper acclimation responses than southern clade populations, resulting in greater differences among populations at the low compared to the high temperature rearing treatment. In the field, adult *W. smithii* are much more likely to encounter an acute cold stress such as an overnight freeze during the spring or fall when average temperatures are relatively low and thermal conditions are similar to those simulated in the low temperature treatment. Selection on cold tolerance should therefore occur primarily during these cooler times of the year. Physiology that depends on diapause status (e.g., postdiapause vs. nondiapause) provides one mechanism to match cold tolerance with the occurrence of extreme cold, but we have shown that *W. smithii* does not use this strategy. Rather, our chill coma recovery data are consistent with the evolution of relatively steeper acclimation responses in the north that confer enhanced adult cold tolerance primarily under thermal conditions (simulated by the cooler rearing treatment) at which tolerance is most beneficial.

In contrast to the geographic patterns we observe in *W. smithii*, studies of geographic variation in thermotolerance rarely identify variation for acclimation responses in *Drosophila* (Yamamoto and Ohba 1982; Kimura 1988; Hoffmann and Watson 1993; Gibert and Huey 2001; Bubliy et al. 2002; Hoffmann et al. 2005). We found only a single study documenting a significant temperature by population interaction for cold tolerance (Ayrinhac et al. 2004). This study compared temperate and tropical populations that presumably represent relative extremes of the range, but other temperate–tropical comparisons have revealed no population differences in acclimation (e.g., Gibert and Huey 2001). Why do we find substantial geographic variation in acclimation responses across 12 degrees of latitude and ~1000 m of elevation in *W. smithii* although acclimation responses are often invariant across 20 or more

degrees of latitude in *Drosophila* (e.g., Hoffmann et al. 2005)? One possibility is that gene flow limits local adaptation in *Drosophila*. Large distances between suitable habitat sites and a weak-flying adult stage limit gene flow between geographic populations of *W. smithii* to effectively zero (Istock and Weisburg 1989), whereas gene flow can often be substantial in *Drosophila*, particularly in cosmopolitan species such as *D. melanogaster* (David and Capy 1988). If acclimation evolves more slowly than thermotolerance per se because of low heritability (low genetic variance or relatively high environmental variance), populations experiencing gene flow may exhibit local adaptation in thermotolerance but not in acclimation.

## DIAPAUSE TIMING, ENVIRONMENTAL TEMPERATURE, AND THERMAL ADAPTATION

Gaston and Chown (1999) used climatic data to demonstrate that extreme winter minima decline more rapidly than extreme summer maximum temperatures with increasing latitude. Such a pattern predicts that geographic populations along a latitudinal cline should be more divergent in cold tolerance than in heat tolerance. This prediction has been important in stimulating empirical research on geographic patterns in cold and heat tolerance (e.g., David et al. 2003; Ayrinhac et al. 2004; Castaneda et al. 2005; Hoffmann et al. 2005; Klok and Chown 2005; Collinge et al. 2006). In addition to several single-taxon examples (e.g., Castaneda et al. 2004; Hoffmann et al. 2005; Terblanche et al. 2006), a broad meta-analysis of insects (Addo-Bediako et al. 2000) lends strong support to this prediction. We observe a similar pattern of extreme temperature variation for geographic populations in *W. smithii*. There is a greater range of variation among populations for extreme minimum temperatures than for maximum temperatures when the entire year is considered (Figs. 3A,B; 4).

Although consistent with empirical data, the prediction of greater geographic variation in cold compared to heat tolerance may be complicated by phenological adaptation. Many species possess dormant physiological states to avoid extreme temperatures during some parts of the year. The frequency and range of temperatures experienced during the active (e.g., nondiapause) growing season may be quite different from those for the entire year (compare Figs. 3 A,B to 3 C,D). As a result, geographic differences in diapause timing can have important effects on how selection acts on thermal responses during the growing season along climatic or latitudinal gradients (Ragland and Kingsolver 2007).

For insects such as *W. smithii*, winter diapause primarily influences the low end of the temperature frequency distribution experienced by actively growing individuals. With the exception of the Massachusetts (MAS) population, frequencies of daily minimum temperatures occurring during the growing season reached zero at about $-3°C$ and converged to similar values between -3

and $+5°C$ (Fig. 3C). Moreover, compared to frequency distributions for the entire year, even the diapause-corrected daily minimum temperature distribution for MAS reaches frequencies of subzero temperatures that are more similar to values for other populations. Direct selection on low temperature tolerance of nondiapause stages is thus more comparable across populations than simple and commonly used environmental proxies such as annual minimum temperature would suggest.

