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Phosphoglucose isomerase genotype affects running speed and heat shock protein expression after exposure to extreme temperatures in a montane willow beetle

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Summary

Eastern Sierra Nevada populations of the willow beetle *Chrysomela aeneicollis* commonly experience stressfully high and low environmental temperatures that may influence survival and reproduction. Allele frequencies at the enzyme locus *phosphoglucose isomerase* (PGI) vary across a climatic latitudinal gradient in these populations, with PGI allele 1 being most common in cooler regions and PGI allele 4 in warmer ones. PGI genotypes differ in heat and cold tolerance and in expression of a 70 kDa heat shock protein. Here we examine genetic, behavioral and environmental factors affecting a performance character, running speed, for willow beetles, and assess effects of consecutive cold and heat exposure on running speed and expression of Hsp70 in the laboratory. In nature, running speed depends on air temperature and is higher for males than females. Mating beetles ran faster than single beetles, and differences among PGI genotypes in male running speed depended on the presence of females. In the laboratory, exposure to cold reduced subsequent running speed, but the amount of this reduction depended on PGI genotype and previous thermal history. Effects of exposure to heat also depended on life history stage and PGI genotype. Adults possessing allele 1 ran fastest after a single exposure to stressful temperature, whereas those possessing allele 4 ran faster after repeated exposure. Larvae possessing allele 4 ran fastest after a single stressful exposure, but running speed generally declined after a second exposure to stressful temperature. The ranking of PGI genotypes after the second exposure depended on whether a larva had been exposed to cold or heat. Effects of temperature on Hsp70 expression also varied among PGI genotypes and depended on type of exposure, especially for adults (single heat exposure, two cold exposures: PGI 1-1>1-4>4-4; other multiple extreme exposures: 4-4>1-4>1-1). There was no consistent association between alleles at other polymorphic enzyme loci and running speed or Hsp70 expression. These data suggest that variation at PGI is associated with considerable plasticity in running speed. Differences in Hsp70 expression among PGI genotypes suggest that the heat-shock response may buffer differences in thermal tolerance and performance among genotypes and help maintain the PGI polymorphism in a thermally variable environment.

Supplementary material available online at
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Key words: adaptation, allozyme, cold, heat, Hsp70, insect, Chrysomelidae, PGI.

Introduction

Over time, changes in Earth's climate have caused local extinctions and shifts in ranges of some species (Barnosky et al., 2003; Bradley, 1999), whereas others have adapted to altered conditions. A species' ability to respond to a new environmental temperature regime depends partly on whether it possesses genetic variation in traits associated with thermal tolerance and temperature adaptation (Bradshaw and Holzapfel, 2001; Bradshaw et al., 2004; Brakefield and Willmer, 1985; Dahlhoff and Rank, 2000; Parmesan and Yohe, 2003; Rank and Dahlhoff, 2002). Scientists are becoming increasingly aware that the environment plays a large role in phenotypic expression of individual genes (DeWitt and Scheiner, 2004), and that genetic variation affects multiple phenotypic characters (Pigliucci, 2004; Pigliucci and Preston, 2004). To fully understand the significance of genetic variation for traits involved in temperature adaptation, their phenotypic expression should be measured under environmental conditions that simulate natural thermal variation (Podrabsky and Somero, 2004). Organisms at the edge of their natural ranges may occur

at the limits of their physiological capacity to cope with environmental change, and are thus ideal for such studies (Ayres and Lombardero, 2000; Ayres and Scriber, 1994; Baskauf and McCauley, 2001; Klok and Chown, 2003; Roy and Thomas, 2003; Sagarin and Somero, 2006; Sorte and Hofmann, 2005).

Many studies suggest that metabolic enzyme loci are associated with an organism's ability to cope with thermal extremes or fluctuations in temperature (Podrabsky and Somero, 2004; Riehle et al., 2001; Riehle et al., 2005). Metabolic enzyme variants differing in functional properties, such as Michaelis-Menten binding constant (K_m) or catalytic efficiency (indexed by k_{cat} or V_{max}/K_m), are often found along environmental temperature gradients, and there are many cases where variation in allozymes (between populations) or orthologous homologs (between closely related species) appear to result from temperature adaptation (Dahlhoff and Somero, 1993; Graves and Somero, 1982; Johns and Somero, 2004; Mitton, 1997; Somero, 2004). For example, in his studies of the *phosphoglucose isomerase* (PGI) polymorphism in *Colias* butterflies, Watt found that PGI genotypes that differ in K_m and V_{max}/K_m also vary in flight performance, and these differences correspond to genotype-based differences in fecundity and mating success (Watt, 1983; Watt, 1992; Watt et al., 1985; Watt et al., 1983). Because these functional properties of metabolic enzymes are strongly temperature dependent, one might expect that the relationship between enzyme genotype and physiological and fitness characters depends on thermal history of the individual organism. However, few studies have addressed this issue (Neargarder et al., 2003; Rank and Dahlhoff, 2002).

The Sierra willow leaf beetle *Chrysomela aeneicollis* (Schaeffer) presents an excellent model organism to gain a better understanding of the relationship between thermal exposure and naturally occurring genetic variation in traits related to temperature adaptation. Sierra Nevada populations of this beetle are found at high elevations (2400–3600 m) and live on the southern edge of the species' range in North America (Brown, 1956). There, they endure greater extremes in temperature than conspecifics in other regions (Dingle et al., 1990). Sierra populations experience wide fluctuations in daily temperatures during summer, from –10°C on cold nights to over 35°C during warm days. Annual and seasonal variation in climatic conditions cause shifts in beetle distribution and abundance (Dahlhoff and Rank, 2000; Fearnley, 2003; McMillan et al., 2005; Rank and Dahlhoff, 2002). Natural selection to temperature appears to act on the PGI locus in Sierra populations of *C. aeneicollis*. PGI allele 1 is present in higher frequency at northern, colder sites, whereas PGI allele 4 is most frequent in sub-populations living in southern, warmer sites. PGI allele 1 frequency increased between 1988 and 1996, coinciding with several years of increasing precipitation and decreasing temperature (Rank and Dahlhoff, 2002). In addition, changes in air temperature during summer 2001 were linked to shifts in PGI allele frequency in populations where alleles 1 and 4 are both common (Fearnley, 2003). By contrast, other polymorphic enzyme loci in these populations do not vary with elevation, latitude, climate change or experimental manipulation in a systematic way (Bruce, 2005; Rank, 1992; Rank and Dahlhoff, 2002). Biochemical and physiological evidence also support the hypothesis that PGI is under temperature selection. PGI allozymes differ in K_m and thermostability (Dahlhoff and Rank, 2000), and heat shock protein (Hsp70) expression and thermal tolerance vary among PGI genotypes (Dahlhoff and Rank, 2000; McMillan et al., 2005; Neargarder et al., 2003; Rank and Dahlhoff, 2002).

Here we evaluate the effect of repeated thermal stress, which mimics temperature fluctuations found in nature, on differences in running speed among PGI genotypes.

Running speed is an important component of fitness for leaf beetle adults and larvae (Gibert et al., 2001; Gilchrist, 1996; Wisco et al., 1997). Male mating success is influenced by the ability to find females more quickly than other males (Rank et al., 2006), whereas females run along willow branches to locate suitable oviposition sites. For larvae, running speed may be related to ability to escape crawling predators (Rank and Smiley, 1994; Rank et al., 1996). Running speed is also useful as a performance character that relates to overall physiological status of an organism (Brana, 2003; Lighton and Duncan, 2002). It is closely related to metabolic rate (Hochachka and Somero, 2002; Lovegrove, 2004; Willmer et al., 2004), and locomotor performance declines after exposure to environmental stress in many species (Folk and Gilchrist, 2005; Klose et al., 2005; Robertson, 2004; Sorensen and Loeschcke, 2004).

