Natural temperature variation affects larval survival, development and Hsp70 expression in a leaf beetle

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Summary

1. Sierra Nevada populations of the beetle *Chrysomela aeneicollis* experience extreme high and low temperatures during summer, which pose special challenges to larvae of limited mobility. In these populations, allele frequency variation at the glycolytic enzyme locus phosphoglucose isomerase (PGI) correlates with differences in temperature between river drainages. PGI allozymes differ in functional properties, and thermal tolerance of adults and larvae depends on PGI genotype.

2. We measured effects of temperature on larval development rate and survival after reciprocal transplantation of populations between drainages. Effects of temperature on growth rate and activity were determined after laboratory acclimation of larvae from Bishop Creek (BC), where PGI alleles 1 and 4 occur in similar frequency. Hsp70 expression levels were measured for free-living larvae of known body temperature, and for laboratory-acclimated BC larvae.

3. Larval mortality was greatest in the coldest drainage and was correlated with minimum night-time air temperature. The frequency of PGI allele 1 declined for BC larvae transplanted to the warmest drainage. Development rate of BC larvae in nature was lowest for experimental groups where PGI-1 allele frequency was highest. Larval growth and activity varied with acclimation temperature and PGI genotype in the laboratory. Hsp70 expression levels in nature were higher for larvae collected later in the day, and varied consistently among PGI genotypes in nature and in the laboratory.

4. These results suggest that daytime temperatures routinely experienced by larvae cause elevated Hsp70 expression levels indicative of physiological stress. Exposure to subzero night-time temperatures appears to cause larval mortality. Up-regulation of Hsp70 may protect larvae from heat and cold stress. Variation in Hsp70 expression among PGI genotypes may result in differential mortality and developmental rates in nature.

Key-words: Chrysomelidae, natural selection, phosphoglucose isomerase, temperature adaptation

Introduction

Temperature is one of the most important abiotic factors influencing survival and reproduction of organisms in nature (Willmer, Stone & Johnston 2000; Hochachka & Somero 2002). Montane organisms are typically confronted with drastic diurnal fluctuations in air temperature in summer, when most species are active. Free-living insect larvae may be particularly vulnerable to fluctuations in temperature, owing to their reduced mobility (limiting thermoregulation), small size (which offers little thermal inertia to buffer changes in ambient temperature) and need to grow rapidly (Danks, Kukal & Ring 1994; Bryant, Thomas & Bale 2002). Temperature variation affects movement, activity and growth through its effects on metabolic rate (Willmer *et al.* 2000; Irwin & Lee 2003). Thus, adults and larvae may have distinct temperature-related performance constraints, as adults typically need to move to find mates or egg-laying sites, whereas larvae need to grow and develop. Also, larval stages frequently have different body forms and dispersal abilities from adults and are exposed to different

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natural enemies. Genes that allow larvae to cope with temperature extremes may incur costs at other life stages through antagonistic pleiotropy (Bradshaw & Holzapfel 1996; Feder *et al*. 1997; Sorensen & Loeschke 2004). Unfortunately, few studies have examined effects of natural temperature extremes on survival and growth of larvae, especially for a species whose adult thermal biology has been investigated.

Here we examine effects of environmental temperature on survival and development rate for larvae of Eastern Sierra Nevada, California, populations of the leaf beetle *Chrysomela aeneicollis*. These beetles occur throughout north-western North America (Brown 1956), but are restricted to high-altitude localities in the Sierra Nevada (Rank 1992b), where they are found on willows in bogs and near lakes and streams. Adults emerge from diapause in early summer to feed, mate and lay eggs. Larvae hatch, mature through three instars, pupate and develop into new adults on the same host plant as their parents (Rank 1992a,b). Larvae are present from early July to late August, when air temperatures vary from daily highs near 30 °C to night-time lows that routinely drop below freezing (Rank & Dahlhoff 2002). Prior work on *C. aeneicollis* suggests that variation at the phosphoglucose isomerase (PGI; glucose-6-phosphate ketol isomerase, EC 5·3·1·9) allozyme locus is associated with differences in environmental temperature. Two PGI alleles predominate in Sierra Nevada beetle populations and frequencies of these alleles vary along a latitudinal thermal gradient, with PGI-1 predominating in the cooler northern drainages and PGI-4 most frequent in warmer, southern subpopulations (Dahlhoff & Rank 2000). In populations where alleles 1 and 4 occur in relatively equal frequency, seasonal or annual shifts in local climate lead to shifts in allele frequency at PGI but not at other polymorphic loci (Rank & Dahlhoff 2002; Fearnley 2004). Thus, PGI (or a closely linked gene) appears to be under temperature selection in these populations.

Up-regulation of heat shock proteins (Hsps), which minimize stress-induced protein aggregation, and play important roles in growth and acquired thermal tolerance, represents an important physiological strategy used by ectotherms to cope with thermal extremes (Feder *et al*. 1992; Dahlgaard *et al*. 1998; Krebs & Feder 1998; Zatsepina *et al*. 2001; Garbuz *et al*. 2003; Sorensen, Kristensen & Loeschke 2003; Ketola *et al*. 2004). The importance of Hsps as a mechanism by which organisms cope with exposure to extreme temperatures in nature has been established in a variety of organisms and habitats (Feder & Hofmann 1999; Kelty & Lee 1999; Tomanek & Somero 1999; Kelty & Lee 2001; Hoffmann, Sorensen & Loeschke 2003; Sorensen *et al*. 2003; Tomanek & Sanford 2003). However, we still know little about how organisms in natural populations balance these benefits of Hsp up-regulation with its costs (Feder *et al*. 1992; Krebs & Feder 1997; Loeschke *et al*. 1997; Sorensen *et al*.

2003; Williams, Shorthouse & Lee 2003). Differential expression of Hsps among adult *C. aeneicollis* of different PGI genotype has been observed in nature and in the laboratory (Dahlhoff & Rank 2000; Rank & Dahlhoff 2002; Neargarder, Dahlhoff & Rank 2003). PGI 1–1 adults up-regulate Hsp70 expression at lower temperatures and express Hsp70 at higher levels than PGI 4–4 genotypes, with 1–4 genotypes typically showing intermediate expression levels and induction temperatures.