Divergence in diapause timing among geographic populations may also alter the relative evolutionary importance of selection at high and low temperatures predicted by Addo-Bediako et al (2000). In particular, if diapause timing evolves to limit exposure to extreme cold temperatures in nondiapause stages, then the thermal habitat of the growing season may exhibit similar geographic variation in extreme high and low temperatures. Figure 4 shows average annual minimum and maximum temperatures (estimated from 35 years of weather data) from weather stations proximal to eight geographic populations of *W. smithii* with available estimates for CP (WI, AL, GS, HC, MM, MC, FV, and PD from Bradshaw and Lounibos 1977). Annual minima and maxima estimated for the entire year show the same trend as that documented in Addo-Bediako et al. (2000): maxima decline faster than minima with increasing latitude. In contrast, geographic variation in annual minimum temperatures for the growing season alone is more comparable to that for annual maxima (compare solid black line to dashed gray line in Fig. 4). This trend suggests that if selection
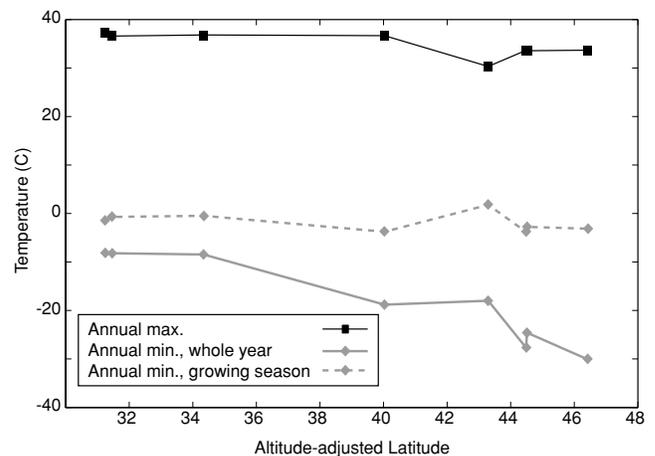
Figure 4. Annual maximum temperatures (—■—) and annual minimum temperatures calculated for the entire year (—◆—, solid line) and for the growing season alone (- ◆ - , dashed line) for eight geographic populations from Bradshaw and Lounibos (1977; see discussion). Annual maxima occur during the growing season: thus, accounting for diapause timing does not change this value. Values are averages across 35 years of data as described in the methods; standard errors of the means are smaller than the symbols. Equivalent latitude (x-axis) was calculated from equation (2) in Bradshaw and Lounibos (1977) to correct for differences in altitude.

on thermotolerance is indeed related to maximum and minimum temperatures experienced by a particular life-history stage, direct selection on high and low temperature stress tolerance of active, nondiapause stages should vary comparably across latitude and altitude in *W. smithii*. Interestingly, Addo-Bediako et al. (2000) point out that interspecific variation in cold tolerance (as measured by lower lethal temperature and supercooling point) tends to increase with increasing latitude of origin. These authors offer several possible explanations for this trend, including the hypothesis that variation in overwintering microhabitat (e.g., below vs. above an insulating snow layer) increases toward the poles. Our data suggest that interspecific variation in overwintering strategies may also contribute to the latitudinal trend in cold tolerance variation.

These considerations complicate interpretation of our results on geographic variation in adult cold tolerance in *W. smithii*. If we consider only the growing season, three of the four populations experience nearly identical frequencies of minimum temperatures below about 5°C (Fig. 3C). The NC Mtn. population exhibits faster chill coma recovery than the two southern clade populations, but only diapausing larvae experience cold temperatures more frequently than do southern clade diapausing larvae. Interannual variation in the frequency of occurrence of temperatures at or below 0°C also supports this conclusion. The interquartile range for the frequency of subzero temperatures during the growing season alone is comparable among NC Mtn. NC coast, and FL populations (data not shown). In contrast, winter cold stress is highly variable among populations, and Zani et al. (2005b) clearly show that northern populations achieve greater fitness than southern populations when exposed to cold stress during the winter. Thus, correlated selection on the diapause stage is a plausible alternative hypothesis to direct selection on the adult stage for the evolution of enhanced adult cold tolerance in northern clade populations. Our data suggest that alternate developmental pathways do not strongly affect cold tolerance in adult *W. smithii*, but this conclusion does not preclude the existence of genetic correlations between larval and adult thermal physiology. Assessing these correlations is beyond the scope of this study. Likewise, rigorously testing whether cold tolerance is associated with low temperature exposure during the growing season would require a broader study incorporating additional northern clade populations. Integrating such a study with a concurrent quantitative genetic analysis would provide a framework to test these alternative hypotheses.

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