We also explore the role that Hsp expression may play in reducing differences in performance after exposure to stressful environmental temperatures. Organisms upregulate Hsps in response to conditions that damage proteins or other cellular structures, and this response enhances survival and thermotolerance in nature (Dahlhoff, 2004; Feder and Hofmann, 1999; Gehring and Wehner, 1995; Sorensen et al., 2003). In addition, Hsp-assisted folding of mutant polypeptides may buffer against the development of phenotypes with reduced fitness as a consequence of exposure to environmental stress (Roberts and Feder, 1999; Rutherford, 2003; Rutherford and Lindquist, 1998). However, stress-inducible Hsp expression competes with housekeeping metabolism and may impose a fitness cost on routinely stressed individuals (Krebs and Feder, 1998; Krebs and Holbrook, 2001; Loeschcke et al., 1997; Robertson, 2004). Previous studies of Sierra willow beetles have shown that genetic variation at PGI results in differential expression and induction of Hsp70 (Dahlhoff and Rank, 2000; McMillan et al., 2005; Rank and Dahlhoff, 2002). Individuals possessing the thermolabile allele 1 upregulate Hsp70 at lower temperatures than individuals homozygous for the thermostable allele 4, and Hsp70 expression levels vary among PGI genotypes over the range of temperatures typically experienced in nature. Thus, the relationship between performance and genotypic variation at a locus under temperature selection (PGI) may be mediated in part by differences in Hsp70 expression. Increased Hsp70 expression in beetles possessing allele 1 could

enhance protection of metabolic enzymes important for locomotor performance, including PGI, and result in higher running speeds after exposure to thermal stress. However, continued exposure to thermal extremes may result in reduced running speed in PGI 1-1 or 1-4 individuals, because of increased cost of maintaining the heat-shock response, relative to individuals homozygous for PGI allele 4. To date, the importance of differential Hsp expression among natural genetic variants of enzymes important for locomotion, such as PGI, has not been demonstrated.

In the present study, we assessed factors that influence running speed and Hsp70 expression of adults and larvae of *C. aeneicollis*. First, we quantified the relationship between beetle body temperature and running speed in nature for both sexes. Second, we measured running speed of males in nature after manipulation of mating frequency. Third, we exposed adults and larvae to stressful temperatures and measured running speeds before and after each exposure. After a final exposure and running speed measurement, we froze beetles for analysis of Hsp70 expression. For all experiments, we obtained genotypes at PGI and two other polymorphic loci, *isocitrate dehydrogenase* (IDH) and *phosphoglucomutase* (PGM), to test whether either trait relates to other metabolic enzyme genotypes.

Materials and methods

Animal collection and maintenance

Animals were collected near Bluff Lake, in the Green Lake sub-drainage of Bishop Creek in the Eastern Sierra Nevada, California, USA (37°11′N, 118°32′W; 3200 m). This locality was chosen because PGI alleles 1 and 4 occur in relatively equal frequency, and variability is also high for other polymorphic enzyme loci (Rank, 1992; Rank and Dahlhoff, 2002). Experiments on adults were performed on individuals recently emerged from winter diapause, which were observed running on willow shoots, feeding, sitting or engaged in reproductive behaviors (mating or fighting over mates in males; ovipositing in females) at collection (June–July). Approximately 40% of females were gravid at collection. Experiments on larvae were performed on third instars, which were feeding or walking at the time of collection (July). After collection, beetles were held in controlled-temperature incubators at natural day-night light cycles (14 h day, 20°C; 10 h night, 4°C) at White Mountain Research Station (WMRS) in Bishop, California. Beetles were fed fresh leaves from their favored host plant (*Salix orestera*) and given moisture by placing a piece of dampened filter paper in the petri dish. Animals were kept in the laboratory for a maximum of 72 h (laboratory running speed experiments) or 24 h (field running speed experiments).

Factors affecting running speed in nature

Experimental design

We measured voluntary running speeds in nature of adult males and females that had been either mating or not mating at the time of collection. We then determined effects of field mating status and mating treatment on male running speed. To perform mating treatment, after an initial measure of running speed in the field, males were randomly assigned to a plastic cup with either two or no females. Pairs were brought to WMRS and kept in these cups throughout the day (20°C, 14 h) and overnight (females removed; 4°C, 10 h), and then returned to the field site for a second measure of running speed, after which males were frozen at –80°C until genotypes at metabolic enzyme loci were determined. These experiments were performed on 23–27 June 2004.

Running speed

Voluntary maximum running speed was measured at ambient temperature (11–24°C) at Bluff Lake. Beetles were placed facing upwards onto the lower portion of a 6 mm diameter vertical wooden dowel. Because they are positively phototactic, beetles voluntarily ran up the dowel. A stopwatch was used to measure the time required to run 10 cm (first experiment, mean=12.1±0.40 s, N=170 females and males) or 5 cm (second experiment, mean=7.2±0.26 s, N=174 males). Experiments were run between 10:00 h and 16:00 h on sunny or partly cloudy days. Immediately after each run, body temperature of each beetle (T_b) and ambient air temperature (T_a) were measured with a digital thermometer (Omega HH-82, Type T thermocouple). T_b was measured by restraining the beetle in a piece of mesh netting and pressing the thermocouple underneath the elytron long enough to obtain a stable temperature reading. This allowed for accurate determination of T_b without damage to the beetle.

Effects of repeated exposure to extreme temperature on running speed

Experimental design

In the laboratory, beetle running speed was measured three times: (1) day 1, within 6 h of field collection (initial run); (2) day 2, 1 h after initial 4 h temperature treatment (–4, 20 or 36°C for adults; –4, 20 or 35°C for larvae; Laboratory run 1), and (3) day 3, 1 h after final 4 h temperature treatment (–4 or 36°C for adults; –4 or 35°C for larvae; Laboratory run 2). Beetles from each initial temperature treatment were exposed to the cold or hot final temperature treatment (Fig. 1). We were unable to include a 20°C ‘control’ group for the second laboratory running speed measure (or subsequent Hsp70 expression level measurements) because the required sample size (N>900) was too large. However, previous experiments on beetles held under ‘control’ (20°C) conditions from these populations showed that Hsp70 expression levels are less than 5 ng g^{-1} total protein. Treatment temperatures were based on temperatures that resulted in differential Hsp expression and thermal tolerance among PGI genotypes in previous studies (McMillan et al., 2005; Neargarder et al., 2003; Rank and Dahlhoff, 2002), and on measures of thermal tolerance for experimental populations (see below). Experiments were performed with 10 sets of adults run from 25 June–7 July 2002 and five sets of third instar larvae run from 27–31 July 2002.

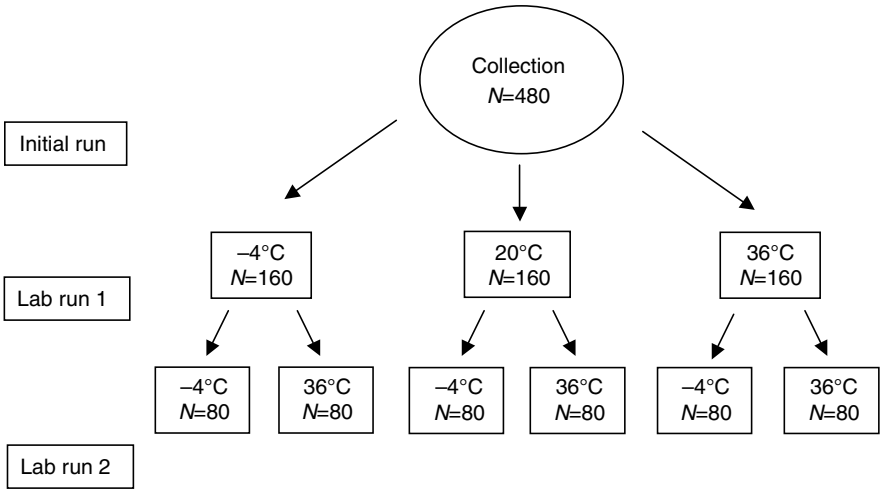

Fig. 1. Experimental design used to determine effects of exposure to thermal extremes on beetle running speed and Hsp70 expression. After field-collection and initial running speed measurement, beetles were divided into three temperature-treatment groups, each of which was sub-divided into two groups. Beetles in first treatment were held at identical or opposite temperatures in the second treatment. Running speed measurements were made immediately after field collection and after two days of consecutive 4 h temperature treatments. Adult sample sizes are shown. Larvae sample sizes were as follows: collection N=240; first treatment: N=80 for each cell; second treatment: N=40 for each cell. Larval heat-treatment temperature was 35°C. Additional details of collection and treatment regime are described in the text. Actual sample sizes used for each analysis are reported in the figure captions.