The mechanistic relationship between PGI genotype and Hsp70 expression in *C. aeneicollis* is not yet known. Nonetheless, PGI variation provides an excellent marker of temperature sensitivity, and this can be used to clarify potential costs and benefits of Hsp70 expression. To accomplish this, one must first determine whether the relationship between PGI genotype and Hsp70 expression is similar in larvae and adults. If this is true, one might find that PGI genotypes associated with greater Hsp expression at lower temperatures have slower developmental rates, but greater tolerance of thermal extremes, than other genotypes. In addition, if Hsp70 protects beetle larvae from exposure to cold, as found in other insects (Kelty & Lee 2001; Yocum 2001), elevated daytime temperatures that lead to greater expression of Hsp70 may enhance survival after exposure to extremely cold temperatures at night.

In this study, effects of environmental temperature variation on survival, development rate and Hsp70 expression were measured for *C. aeneicollis* larvae. Mortality rates were measured in nature after reciprocal transplantation of young larvae between otherwise similar sites differing in thermal regime. In addition, the relationship between PGI genotype, development rate and survival of larvae from a single population (Bishop Creek), where the most common PGI alleles are found in relatively equal frequency, were measured in three drainages in nature. Third, the relationship between PGI genotype and larval growth was measured at different temperatures in the laboratory. Finally, effects of temperature on Hsp70 expression were measured in the field and in the laboratory for different PGI genotypes.

Materials and methods

study populations

Experiments focused on populations from three drainages in the Eastern Sierra Nevada that differ in typical thermal regime and in PGI frequency: Big Pine Creek (BPC: 37°11′ N, 118°32′ W), Rock Creek (RC: 37°25′ N, 118°24′ W) and Bishop Creek (BC: 37°11′ N; 118°32′ W). Habitats in BPC, where allele 4 is common, tend to have higher daytime temperatures and fewer freezing nights; sites in RC, where allele 1 predominates, tend to have lower high temperatures and night-time temperatures that drop below freezing regularly in summer. In BC, where PGI alleles occur in

relative parity (Rank 1992b; Dahlhoff & Rank 2000; Rank & Dahlhoff 2002), thermal conditions are intermediate between BPC and RC.

LARVAL SURVIVAL AND DEVELOPMENT RATES IN NATURE

Experimental localities were selected that shared overall similar physical characteristics: sunny, open meadows at about 3000 m in elevation where plants grow on thin, rocky soils typical of the Eastern Sierra Nevada. Experimental willows at each site grew near a small creek. In July 1999, family groups of late 1st and early 2nd instar larvae were collected from source localities in BPC (3rd Falls: 2950 m), RC (Mosquito Flat: 3110 m) and BC (South Lake: 2996 m) and brought to the Owens Valley laboratory of the White Mountain Research Station (WMRS) in Bishop, CA, and held at 4 °C for 24–48 h. Larvae from each source population were transplanted onto one of ten clones of their favoured host willow (*Salix orestra*) at each site, and confined to their experimental branch with resin (Tanglefoot™). Two groups of larvae from each source population were placed on separate experimental branches of each willow. Most groups (87%) ranged from 10 to 15 individuals, similar to natural group sizes of second instar larvae (N. E. Rank, unpublished data). Ambient air temperature was recorded every 30 min at each locality using 'Tidbit' temperature loggers (Onset Computer Co., Pocaset, MA) suspended in white, plastic thermal shields secured to willow branches. Daily minimum air temperature was obtained from logger output using Boxcar Pro software (Version 4·0, Onset Comp.). Number of surviving larvae belonging to each instar was recorded every 1–3 days for 21 days. At the end of the experiment, surviving larvae were flash frozen on dry ice and stored at −70 °C until biochemical analysis. Willow leaves were collected throughout the experiment, weighed and dried to measure water content.

Mortality rates were quantified by subtracting number of survivors at each count from the previous count and dividing by the previous number. This assumes that larvae that had disappeared were dead, which is reasonable because larvae do not leave the host before pupation. Before statistical analysis, average survivorship was calculated for each branch by determining the area beneath the survival curve and dividing it by the total area if all larvae had survived to the last count (Rank 1994). To estimate larval development rate, we first calculated average instar at each count by summing the number of larvae in each developmental stage and dividing by the total per branch. Linear regressions of log number of days since initiation of transplant (X) *vs* average instar (Y) were used to calculate slopes that estimated rate of instar change on each branch (Rank *et al.* 1998). Branches were excluded if fewer than two individuals survived to the end of the experiment.

Mixed model ANOVA was used to evaluate effects of source drainage, target drainage and host plant on larval survival and development rate. All effects that included host plant were considered a random factor in the ANOVA. Linear regression was used to relate minimum temperature between counts to the site average of larval mortality for that interval. Survival of PGI genotypes from BC among target drainages was evaluated using a frequency test. Weighted linear regression (X = frequency of the PGI allele 1 among survivors per branch, Y = larval development rate) was used to determine whether PGI genotype was related to larval growth. Values were weighted by number of surviving larvae to account for variation among samples in precision of the allele frequency estimate. All statistical analyses were conducted using Jmp In software (Version 5·0 for PC, SAS Institute, Inc., Cary, NC).

DEVELOPMENT AND ACTIVITY AFTER LABORATORY TEMPERATURE ACCLIMATION

During July 2001, first instar larvae were collected from different family groups on several willow clones from Bluff Lake in BC. Larvae were reared on fresh leaves from *S. orestera* for about 3 days at WMRS (natural day–night light cycles, 20 °C day, 4 °C night), until they moulted. Host foliage was acquired from four different willow clones and leaves were changed daily. Once moulting occurred, beetles were weighed to the nearest 0·01 mg and removed from the plant for a 4-h temperature treatment (20, 27 or 34 °C; 12–4 p.m.). After treatment, larvae were returned to plants at 20 °C until midnight, held at 4 °C until 8 a.m., and returned to 20 °C until the next treatment. Treatments were imposed for 2 (n = 21) or 3 (n = 113) days. Activity was scored after each heat treatment (1 = not moving, 2 = moving, 3 = walking). Beetles were weighed again after the last treatment, reared at moderate temperatures until moulting to third instar, and flash-frozen for genotype analysis.

Before analysis, relative larval growth was calculated by dividing the natural log-transformed gain in body mass by time in treatment (Rank 1994). Larvae were excluded from analysis if they moulted or died during the 3-day treatment or if they lost body mass. Activity values were averaged across days for each larva. Activity was analysed by mixed model ANOVA including grouping factors of PGI genotype and acclimation temperature (preliminary analyses based on host plant revealed no host plant effects on the dependent variables). Growth rates were analysed by ANCOVA, including grouping factors described above and initial mass and days in treatment as covariates in the final model.

MEASURING BODY TEMPERATURE AND EXPRESSION OF HSP70 IN NATURE

Larval body temperature and ambient air temperature were measured during August 1998 at five time intervals:

early morning (7–8 a.m.); mid-morning (10–11 a.m.) mid-day (12–1 p.m.) mid-afternoon (2–3 p.m.) and late afternoon (5–6 p.m.). Larvae in sun and shade were measured at each time point. For each larva, body (T_b) and air temperature (T_a) were measured using a hand-held digital thermometer (HH-82, Omega Engineering Inc., Stamford, CT) equipped with a T-type Teflon insulated 36 AWG fine wire thermocouple. Immediately after T_b determination, larvae were flash-frozen on dry ice for biochemical analysis.

For analysis of variation in T_a and T_b, three sites in Big Pine Creek and two sites in Rock Creek where conditions had been sunny throughout the day were selected. Both variables were analysed by multivariate analysis of covariance, with between subjects factors of time interval and exposure (sun or shade). Site elevation was used as a covariate, and T_a and T_b were used as dependent variables in a repeated measures design that allowed for tests of interactions among between- and within-subjects factors.

Hsp70 expression levels were determined for a subset of beetles of known body temperature. Individuals used for this analysis were collected on different days at similar elevation localities in RC (Heart Lake, 3190 m), BC (Bluff Lake, 3203 m) and BPC (Sam Mack Creek, 3229 m). PGI genotypes were subsequently determined for 43 of these individuals. Because not all PGI genotypes were represented at all time points in each drainage, and because it was not always possible to obtain both Hsp70 expression level and PGI genotype for every individual, differences in Hsp70 expression among PGI genotypes were analysed separately from effects of time of day. Thus, PGI genotype was used as a grouping factor in a one-way ANOVA, and effects of drainage and time were used as factors in a two-way ANOVA.

LABORATORY HEAT SHOCK INDUCTION

During July 2000, 3rd instar larvae were collected from Bluff Lake, and transported to WMRS, where they were held at common garden conditions (light, 20 °C: 8 a.m. to 8 p.m.; dark, 4 °C: 8 p.m. to 8 a.m.) for 8 days. After acclimation, larvae were held for 4 h at one of four temperatures (20, 30, 33 or 35 °C) in 1·5 ml microfuge tubes in a temperature-controlled heating block (Boekel Scientific, Feasterville, PA). After heat treatment, all larvae were held at 20 °C for 1 hour, weighed and flash frozen on dry ice.

BIOCHEMICAL ANALYSIS

PGI genotypes were determined by starch gel electrophoresis following published methods (Rank 1992b). Expression of a 72 kDa heat shock protein was quantified according to published methods (Rank & Dahlhoff 2002; Neargarder *et al.* 2003), with the exception that larval body wall was homogenized in 250 µl 50 mM Tris-HCl, pH 7·4 at 20 °C.
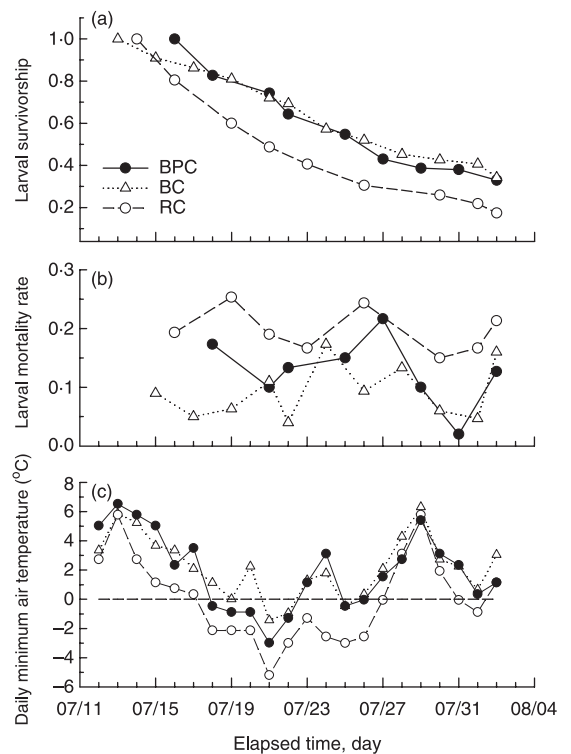
Fig. 1. (a) Survival of transplanted *C. aeneicollis* larvae targeted to Big Pine Creek (BPC), Bishop Creek (BC) and Rock Creek (RC). Data shown are least-squares means of proportion of individuals surviving between counts in each target drainage (*n* = 60 groups per source per drainage). (b) Larval mortality rates (proportion of individuals that died between censuses) in each drainage. (c) Daily minimum air temperatures recorded by temperature loggers at each site during larval survival and growth experiment.