Ambient air temperature was recorded every 30 min near the Bluff Lake collection site using 'Tidbit' temperature loggers (Onset Computer Co., Pocaset, MA, USA) suspended in a white plastic thermal shields, following the methods of McMillan et al. (McMillan et al., 2005). Mean daily air temperature (measured from 08:00 h–20:00 h each day) for this period was 20.7±0.2°C, determined using Jmp In software (Version 5.0 for PC; SAS Institute Inc., Cary, NC, USA). This temperature (±2°C) was used as 'control' temperature for running speed experiments.

Determination of sub-lethal stressful (extreme) temperatures

Stressful temperatures for adults and larvae were confirmed by assessing temperature at which 50% of animals died from exposure to a thermal extreme (LT_{50}). For LT_{50} assessment, 10–30 individuals were exposed to one of 2–3 temperatures for 4 h, held at 20°C for 1 h and then examined for recovery using an index described in Neargarder et al. (Neargarder et al., 2003). LT_{50} was interpolated between temperatures at which beetle recovery was higher or lower than 50%. Treatment temperatures were 1°C warmer than LT_{50} cold and 1°C cooler than LT_{50} heat. Few individuals died during running speed experiments (six adults and one larva), indicating that treatment temperatures were generally below the maximum value and above the minimum value for *C. aeneicollis* thermal tolerance.

Running speed

Voluntary maximum running speed was measured in the laboratory at 20±2°C. Beetles were placed onto the lower portion of a 6 mm diameter vertical wooden dowel, facing a 100-watt incandescent bulb 60 cm above the dowel. Runs were filmed with a Sony digital video camera and videos were analyzed in iMovie for Macintosh (Version 3.0.3). The fastest portion of each run (10 cm for adults, 2 cm for larvae) was measured. Running speed was determined by subtracting the start from the end time on the video clip. Occasionally, smaller segments (5 cm for adults, 1 cm for larvae) were used if an individual stopped moving in a straight line during the run. For each run, T_a was measured with a digital thermometer. During a practice trial of this experiment, it was observed that T_b was within 1°C of T_a under the artificial lighting conditions in the laboratory.

Biochemical analysis

Determination of allozyme genotype

Adults and larvae from laboratory running speed measurements were weighed and frozen at –80°C exactly 1 h after the final temperature treatment and running speed measurement. Enzyme genotypes for three allozyme loci, IDH, PGI and PGM, were determined using established starch gel electrophoresis protocols (Murphy et al., 1996; Rank, 1992). Adult males used in the field running speed experiment were genotyped at IDH and PGI. Genotypes at PGM were unavailable for these beetles because samples were degraded by an accidental freezer thaw.

Determination of Hsp70 expression

Hsp70 expression was quantified for thorax tissue in 166 adults sampled randomly from each combination of experimental treatment, sex and PGI genotype from the original sample of 480 individuals for which running speed was measured. It was quantified for body wall in 110 larvae

sampled from each combination in a similar, random way. Hsp70 expression was measured by western blot analysis using precast polyacrylamide gels (Tris-HCl 10-4% PAGE Ready Gels; Bio-Rad Laboratories, Hercules, CA, USA) loaded with 40 μg total protein. Samples were electrophoresed and transferred using standard protocols (e.g. Rank and Dahlhoff, 2002). To detect Hsp70, blots were treated with a mouse monoclonal anti-Hsp70 antibody (SPA-822; StressGen Biotechnologies, Victoria, BC, Canada). Prior studies of beetle Hsp70 suggest that this antibody is specific for stress-inducible Hsp70, as bands rarely appear on western blots run for beetles held at 20°C by day, 4°C at night, but expression reaches high levels after exposure to elevated temperatures (McMillan et al., 2005; Rank and Dahlhoff, 2002). Location of bound primary antibody was determined using an anti-mouse immunoglobulin G (IgG) conjugated with peroxidase, which was reacted using ECL-Plus (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) and the presence of a 72-kDa band was detected using a Storm 860 Molecular Imager (GE Healthcare).

Gels were run in blocks of eight (adults, 48 gels; larvae, 28 gels). On each gel, proteins of known genotype from four randomly determined individuals were electrophoresed, in duplicate, along with a positive control (human recombinant Hsp72; SPP-855, StressGen Biotechnologies). On each gel, 2–3 different concentrations of positive control were run, so that an eight-gel array would produce a serial dilution (0.5–4 ng) of pure Hsp70. The resulting blot array was treated and scanned on the phosphoimager as a single dataset. Intensity and size of control bands were background corrected using ImageQuant software (GE Healthcare) and used to generate a non-linear (logarithmic) 'standard expression curve' for each blot array. To quantify relative Hsp70 expression, intensity/size values of each duplicate sample were averaged and Hsp70 values determined by extrapolation using ImageQuant. Resulting Hsp70 expression levels are reported as ng of Hsp70 per g of total muscle protein.

Statistical analysis

All statistical analyses were performed using Jmp In software (Version 5.0 for PC; SAS Institute). For brevity, statistics supporting each experimental result are cited parenthetically in the text or figure captions, with the exception of the Hsp70 analyses (Table 1). Data from the first experiment on running speed in the field was analyzed by two-way analysis of covariance (ANCOVA), using sex and mating status as main effects and beetle T_b as a covariate. In the second field experiment, three-way ANCOVA was performed, with running speed after the laboratory treatment as a dependent variable. Main effects included enzyme genotype, initial field mating status (mated or single), laboratory mating treatment (no female or two females) and interactions. Beetle T_b was used as a covariate.

We have provided tables summarizing statistical analyses of laboratory running speed of all three polymorphic enzyme loci in the supplementary material (supplementary material Tables S1–S5). Rare IDH, PGI or PGM genotypes were omitted from these analyses, because including them would create missing cells in analysis of variance (ANOVA) or ANCOVA models (supplementary material Table S1). Analyses of laboratory running speed were performed using these enzyme genotypes, sex (for adults) and exposure temperature as main effects (supplementary material Tables S2–S5). Preliminary analyses showed that variation in T_a during laboratory runs did not significantly affect running speed. Running speed of adults and larvae after field collection was analyzed using two-way ANOVA (genotype and sex as main effects; supplementary material Table S2). The effect of PGI genotype on the relationship between body mass and running speed was analyzed using heterogeneity of slopes. Adult running speed after one laboratory treatment was analyzed with ANOVA (supplementary material Table S3), larval running speed with ANCOVA (genotype as a main