Results

LARVAL SURVIVAL AND DEVELOPMENT RATE IN NATURE

Mortality of transplanted *Chrysomela aeneicollis* larvae occurred in all drainages (Fig. 1a); however, mortality rates were typically lower for individuals targeted to Bishop Creek (BC) and Big Pine Creek (BPC) than individuals targeted to Rock Creek (RC) (Fig. 1b; Table 1). Larval survivorship did not depend on source population (Table 1). A cooling trend occurred in the entire region shortly after the experiment was initiated, especially from 13–21 July (Fig. 1c). Minimum air temperature was significantly lower in RC than BC or BPC during the measurement period (Fig. 1c; randomized-blocks ANOVA, $F_{2,40} = 24·8$; $P < 0·001$). Much larval mortality occurred during a 9-day period (18–26 July) where minimum temperatures fell below zero every night in RC, but less frequently in BPC (five nights) and BC (two nights; Fig. 1c). On several mornings during that time, larvae that appeared to have frozen on experimental branches were observed. Minimum air temperature was significantly related to mortality rate in all drainages (Fig. 2). Larval development rate in the field differed

Table 1. Mixed-model nested ANOVA results for effects of source population, target drainage, and plant on larval survivorship and development rate in the field. Drainage and source were treated as fixed effects. Plant was treated as a random effect and was nested in target drainage

Source of variation	Average survivorship			Larval development rate		
	df	SS	$F(P)$	df	SS	$F(P)$
Source drainage	2	0·113	1·4	2	0·0004	0·5
Target drainage	2	1·498	7·2***	2	0·0007	0·5***
Plant[Target]	27	2·808	2·6***	24	0·0198	2·1*
Source × Target	4	0·144	0·9	4	0·0024	1·6
Source × Plant[Target]	54	2·153	1·0	48	0·0193	1·5
Error	89	3·568		59	0·0158	

***$P < 0.005$, *$P < 0.05$.

Fig. 3. Relationship between development rate and PGI-1 allele frequency in groups of BC larvae targeted to BPC, BC or RC (symbols as shown in Fig. 1 legend). Development rates were calculated as described in Methods. Development rate was significantly related to PGI allele frequency ($Y = -0.041X + 0.072$; $R^2 = 0.24$; $F_{1,22} = 7.0$, $P = 0.01$).
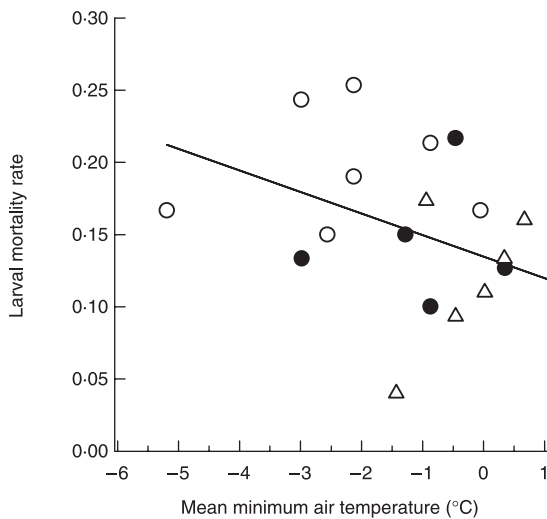

Fig. 2. Relationship between minimum air temperature and mean mortality rate of *C. aeneicollis* larvae targeted to BPC, BC or RC (symbols as shown in Fig. 1 legend). Mortality was negatively related to minimum air temperature ($Y = -0.015X + 0.13$; $R^2 = 0.31$; $F_{1,25} = 11.4$; $P = 0.002$).

Fig. 4. Effects of acclimation temperature on larval growth rate (a) and activity (b) of laboratory reared BC larvae of three common PGI genotypes (1–1, 1–4, 4–4). Data shown are least-squares means (± SE) of growth and activity of each PGI genotype at three treatment temperatures (1–1, $n = 71$; 1–4, $n = 79$; 4–4, $n = 21$). Additional statistical analyses shown in Table 2.

among host plants, but did not depend on source or target drainage (Table 1).

PGI-1 allele frequency of surviving BC larvae targeted to BC (0·51) and RC (0·45) was similar to BC source population frequency (0·55) (Rank & Dahlhoff 2002). However, PGI-1 frequency for surviving BC larvae targeted to BPC (0·29) was significantly lower than those targeted to BC or RC (G-test of genotype frequency heterogeneity among drainages, $G = 9.8$, df = 4, $P = 0.044$). Development rates of BC larvae did not differ among target drainages ($F_{2,22.7} = 0.3$; $P > 0.73$). However, development rate was slowest for larval groups with the highest frequency of PGI allele 1 (Fig. 3).

GROWTH AND ACTIVITY AFTER LABORATORY TEMPERATURE ACCLIMATION

Relative larval growth varied among PGI genotypes and depended on acclimation temperature (Fig. 4; Table 2). Larval growth depended on number of days
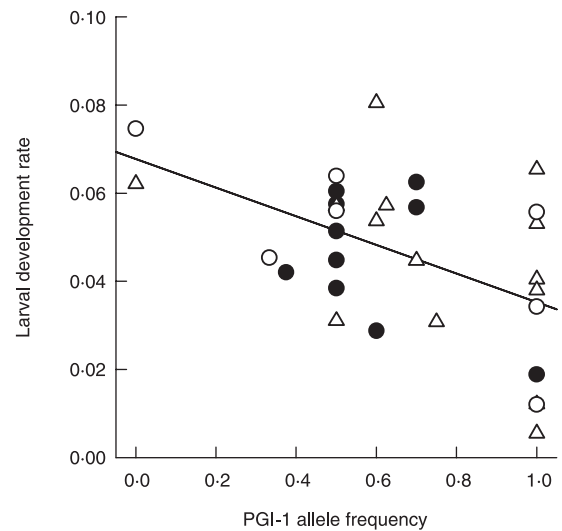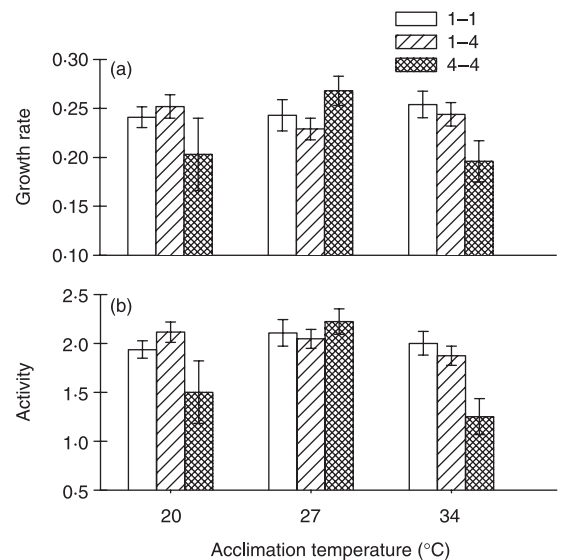
in the experiment and was negatively related to initial body mass (Table 2). Growth rates for PGI 1–1 and PGI 1–4 individuals did not depend on acclimation temperature (Fig. 4a). However, PGI 4–4 homozygotes grew faster at 27 °C than at either 20 or 34 °C. Similar patterns were found with respect to larval activity (Fig. 4b, Table 2). Larval growth and activity were weakly correlated with each other ($r^2 = 0.05$, $n = 133$, $P < 0.01$).

Table 2. ANCOVA and ANOVA showing effects of PGI genotype and laboratory acclimation temperature on growth and activity. PGI genotype (1–1, 1–4, 4–4) and treatment temperature (20, 27 and 34 °C) were treated as fixed effects. Initial body mass and days in treatment were used as covariates in growth analysis (not significant in activity analysis)

	Growth			Activity after treatment		
Source of variation	df	SS	$F(P)$	df	SS	$F(P)$
Acclimation temperature (T)	2	0·0050	0·9	2	3·2048	7·8***
PGI genotype (G)	2	0·0052	1·0	2	1·3576	3·3*
$G \times T$	4	0·0301	2·8*	4	2·9633	3·6**
Initial body mass	1	0·1545	56·5***	–	–	–
Days in experiment	1	0·1180	43·2***	–	–	–
Error	123	0·3363		124	25·365	

***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

Table 3. MANCOVA results for effects of time of day and exposure to sun or shade on body temperatures of field-acclimatized larval *Chrysomela aeneicollis*. Analysis included larvae in sun or shade at five locations at different elevations, five times of day. Body and air temperature are dependent variables. Elevation is a covariate

Source of variation	df	F
Between-subjects factors		
Interval (I)	4	76·37****
Light exposure (L)	1	3·37[1]
$I \times L$	4	0·55
Elevation (E)	1	16·81****
Error	198	
Within-subjects factors		
T_b *vs* T_a (T)	1	5·79**
$T \times I$	4	2·67*
$T \times L$	1	26·40****
$T \times L \times I$	4	0·90
$T \times E$	1	0·26
Error	198	

****$P < 0.0001$, **$P < 0.01$, *$P < 0.05$, [1]$P < 0.10$.

Table 4. Effects of PGI genotype and treatment temperature on Hsp70 expression for Bishop Creek *C. aeneicollis* larvae held at common garden conditions in the laboratory. Data shown are least squares means (± SE)

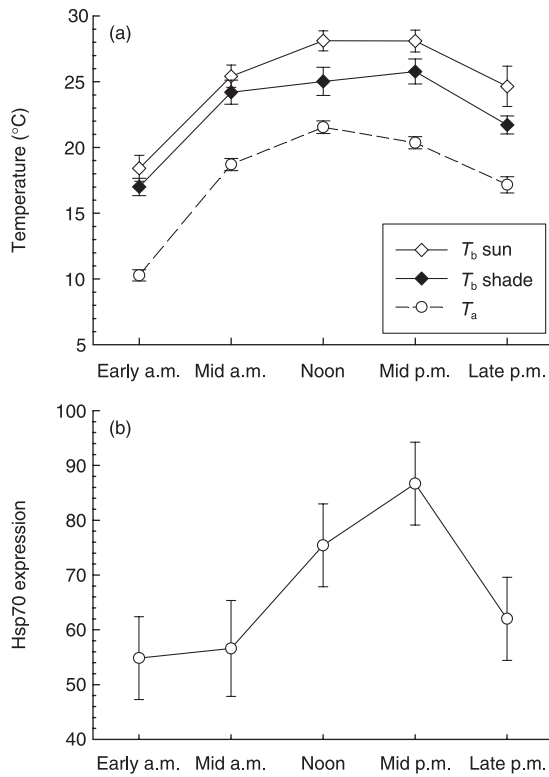|	Hsp70 expression Treatment temperature (°C)			
PGI genotype	0	30	33	35
1–1 ($n = 29$)	14·8 (3·7)	35·8 (3·2)	21·9 (3·2)	20·0 (3·4)
1–4 ($n = 31$)	12·4 (3·7)	27·7 (3·2)	24·6 (3·2)	25·6 (3·0)
4–4 ($n = 27$)	13·7 (3·3)	24·1 (3·0)	23·6 (3·4)	31·7 (3·2)

Fig. 5. (a) Changes in air temperature (T_a) throughout the day in nature, and its effect on body temperature (T_b) of larvae sitting in sun (open diamonds) or shade (solid diamonds). Data shown are least squares means (± SE) for larvae at sites in BPC and RC. (b) Hsp70 expression levels of field acclimatized larvae, reported as nanograms of a 72-kDa isoform of Hsp70 per g total thorax muscle protein. Data shown are least-squares means (± SE) for $n = 9$ larvae collected at each time point.

LARVAL BODY TEMPERATURE AND INDUCTION OF HSP70 IN NATURE

In nature, larval body temperature was significantly correlated with air temperature ($Y = 8·30 + 0·87X$; $F_{1,157} = 290·2$; $P < 0·0001$), though average T_b was 3–9 °C higher than average T_a (Fig. 5a). Body temperature of beetles in sun and shade increased until mid-day and remained elevated until the late afternoon. Beetles measured in the sun were on average 10% warmer than those in the shade, and the difference between sun and shade varied over the day (Table 3, Fig. 5a). Maximal body temperature measured was 36·7 °C, and 9·4% of larvae showed body temperatures at or above 30 °C (16·7% of larvae at mid-day). Hsp70 expression levels were higher for larvae collected in the afternoon than earlier in the day (Fig. 5b; two-way ANOVA $F_{4,43} = 2·9$; $P < 0·05$). PGI 1–1 ($n = 16$) and 1–4 ($n = 17$) individuals expressed nearly twice as much Hsp70 ($79·9 \pm 6·7$ ng g^{-1}; $74·4 \pm 6·7$ ng g^{-1}, respectively) than 4–4 ($n = 10$) individuals ($41·7 \pm 8·5$ ng g^{-1}; one-way ANOVA $F_{2,41} = 6·8$; $P < 0·005$).