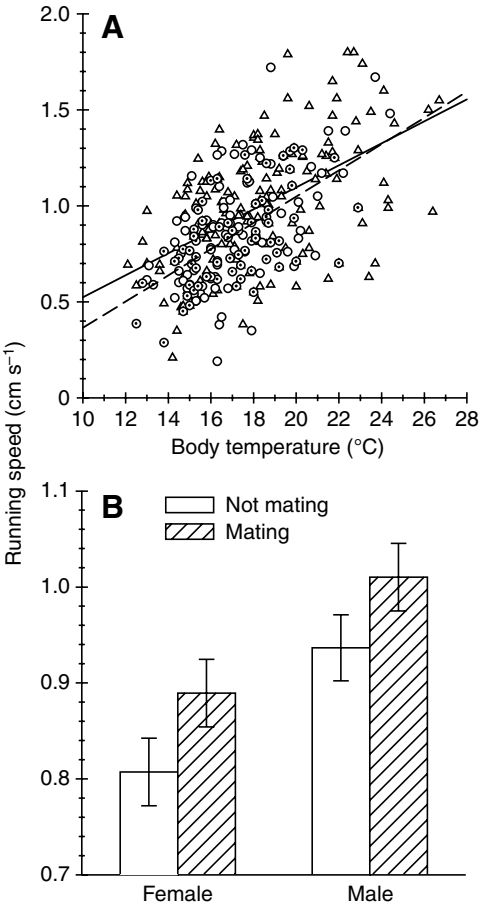

Fig. 2. (A) Effects of body temperature on field running speed for males (N=86, open triangles) and females (N=84, circles; gravid individuals are indicated by dotted circles). Adult running speed was related to beetle T_b in nature for females (broken line: y=–0.29+0.068x; R^2=0.31; $F_{1,82}$=37.1; P<0.0001) and males (solid line: y=–0.22+0.072x; R^2=0.28; $F_{1,84}$=32.3; P<0.0001). (B) Field running speed of males and females mating or not mating in nature. Data are least square means (±s.e.m.) of running speed of N=42 individuals for all treatments but field-mating males (N=44). The sex difference and mating status effect were significant (see Results).

effect, body mass as a covariate, and the interaction term; supplementary material Tables S2, S3).

For analysis of adult running speed after the second temperature exposure, main effects included initial temperature exposure, final temperature exposure, genotype and sex. All three-way interactions were tested, but four-way interaction among between-subjects factors could not be estimated because of missing cells (supplementary material Table S4). For analysis of larval running speed, factors included initial and final temperature exposure, genotype, all three-way interactions and larval body mass as a covariate (supplementary material Table S5). Finally, Hsp70 expression in adults and larvae was analyzed by ANOVA (adults) and ANCOVA (larvae) using the same grouping factors as in the analysis of running speed (Table 1).

Results

Factors affecting running speed in nature

Adult running speed was closely related to beetle T_b in nature (Fig. 2A). Males ran 12% faster than females ($F_{1,164}$=12.7, P=0.0005), although obviously gravid females ran at a similar velocity to those that were not gravid ($F_{2,81}$=1.6, P>0.2). Beetles of both sexes that were mating at the time of field collection ran faster than single beetles (Fig. 2B; $F_{1,164}$=4.9, P=0.028). Beetle T_b was directly correlated with, but significantly greater than, T_a (y=1.01x+1.18, R^2=0.65, $F_{1,168}$=315, P<0.0001; mean T_b–T_a=1.33±0.09°C, paired comparisons t=14.1, N=170, P<0.0001). Beetle T_b did not depend on sex or mating status (P>0.4 for all comparisons).

Male running speed was correlated with T_b before and after the mating treatment (N=174, P<0.0001 for both treatment groups), in which a fresh collection of males were held for 14 h with either two females or without females. Pretreatment running speed was not significantly different between mating and non-mating males in this smaller sample ($F_{1,167}$=1.3, P=0.26), as it was in the larger experiment described above. It also did not depend on enzyme genotype (PGI: $F_{2,167}$=1.9, P=0.15; IDH: $F_{4,155}$=1.9, P=0.11), nor was there a significant interaction between pretreatment running speed and genotype (PGI: $F_{2,167}$=0.3, P>0.7; IDH: $F_{4,155}$=2.1, P=0.08). However, male running speed after 14 h exposure to females varied among PGI genotypes (Fig. 3; 1-1>1-4=4-4). PGI 1-1 and PGI 4-4 males that had been kept with two females ran faster than those that had been kept singly, but there was no difference for PGI 1-4 males (Fig. 3; PGI genotype × treatment interaction: $F_{1,163}$=4.5, P=0.013). There was no significant effect of IDH genotype ($F_{4,145}$=0.97, P=0.43) or interactions with other factors (P>0.2 for all comparisons) on post-treatment running speed. Pretreatment running speed was related to post-treatment running speed (y=0.28x+1.03, R^2=0.09, $F_{1,172}$=16.4, P<0.0001; both adjusted by T_b), indicating that although there is a large amount of variation in running speed among treatment groups, individual running speed measures are repeatable.

Factors affecting running speed of field-collected beetles in the laboratory

Laboratory running speed (measured shortly after collection) of adult males was 12% greater than females ($F_{1,451}$=28.1, P<0.0001), as was observed in the field (Fig. 2), and this difference varied among PGI genotypes (Fig. 4; $F_{2,451}$=4.2, P=0.016). PGI 1-1 and 1-4 males ran much faster than females, but PGI 4-4 females and males ran at similar speeds. Running speed of third instar larvae was positively related to body mass ($F_{1,179}$=16.8, P<0.0001), a relationship

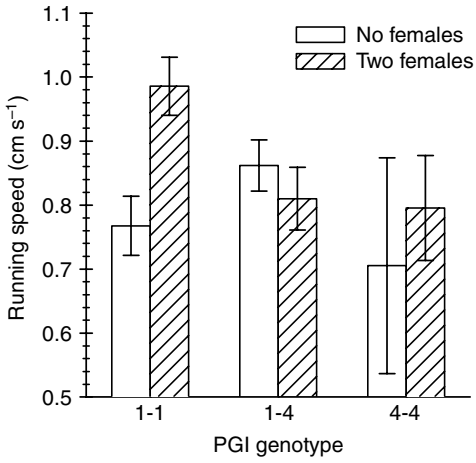

Fig. 3. Field running speed of males after being held in the laboratory in either the presence or absence of two females. Data are least square means (±s.e.m.) of running speed for the three predominant phosphoglucose isomerase (PGI) genotypes, reported as a function of mating treatment (open bars, no female, N=37, 48, 4; striped bars, two females, N=39, 32, 14).

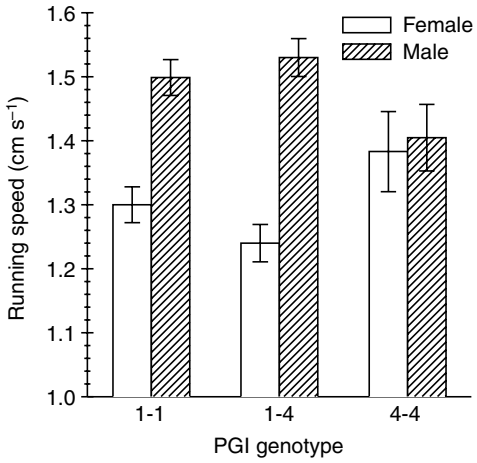

Fig. 4. Laboratory running speed of field-collected male and female adults of three predominant PGI genotypes. Data shown are least square means (±s.e.m.) of running speed for PGI 1-1, 1-4 and 4-4 genotypes (female, N=98, 92, 20; male, N=100, 88, 29). Statistical analyses were performed using ANOVA and the results are described in the text. See Table S2 in supplemental material for analysis of other polymorphic enzyme genotypes.

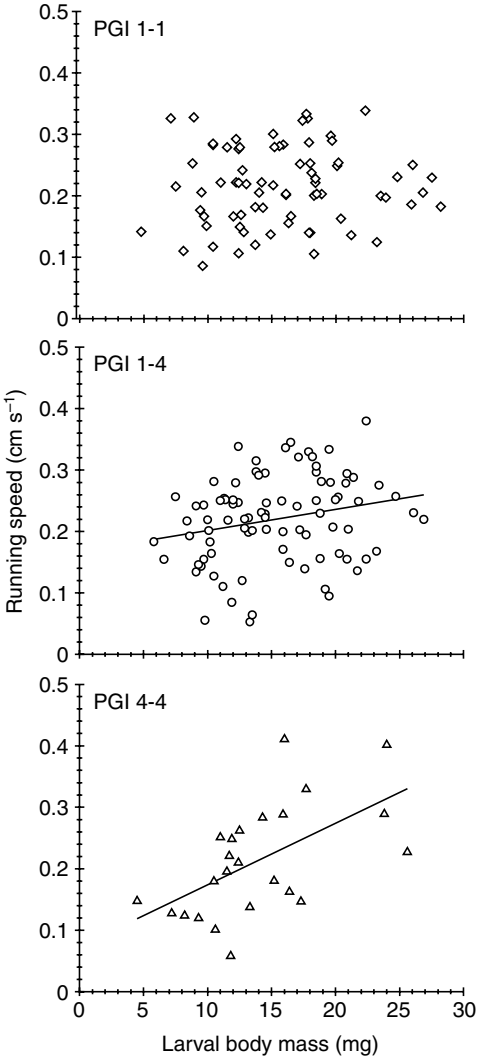

Fig. 5. Variation among PGI genotypes in the effects of body mass on running speed for field-collected larvae. Data shown are running speeds for PGI 1-1, 1-4 and 4-4 genotypes (N=74, 87, 24, respectively). Running speed was significantly related to body mass for PGI 1-4 and 4-4 (1-4: y=3.45x+0.17; R^2=0.05; $F_{1,85}$=4.9; P=0.03; 4-4: y=10.03x+0.07; R^2=0.33; $F_{1,22}$=10.7; P=0.003), but not 1-1 genotypes (not significant).

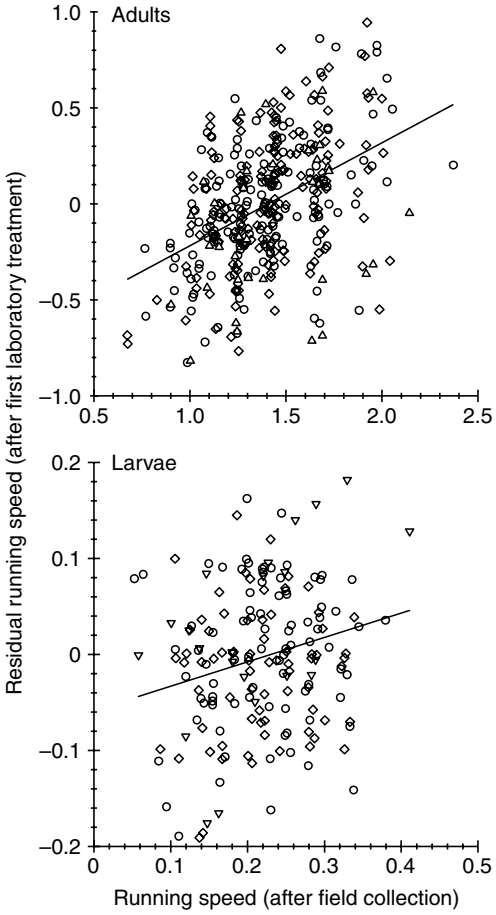

Fig. 6. Repeatability of laboratory running speed measures for adults and larvae. Data shown are running speeds measured immediately after field collection and after first temperature treatment for beetles of three predominant PGI genotypes (1-1: diamonds; 1-4: circles; 4-4: triangles). Residuals of one-way ANOVA (effects of treatment temperature on running speed) were used as dependent variables in regression analysis, to factor out direct effects of treatment temperature on laboratory running speed. Regression analyses, y=–0.75+0.54x, R^2=0.20, $F_{1,368}$=88.0; P<0.0001 (adults); y=–0.05+0.23x, R^2=0.05, $F_{1,179}$=8.3, P<0.005 (larvae).

that was significant for PGI 4-4 larvae, weak for PGI 1-4 larvae and not significant for PGI 1-1 larvae (Fig. 5; heterogeneity of slopes analysis: $F_{2,179}$=4.3, P=0.015). Adult PGM and larval IDH genotypes varied significantly in running speed after field collection (supplementary material Table S2). Adults possessing PGM allele 1 ran slower than other genotypes, but there was no consistent association between any IDH allele and running speed. Not surprisingly, adults ran over six times faster than larvae (adults: 1.39±0.014 cm s^{-1}; larvae: 0.217±0.005 cm s^{-1}). Initial running speeds of adults and larvae were positively correlated to residual running speeds after the first temperature-exposure treatment, although a high degree of unexplained variation in individual running speed was observed (Fig. 6).

Effects of exposure to heat and cold on running speed

Effects of first temperature treatment

After one day in the laboratory at 20°C, running speed increased for adults (8%) and larvae (21%). Exposure to heat (35–36°C) reduced adult running speed by 11%, and larval running speed increased less among individuals exposed to heat (15%) than those exposed to the control (20°C) treatment. Exposure to –4°C reduced running speed by 23% in adults and larvae (adults: $F_{2,379}$=17.6, P<0.0001; larvae: $F_{2,207}$=21.2, P<0.0001). Running speed was also influenced by sex and PGI genotype in adults. Males ran 13% faster than females ($F_{1,354}$=11.3, P=0.0009). PGI 1-1 and 1-4 individuals ran faster than 4-4 individuals after exposure to heat or cold (Fig. 7; $F_{2,379}$=3.0, P=0.051; supplementary material Table S3). The interaction between sex and PGI genotype was not significant

($F_{2,379}$=0.7, P>0.50), and genotype effects were not observed for other polymorphic loci. Among larvae, PGI 4-4 individuals ran slower than other genotypes when held at 20°C, but faster than PGI 1-4 or 1-1 individuals after exposure to cold or heat (Fig. 7; PGI by treatment-temperature interaction; $F_{4,207}$=3.7, P=0.006). Differences in larval running speed among PGI genotypes depended on body mass after the treatment (supplementary material Table S3).

Effects of repeated temperature treatment

Mean running speed of adults and larvae declined after the second exposure, in which all individuals were exposed to either cold or heat (adults: 5%, paired comparisons t=–2.64, N=341, P=0.009; larvae: 7%, paired comparisons t=–2.45, N=212, P=0.01). Beetles exposed to cold ran more slowly than those exposed to heat (adults: 11%, $F_{1,332}$=6.9, P=0.009; larvae: 24%, $F_{1,192}$=23.9, P<0.0001; supplementary material Tables S4, S5). Body mass was positively related to larval running speed ($F_{1,192}$=11.8, P=0.0007), but this relationship also depended on PGI genotype and second temperature exposure (PGI genotype × final exposure; $F_{2,192}$=5.5, P=0.005; supplementary material Table S5). For PGI 1-1 larvae, the positive relationship between mass and running speed was more pronounced when larvae were exposed to cold than heat, whereas for PGI 1-4 and 4-4 larvae, the positive relationship was most pronounced when larvae were exposed to heat.