LABORATORY HEAT SHOCK

Hsp70 expression was lower for laboratory-acclimated than field-acclimatized larvae (Fig. 5b, Table 4). Hsp70 expression after laboratory heat shock depended on acclimation temperature (two-way ANOVA $F_{3,75} = 8·8$; $P < 0·001$) and varied among PGI genotypes (temperature–PGI genotype interaction: $F_{6,75} = 2·3$; $P < 0·05$). At

30 °C, PGI 1–1 homozygotes expressed higher levels of Hsp70 than 1–4 heterozygotes or 4–4 homozygotes, whereas after exposure to 35 °C, these differences were reversed: 4–4 > 1–4 > 1–1. At 20 °C, Hsp70 expression levels were not zero; thus, a non-inducible (cognate) form of Hsp70 may also have been detected.

Discussion

These results suggest that thermal extremes have profound effects on survival and rates of development of Sierra Willow Beetle larvae. While previous studies have demonstrated the importance of cold exposure on growth, development rate and survival (Cooke & Roland 2003; Renault *et al.* 2003; Williams *et al.* 2003), the present study is one of the first to demonstrate natural cold mortality in the summer. In the Eastern Sierra Nevada, beetle larvae are routinely exposed to subzero night-time temperatures in the midst of development, and this appears to cause mortality. Larval mortality was highest in the drainage that was coldest during the transplant experiment (RC), and was significantly correlated with minimum night-time air temperature in all drainages. In contrast to the effects of cold, elevated temperatures did not appear to cause rapid mortality, since we never observed dead or dying larvae in the late afternoon, when temperatures were highest. However, repeated exposure to elevated temperatures may impose other costs, via the heat shock response as discussed below.

Several possibilities may explain the relationship between colder temperatures and larval mortality. Slow-growing larvae may have higher mortality, because they remain vulnerable to attack from predators for an extended period of time (Clancy & Price 1987). However, we excluded crawling predators from our experiment, and the most important flying predator, the wasp *Symmorphus cristatus*, specializes on larger, 3rd instar larvae (Sears *et al.* 2001). Thus, it is unlikely that differential survival was due to variation in predation. Alternately, small, slower-growing larvae may be more susceptible to freezing events, owing to lower energy stores and higher surface area to volume ratios (Fordyce & Shapiro 2003). This may explain much of the mortality observed in our experiment. We also observed differences in survival and development rates for individuals possessing differing forms of the glycolytic enzyme phosphoglucose isomerase (PGI; discussed below), an enzyme critical for allocation of energy to storage and activity.

Recent studies of causes of variation in larval survival and development rates for insect herbivores in nature have found that temperature-dependent effects on host plant may affect larval survival (Ayres & Scriber 1994; Virtanen & Neuvonen 1999; Hellmann 2002). Generally, high-quality host plants support rapid growth, which may ameliorate detrimental effects of thermal extremes in marginal habitats (Alonso 1999; Hellmann 2002; Levesque, Fortin &

Mauffette 2002). Previous studies of Sierra populations of *C. aeneicollis* showed that larvae survived best on plants with highest water content, an index of plant quality (Rank 1994). In the present study, development rates varied among target host plants within, but not between drainages; in addition, we did not observe differences in water content between experimental host plants (D. McMillan, unpublished data). We are not able to completely rule out abiotic differences, besides temperature, among drainages, though we selected sites with similar physical characteristics to minimize these differences. Our data suggest that differences in larval survival between drainages are caused primarily by variation in exposure to thermal extremes.

On sunny days, larvae sitting in the sun had significantly higher body temperatures than those in shade. Daytime temperatures routinely experienced by larvae caused elevation of heat shock protein (Hsp70) expression levels, especially later in the afternoon. Interestingly, Hsp70 expression was elevated by 8 a.m. in some individuals. Three possibilities may explain this result. First, the antibody used, which detects an inducible isoform of Hsp70 in adult beetles, may also detect 'background' levels of a non-inducible cognate form (Hsc70) of similar molecular weight in larvae. While expression levels of Hsc70 may change with long-term acclimatization or during development, the dramatic diurnal shifts imply rapid changes in expression and are probably due to expression of a stress-inducible Hsp. Second, Hsp70 present in tissue early in the day may be the result of the previous day's exposure to elevated temperature. It may take up to 32 h to clear cells of Hsp70 after an induction (Feder *et al.* 1992; Dahlgaard *et al.* 1998). Third, Hsp70 expression may be induced directly by exposure to night-time cold. Recent studies suggest that Hsps enhance cold tolerance in insects (Kelty & Lee 1999, 2001; Yocum 2001; Hoffmann *et al.* 2003). Although cold tolerance is thought to be uncoupled from the ability to tolerate elevated temperatures (Chown 2001; Klok & Chown 2003), few studies have examined a natural system in which the animal is exposed to elevated and subzero temperatures over a single diurnal cycle. Thus, up-regulation of Hsp70 expression during the day, perhaps coupled with expression induced by night-time cold, may confer enhanced larval survival after exposure to freezing temperatures in summer. This may partially account for the enhanced tolerance to cold exposure observed by Rank & Dahlhoff (2002).

Our results suggest that PGI is under temperature selection in beetle larvae, as it appears to be in adults. In nature, mortality of larvae possessing the PGI-1 allele was highest in BPC, the drainage where daytime temperatures tend to be highest, and the natural frequency of allele 1 is relatively low. Also, experimental groups from BC with high frequencies of the PGI-1 allele developed most slowly under all field conditions. Laboratory results also suggested that differences in growth rate and activity among PGI genotypes depend

on thermal history. PGI 4–4 genotypes had higher growth rates and activities than 1–1 or 1–4 genotypes after exposure to a moderately high temperature typical of natural habitats (27 °C), but not after exposure to extreme temperatures rarely experienced in nature (35 °C). Differences in survival and development among PGI genotypes may be due in part to differential patterns of Hsp70 expression (Rank & Dahlhoff 2002; Neargarder *et al*. 2003). PGI 1–1 genotypes expressed higher levels of Hsp70 at moderate temperatures than 1–4 or 4–4 genotypes under both natural and laboratory-controlled conditions. The mechanistic relationship between PGI genotype and Hsp70 expression in *C. aeneicollis* is not yet clearly understood. One possibility is that PGI is closely linked to one or more other loci that are under natural selection. Another possibility is that PGI itself is under selection, which is consistent with functional differences in thermal stability and binding constant that exist among PGI genotypes (Dahlhoff & Rank 2000; E. Dahlhoff, unpublished data), and consistent with results found for a variety of other taxa (Shihab & Heath 1987; Johannesson, Kautsky & Tedengren 1990; Watt 1992; Katz & Harrison 1997; Watt *et al*. 2003). In either case, higher expression levels of Hsp70 for individuals possessing the 1 allele may lead to greater cold tolerance in nature, at the cost of reduced developmental rate.