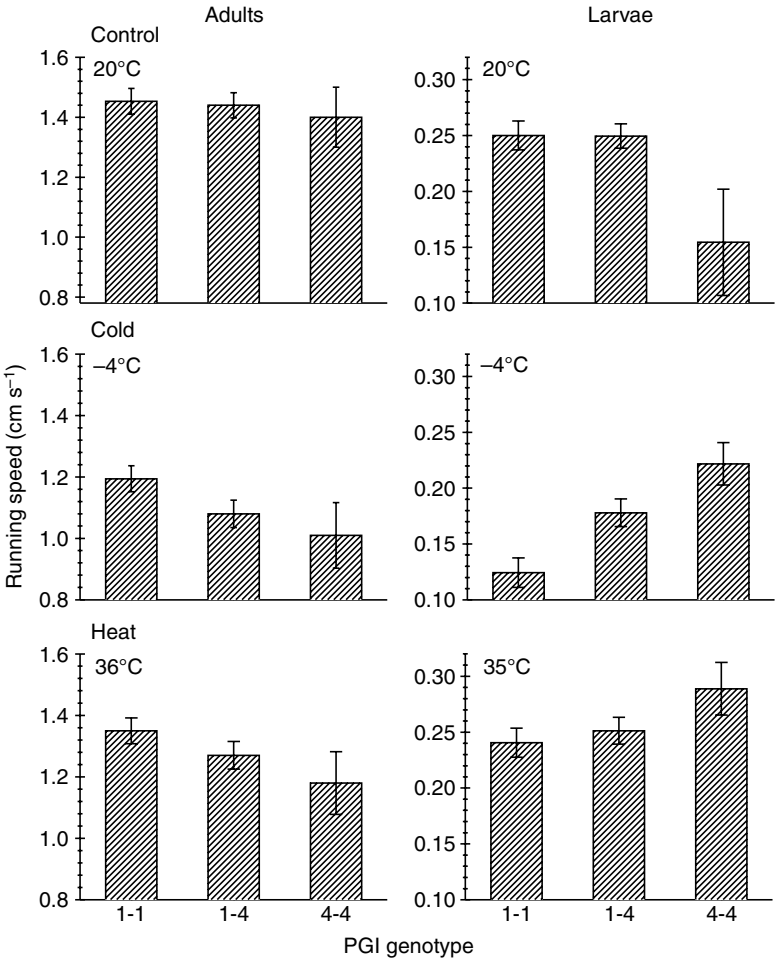

Fig. 7. Differences among PGI genotypes in effect of first temperature treatment on running speed in adults and larvae. Data are least square means (±s.e.m.) of running speed for PGI 1-1, 1-4 and 4-4 genotypes (adults: –4°C: N=72, 62, 18; 20°C: N=68, 71, 15; 36°C: N=72, 62, 19. Larvae: 20°C: N=31, 41, 6; –4°C: N=28, 32, 14; 35°C: N=29, 34, 10). Statistical analyses of effects of single treatment temperature were performed using ANOVA (adults) and ANCOVA (larvae) and results are described in the text. Additional analyses of effects of other enzyme genotypes are described in Tables S2, S3 in supplementary material.

Analysis of the change in running speed between the first and second temperature-exposure treatments revealed that individuals that had been initially exposed to cold ran faster after the second exposure to heat or cold (Fig. 8; adults: $F_{2,309}$=13.9, P<0.0001; larvae: $F_{2,194}$=29.1, P<0.0001; supplementary material Tables S4, S5). PGI 4-4 adults ran faster after the second exposure to heat or cold than they had after the first temperature exposure ($F_{2,309}$=3.1, P=0.045), whereas other genotypes ran more slowly (Fig. 8). For larvae, the relationship between PGI genotype and the change in running speed between the first and second exposure treatment was somewhat different (Fig. 8). After the second exposure, most larvae ran slower. PGI 1-1 larvae ran more quickly than other genotypes after heat exposure, but PGI 1-1 individuals also ran more slowly than other genotypes after exposure to cold (PGI genotype × final exposure; $F_{2,194}$=4.7, P=0.01; supplementary material Table S5). No effects were observed for other polymorphic loci (supplementary material Tables S4, S5).

Hsp70 expression levels

Most adults (86%) and all larvae showed evidence of Hsp70 upregulation relative to 'control' (20°C) conditions (<5 ng Hsp70 g^{-1} total muscle protein), regardless of whether they experienced heat or cold stress. Adults that had received the control temperature (20°C) during the initial exposure expressed lower levels of Hsp70 after the second (stress) exposure than those that had been exposed to cold or heat twice (top row *versus* two bottom rows, Fig. 9). Adults and larvae that were exposed to heat for the second exposure expressed more Hsp70 than those exposed to cold (Figs 9, 10, Table 1). All larvae induced Hsp70 above 'background' levels typically observed under mild thermal conditions in nature and the laboratory, even after two exposures to cold (Fig. 10, Table 1).

Effects of exposure temperature on Hsp70 expression varied among PGI genotypes. Adults of all PGI genotypes that received a single cold treatment (Fig. 9; top-left panel) expressed low levels of Hsp70. However, PGI genotype was related to Hsp70 expression (1-1>1-4>4-4) for beetles exposed once to heat (Fig. 9, top-right panel). Adults that received two cold exposures showed a similar ranking among PGI genotypes

as those given a single heat exposure, whereas those that were exposed to cold and then heat showed the opposite ranking (4-4>1-4>1-1; Fig. 9, middle panels). Adults exposed to heat and then cold showed a similar ranking among PGI genotypes as those that were exposed to heat twice (Fig. 9, bottom panels; Table 1). Adults exposed to two stress exposures (Fig. 9, bottom-four panels) had 2–6-fold greater Hsp70 expression levels, depending on PGI genotype, than adults exposed to one stress (Fig. 9, top-two panels). Larval Hsp70 expression also varied among PGI genotypes (1-1>1-4>4-4 overall; Fig. 10, Table 1; trend reversed for two exposures to heat). No effects were observed for other polymorphic loci.

Discussion

We detected substantial effects of environmental temperature, mating and thermal history, sex and genetic variation at the glycolytic enzyme locus PGI on voluntary running speed and the heat-shock response in *C. aeneicollis* adults and larvae. Males ran faster than females, and running speed for both sexes depended strongly on ambient temperature in the field. Environmental effects on running speed, including those of recent mating and thermal history, depended on PGI genotype. Exposure to cold caused greater reductions in running speed than exposure to heat, and larvae were more sensitive to thermal extremes than adults, as reported in earlier studies (Neargarder et al., 2003). Responses to thermal stress varied significantly among genotypes at PGI, but rarely at other enzyme loci. Adults possessing the PGI 1 allele ran faster than PGI 4-4 homozygotes in nature and after one exposure to extreme temperature, but repeated exposure to extreme temperature in adults, or a single extreme exposure for larvae, resulted in greater running speed for 4-4 homozygotes. Hsp70 expression depended on recent thermal history and on PGI

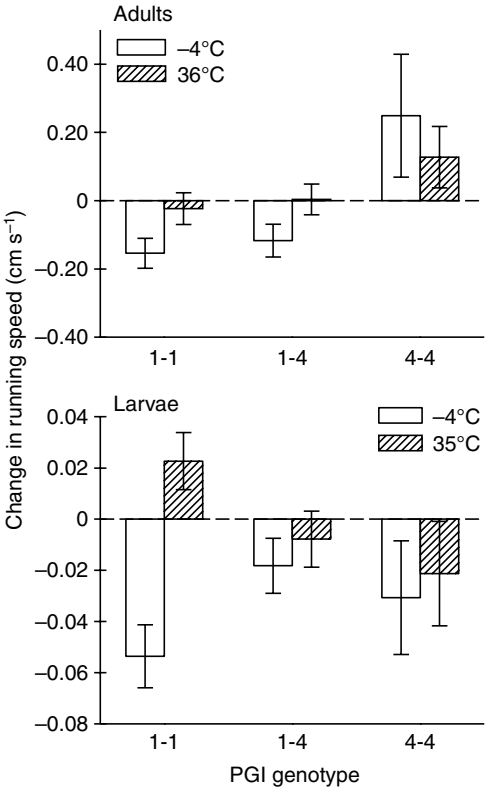

Fig. 8. Differences in adult and larval running speed measured after first and second temperature treatments. Data are least square means (±s.e.m.) of running speed for the three main PGI genotypes, reported as a function of second temperature treatment [–4°C (open bars), 36 or 35°C (shaded bars)]. Sample sizes are as follows. Adults: –4°C second treatment: *N*=85, 72, 9; 36°C second treatment: *N*=73, 79, 23. Larvae: –4°C second treatment: *N*=39, 52, 14; 35°C second treatment: *N*=48, 48, 15. Statistical analyses of difference in running speed between two temperature treatments were performed using ANOVA (adults) and ANCOVA (larvae) and results are described in the text and in the supplemental tables (supplementary material Tables S4, S5).

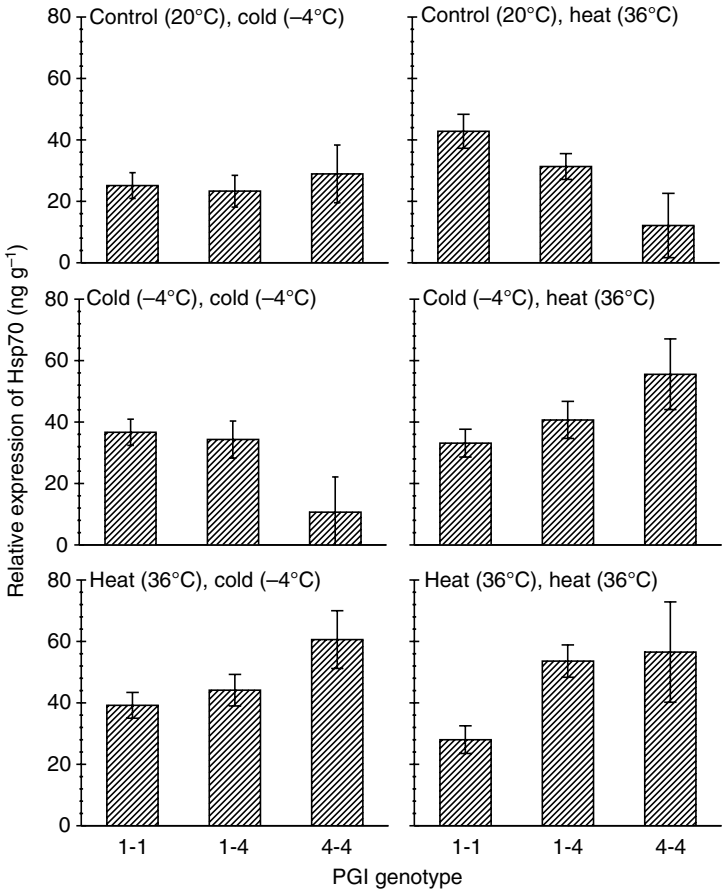

Fig. 9. Differences between PGI genotypes in effects of two 4 h temperature treatments on Hsp70 expression in adult beetles. Treatment temperatures, in order, are shown in each panel. Data are least square means (±s.e.m.) of Hsp70 expression (ng of Hsp70 per g of thoracic muscle), measured by western blot analysis of PGI 1-1, 1-4 and 4-4 genotypes [left panels (–4°C second treatment): 20°C: *N*=15, 10, 2; –4°C: *N*=17, 9, 2; 36°C: *N*=15, 10, 2. Right panels (36°C second treatment): 20°C: *N*=10, 18, 3; –4°C: *N*=15, 9, 2; 36°C: *N*=15, 11, 1]. Statistical analysis was performed on Hsp70 data using ANOVA (Table 1).

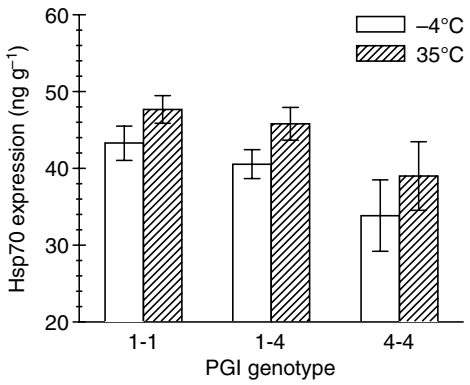

Fig. 10. Differences in larval Hsp70 expression after two 4 h laboratory temperature treatments. Data are least square means (±s.e.m.) of Hsp70 expression for the three main PGI genotypes, reported as a function of second temperature treatment (–4°C second treatment: *N*=21, 26, 6; 35°C second treatment: *N*=28, 24, 5). Statistical analysis was performed on Hsp70 data using ANOVA (Table 1).

genotype, as found in previous studies (Dahlhoff and Rank, 2000; McMillan et al., 2005; Rank and Dahlhoff, 2002). Nevertheless, the ranking of each PGI genotype with respect to Hsp70 expression depended on whether the animal had been exposed to a single stress or to repeated bouts of extreme temperature. Thus, phenotypic expression of genetic differences at PGI depended on recent environmental conditions experienced by the beetle, suggesting phenotypic plasticity in performance characters and the stress response. This is consistent with predictions that high levels of plasticity may evolve in response to thermally heterogeneous environments (Garland, Jr and Kelly, 2006), such as the challenging montane conditions where *C. aeneicollis* occurs.

Males run faster than females (Figs 2, 4). Running speed may be especially important for adult male willow beetles, because a male's mating success is related to his ability to locate and mate with as many females as possible (Rank et al., 2006). Difference in running speed among males varies among PGI genotypes and depends on prior mating activity (Figs 3, 4). Alleles at any locus that are associated with enhanced locomotor performance may increase in frequency as a consequence of increased mating success of individuals that possess them.

The relationship between larval body size and running speed also varied among PGI genotypes (Fig. 5; supplementary material Tables S2, S3 and S5). In field-collected larvae, PGI 1-1 individuals showed no evidence of a body size/running speed relationship, whereas PGI 4-4 individuals showed a strong positive one. After exposure to stressful temperatures in the laboratory, the effect of PGI genotype on the mass/running speed relationship depended on the type of stress. For PGI 1-1 larvae, the positive relationship between mass and running speed was strongest when larvae were exposed to cold, whereas for PGI 1-4 and 4-4 larvae, the positive relationship was most pronounced when larvae were exposed to heat. These data suggest that selection could act on PGI for small larvae, if some genotypes fail to evade crawling predators such as syrphid fly larvae (Rank and Smiley, 1994; Smiley and Rank, 1986). Previous studies of *C. aeneicollis* have demonstrated that variation in larval thermal tolerance among genotypes depended on the type (warm *versus* cold) and degree of thermal stress (McMillan et al., 2005; Neargarder et al., 2003).