Acknowledgements

References

Alonso, C. (1999) Variation in herbivory by *Yponomeuta mahalebella* on its only host plant *Prunus mahaleb* along an elevational gradient. *Ecological Entomology* **24**, 371–379.

Ayres, M.P. & Scriber, J.M. (1994) Local adaptation to regional climates in *Papilio canadensis* (Lepidoptera, Papilionidae). *Ecological Monographs* **64**, 465–482.

Bradshaw, W.E. & Holzapfel, C.M. (1996) Genetic constraints to life-history evolution in the pitcher-plant mosquito, *Wyeomyia smithii*. *Evolution* **50**, 1176–1181.

Brown, W.J. (1956) The New World species of *Chrysomela* L. (Coleoptera: Chrysomelidae). *Canadian Entomologist* **88**, 1–54.

Bryant, S.R., Thomas, C.D. & Bale, J.S. (2002) The influence of thermal ecology on the distribution of three nymphalid butterflies. *Journal of Applied Ecology* **39**, 43–55.

Chown, S.L. (2001) Physiological variation in insects: hierarchical levels and implications. *Journal of Insect Physiology* **47**, 649–660.

Clancy, K.M. & Price, P.W. (1987) Rapid herbivore growth enhances enemy attack: sublethal plant defenses remain a paradox. *Ecology* **68**, 733–737.

Cooke, B.J. & Roland, J. (2003) The effect of winter temperature on forest tent caterpillar (Lepidoptera: Lasiocampidae) egg survival and population dynamics in northern climates. *Environmental Entomology* **32**, 299–311.

Dahlgaard, J., Loeschcke, V., Michalak, P. & Justesen, J. (1998) Induced thermotolerance and associated expression of the heat-shock protein Hsp70 in adult *Drosophila melanogaster*. *Functional Ecology* **12**, 786–793.

Dahlhoff, E.P. & Rank, N.E. (2000) Functional and physiological consequences of genetic variation at phosphoglucose isomerase: heat shock protein expression is related to enzyme genotype in a montane beetle. *Proceedings of the National Academy of Sciences USA* **97**, 10056–10061.

Danks, H.V., Kukal, O. & Ring, R.A. (1994) Insect cold-hardiness: insights from the Arctic. *Arctic* **47**, 391–404.

Fearnley, S. (2004) *Adaptation at an enzyme locus in* Chrysomela aeneicollis: *Situating the PGI polymorphism in a functional and historical context*. Master's Thesis, Sonoma State University, Rohnert Park, CA.

Feder, M.E. & Hofmann, G.E. (1999) Heat-shock proteins, molecular chaperones and the heat-shock response: evolutionary and ecological physiology. *Annual Review of Physiology* **61**, 243–282.

Feder, J.H., Rossi, J.M., Solomon, J., Solomon, N. & Lindquist, S. (1992) The consequences of expressing hsp70 in *Drosophila* cells at normal temperatures. *Genes and Development* **6**, 1402–1413.

Feder, J.L., Roethele, J.B., Wlazlo, B. & Berlocher, S.H. (1997) Selective maintenance of allozyme differences among sympatric host races of the apple maggot fly. *Proceedings of the National Academy of Sciences USA* **94**, 11417–11421.

Fordyce, J.A. & Shapiro, A.M. (2003) Another perspective on the slow-growth/high-mortality hypothesis: chilling effects on swallowtail larvae. *Ecology* **84**, 263–268.

Garbuz, D., Evgenev, M.B., Feder, M.E. & Zatsepina, O.G. (2003) Evolution of thermotolerance and the heat-shock response: evidence from inter/intraspecific comparison and interspecific hybridization in the *virilis* species group of *Drosophila*. I. Thermal phenotype. *Journal of Experimental Biology* **206**, 2399–2408.

Hellmann, J.J. (2002) The effect of an environmental change on mobile butterfly larvae and the nutritional quality of their hosts. *Journal of Animal Ecology* **71**, 925–936.

Hochachka, P.W. & Somero, G.N. (2002) *Biochemical Adaptation: Mechanism and Process in Physiological Evolution.* Oxford University Press, New York.

Hoffmann, A.A., Sorensen, J.G. & Loeschcke, V. (2003) Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. *Journal of Thermal Biology* **28**, 175–216.

Irwin, J.T. & Lee, R.E. (2003) Cold winter microenvironments conserve energy and improve overwintering survival and potential fecundity of the goldenrod gall fly, *Eurosta solidaginis*. *Oikos* **100**, 71–78.

Johannesson, K., Kautsky, N. & Tedengren, M. (1990) Genotypic and phenotypic differences between Baltic and North Sea populations of *Mytilus edulis* evaluated through reciprocal transplantations II. Genetic variation. *Marine Ecology Progress Series* **59**, 211–220.

Katz, L.A. & Harrison, R.G. (1997) Balancing selection on electrophoretic variation of phosphoglucose isomerase in two species of field cricket: *Gryllus veletis* and *G. pennsylvanicus*. *Genetics* **147**, 609–621.

Kelty, J.D. & Lee, R.E. (1999) Induction of rapid cold hardening by cooling at ecologically relevant rates in *Drosophila melanogaster*. *Journal of Insect Physiology* **45**, 719–726.

Kelty, J.D. & Lee, R.E. (2001) Rapid cold-hardening of *Drosophila melanogaster* (Diptera: Drosophilidae) during ecologically based thermoperiodic cycles. *Journal of Experimental Biology* **204**, 1659–1666.

Ketola, T., Laakso, J., Kaitala, V. & Airaksinen, S. (2004) Evolution of Hsp90 expression in *Tetrahymena thermophila* (Protozoa, Ciliata) populations exposed to thermally variable environments. *Evolution* **58**, 741–748.

Klok, C.J. & Chown, S.L. (2003) Resistance to temperature extremes in sub-Antarctic weevils: interspecific variation, population differentiation and acclimation. *Biological Journal of the Linnean Society* **78**, 401–414.

Krebs, R.A. & Feder, M.E. (1997) Deleterious consequences of Hsp70 overexpression in *Drosophila melanogaster* larvae. *Cell Stress Chaperones* **2**, 60–71.

Krebs, R.A. & Feder, M.E. (1998) Hsp70 and larval thermotolerance in *Drosophila melanogaster*: how much is enough and when is more too much? *Journal of Insect Physiology* **44**, 1091–1101.

Levesque, K.R., Fortin, M. & Mauffette, Y. (2002) Temperature and food quality effects on growth, consumption and post-ingestive utilization efficiencies of the forest tent caterpillar *Malacosoma disstria* (Lepidoptera: Lasiocampidae). *Bulletin of Entomological Research* **92**, 127–136.

Loeschcke, V., Krebs, R.A., Dahlgaard, J. & Michalak, P. (1997) High-temperature stress and the evolution of thermal resistance in *Drosophila*. *Environmental Stress, Adaptation and Evolution* (eds R. Bijlsma & V. Loeschcke), pp. 175–190. Birkhauser Verlag, Basel.

Neargarder, G.G., Dahlhoff, E.P. & Rank, N.E. (2003) Variation in thermal tolerance and HSP70 expression is linked to phosphoglucose isomerase genotype in a montane leaf beetle. *Functional Ecology* **17**, 213–221.

Rank, N.E. (1992a) A hierarchical analysis of genetic differentiation in a montane leaf beetle (*Chrysomela aeneicollis*). *Evolution* **46**, 1097–1111.

Rank, N.E. (1992b) Host plant preference based on salicylate chemistry in a willow leaf beetle (*Chrysomela aeneicollis*). *Oecologia (Berlin)* **90**, 95–101.

Rank, N.E. (1994) Host plant effects on larval survival in a salicin-using leaf beetle *Chrysomela aeneicollis* (Coleoptera: Chrysomelidae). *Oecologia (Berlin)* **97**, 342–353.

Rank, N.E. & Dahlhoff, E.P. (2002) Allele frequency shifts in response to climate change and physiological consequences of allozyme variation in a montane insect. *Evolution* **56**, 2278–2289.

Rank, N.E., Köpf, A., Julkunen-Tiitto, R. & Tahvanainen, J. (1998) Host preference and larval performance of the salicylate-using leaf beetle *Phratora vitellinae*. *Ecology* **79**, 618–631.

Renault, D., Hance, T., Vannier, G. & Vernon, P. (2003) Is body size an influential parameter in determining the duration of survival at low temperatures in *Alphitobius diaperinus* Panzer (Coleoptera: Tenebrionidae)? *Journal of Zoology* **259**, 381–388.

Sears, A.L.W., Smiley, J.T., Hilker, M., Muller, F. & Rank, N.E. (2001) Nesting behavior and prey use in two geographically separated populations of the specialist wasp *Symmorphus cristatus* (Vespidae: Eumeninae). *American Midland Naturalist* **145**, 233–246.

Shihab, A.F. & Heath, D.J. (1987) Components of fitness and the PGI polymorphism in the freshwater isopod *Asellus aquaticus* L. 1. Fecundity selection. *Heredity* **58**, 69–74.

Sorensen, J.G. & Loeschcke, V. (2004) Effects of relative emergence time on heat stress resistance traits, longevity and hsp70 expression level in *Drosophila melanogaster*. *Journal of Thermal Biology* **29**, 195–203.

Sorensen, J.G., Kristensen, T.N. & Loeschcke, V. (2003) The evolutionary and ecological role of heat shock proteins. *Ecology Letters* **6**, 1025–1037.

Tomanek, L. & Sanford, E. (2003) Heat-shock protein 70 (Hsp70) as a biochemical stress indicator: an experimental field test in two congeneric intertidal gastropods (Genus: *Tegula*). *Biology Bulletin* **205**, 276–284.

Tomanek, L. & Somero, G.N. (1999) Evolutionary and acclimation-induced variation in the heat-shock responses of congeneric marine snails (genus *Tegula*) from different thermal habitats: implications for limits of thermotolerance and biogeography. *Journal of Experimental Biology* **202**, 2925–2936.

Virtanen, T. & Neuvonen, S. (1999) Performance of moth larvae on birch in relation to altitude, climate, host quality and parasitoids. *Oecologia* **120**, 92–101.

Watt, W.B. (1992) Eggs, enzymes, and evolution: natural genetic variants change insect fecundity. *Proceedings of the National Academy of Sciences USA* **89**, 10608–10612.

Watt, W.B., Wheat, C.W., Meyer, E.H. & Martin, J.F. (2003) Adaptation at specific loci. VII. Natural selection, dispersal and the diversity of molecular-functional variation patterns among butterfly species complexes (Colias: Lepidoptera, Pieridae). *Molecular Ecology* **12**, 1265–1275.

Williams, J.B., Shorthouse, J.D. & Lee, R.E. (2003) Deleterious effects of mild simulated overwintering temperatures on survival and potential fecundity of rose-galling *Diplolepis* wasps (Hymenoptera: Cynipidae). *Journal of Experimental Zoology Part A Comparative Experimental Biology* **298A**, 23–31.

Willmer, P., Stone, G. & Johnston, I. (2000) *Environmental Physiology of Animals*. Blackwell Science, Oxford.

Yocum, G.D. (2001) Differential expression of two HSP70 transcripts in response to cold shock, thermoperiod, and adult diapause in the Colorado potato beetle. *Journal of Insect Physiology* **47**, 1139–1145.

Zatsepina, O.G., Velikodvorskaia, V.V., Molodtsov, V.B., Garbuz, D., Lerman, D.N., Bettencourt, B.R., Feder, M.E. & Evgenev, M.B. (2001) A *Drosophila melanogaster* strain from sub-equatorial Africa has exceptional thermotolerance but decreased Hsp70 expression. *Journal of Experimental Biology* **204**, 1869–1881.