Experimental exposure to cold affected running speed more severely than exposure to heat. This cold sensitivity was evident in running speeds of adults and larvae after the first exposure, in the reduction of running speed after the second exposure, and in upregulation of Hsp70 in response to cold. Cold exposure may cause physical damage to cells and tissues because of ice crystal formation, and temporarily lower metabolic rate. Most Northern hemisphere insects are exposed to sub-zero temperatures in winter, and avoid tissue freezing by rapid cold-hardening and avoidance of ice formation in tissues (Bradshaw et al., 2004; David et al., 2003; Duman and Horwath, 1983; Kelty and Lee, 1999; Van der Laak, 1982). Conditions in the Eastern Sierra Nevada may be more similar

Table 1. *Three-way analysis of variance showing effect of exposure temperature and phosphoglucose isomerase (PGI) genotype on Hsp70 expression levels in adults and larvae**

	Adults				Larvae			
Source	d.f.	SS	*F*	*P*	d.f.	SS	*F*	*P*
Initial exposure (I)	2	5887.0	10.0	0.001	2	47.1	0.3	n.s.
Final exposure (F)	1	332.0	1.1	n.s.	1	414.5	4.8	0.031
PGI genotype (G)	2	675.6	1.1	n.s.	2	530.3	3.1	0.052
I × F	2	984.1	1.7	n.s.	2	81.1	0.5	n.s.
I × G	4	4850.9	4.1	0.003	4	383.9	1.1	n.s.
F × G	2	472.4	0.8	n.s.	2	6.7	0.0	n.s.
I × F × G	4	3844.4	3.3	0.014	4	210.1	0.6	n.s.
Error	148	43601.8			92	7975.9		

*Initial exposure (I): –4, 20 or 36°C (adults), 35°C (larvae). Final exposure temperature (F): –4 or 36°C (adults), 35°C (larvae). All factors treated as fixed effects.

n.s., not significant.

to temperate habitats in the Southern hemisphere, where insects are typically exposed to both freezing cold and warm temperatures in the same season (Bonan, 2002; Sinclair and Chown, 2005). Insects from these habitats have evolved the ability to tolerate routine ice formation in tissues (Sinclair et al., 2003; Sinclair and Chown, 2005; Sinclair et al., 2004). The fact that *C. aeneicollis* can survive exposure to sub-zero temperatures suggests that they have some tolerance to freezing in summer (Neargarder et al., 2003; Rank and Dahlhoff, 2002), and mechanisms of cold tolerance may include upregulation of Hsps (Burton et al., 1988; Hoffmann et al., 2003; Michaud and Denlinger, 2005; Yiangou et al., 1997; Yocum, 2001). Although we found no evidence of ice formation in cold-treated individuals in the laboratory, we have detected cold-induced mortality and evidence of ice formation in tissue for beetles in nature (McMillan et al., 2005; Rank, 1994; Smiley and Rank, 1986). Thus, cold is probably a significant selective force in these populations. By contrast, we have not observed mortality as the result of exposure to daytime high temperatures. It is likely that heat has more subtle effects on survival and reproductive success than cold over the range of temperatures experienced by *C. aeneicollis*.

The relationship between extreme temperature treatment and differences among PGI genotypes in adult and larval running speed suggests considerable phenotypic plasticity in response to temperature stress. For adults, PGI allele 1 was associated with faster running speed after a single exposure to stressful temperature, but PGI allele 4 was associated with increases in running speed after a second exposure. By contrast, for larvae, PGI allele 4 was associated with faster running after a single exposure to stressful temperature, and PGI 1-1 individuals experienced greater declines in running speed than other genotypes after a second cold exposure. Overall, the results in larvae are complementary to those with adults, and show that genotypes that suffer declines after a single stress may recover after repeated exposure.

There was no direct relationship between the running speed of an individual beetle and Hsp70 expression, but differences among exposure treatments showed correspondence between the two traits. Adults exposed to two stresses typically ran more slowly and expressed more Hsp70 than those exposed to a single treatment of heat or cold, suggesting that upregulation of Hsps may enhance survival at a cost of reduced activity, as has been observed in other insects (Feder et al., 1992; Krebs and Feder, 1998; Krebs and Holbrook, 2001; Sorensen et al., 2001). The up to fivefold differences in Hsp70 expression level among PGI genotypes reported here (1-1>1-4>4-4 for a single exposure to heat) are consistent with findings of our earlier studies. In addition, differences in Hsp expression level are similar to those observed for other natural populations of insects (Sarup et al., 2006; Sorensen et al., 2005). However, as was found for running speed, Hsp70 expression was greater for adults possessing allele 4 that had been exposed to two stressful temperature treatments (except those exposed to cold twice). In larvae exposed to heat, allele 1 was associated with faster running speeds and greater levels of Hsp70 expression after one exposure, but allele 4 was after the second exposure. These Hsp data suggest that individuals possessing allele 4 are more able to tolerate repeated or more extreme exposure to cold or heat, whereas individuals possessing allele 1 perform best under moderate conditions. Thus, repeated exposure to extreme temperatures influences Hsp70 expression in a fundamentally different way than do single exposures to heat or cold. This may be especially important to an insect that is exposed in nature to elevated and sub-zero temperatures within 12 h of each other (McMillan et al., 2005; Neargarder et al., 2003). The finding that individuals that possess the 4 allele upregulate Hsps after repeated exposure to extreme conditions is also consistent with geographic variation in PGI allele frequency. Allele 4 occurs more commonly in warmer, southern drainages of the Eastern Sierra, whereas allele 1 predominates in cooler drainages. If PGI 4-4 individuals initiate their heat-shock response only after repeated hot/cold thermal stress, then this might resolve the apparent paradox, discussed in previous papers (Neargarder et al., 2003; Rank and Dahlhoff, 2002), that allele 4 appeared to be associated with a ‘less vigorous’ heat-shock response.

Taken together with earlier studies of Sierra populations of *C. aeneicollis*, data presented here suggest that PGI may be under temperature selection. PGI is important in the metabolism of glucose, as it is located near the branch point of several pathways utilizing glucose-6-phosphate, including glycolysis and gluconeogenesis (Dykhuizen and Hartl, 1983). Evidence for temperature selection at PGI has been demonstrated in a variety of species (Eanes et al., 1993; Katz and Harrison, 1997; Watt, 1977; Zera, 1987), and previous studies of PGI kinetics in *C. aeneicollis* have shown that there are small differences among PGI allozymes in the Michaelis–Menten binding constant (K_m) and enzyme thermal stability (4-4>1-4>1-1) (Dahlhoff and Rank, 2000). Recent studies of partially purified enzyme are consistent with these early data, and suggest that catalytic efficiency, indexed by V_{max}/K_m, is higher for 1-1 than 4-4 allozymes at moderate temperature (E.P.D., unpublished data). These data suggest that PGI allele 4 (a slow-migrating allele) is more thermostable, and thus less efficient at moderate temperatures, than allele 1 (a fast-migrating allele).

These results are consistent with other functional studies of PGI allozymes in ectotherms (Hoffmann, 1981a; Hoffmann, 1981b; Watt, 1977; Watt, 1983). In Atlantic populations of the sea anenome *Metridium senile*, there are two common PGI alleles that vary in frequency across a biogeographic thermal gradient. Homozygotes of a fast allele have greater sensitivity of K_m to temperature and higher V_{max}/K_m ratios than homozygotes of a slow allele (Hoffmann, 1981a; Hoffmann, 1981b). Heterozygotes are intermediate, as is the case for *C. aeneicollis* PGI allozymes (Dahlhoff and Rank, 2000). In *Colias eurytheme*, enzymes from fast-migrating homozygotes (notably 2/2) have greater sensitivity of K_m to temperature, and higher V_{max}/K_m ratios than allozymes from ‘slow’ homozygotes (4/4, 5/5). In *C. eurytheme*, some heterozygotes (especially 3/4) have most favorable kinetics and flight performance under moderate conditions, but unusually hot weather favors

thermostable homozygotes possessing slow alleles (Watt, 1977; Watt, 1983; Watt et al., 1983). Homologous charge substitutions across multiple taxa could be responsible for the observation that PGI 'fast' alleles tend to make thermolabile allozymes, 'slow' alleles thermostable ones (Riddoch, 1993; Wheat et al., 2006).

Functional differences among PGI allozymes suggest a mechanism for temperature selection in *C. aeneicollis.* Beetles possessing the 'thermolabile but efficient' allele 1 run faster and have higher metabolic rates than other genotypes under moderate thermal conditions. Furthermore, adults possessing the 1 allele upregulate Hsps rapidly after a single stress, leaving cellular proteins protected from deleterious effects of typical thermal variation. Natural selection may favor these individuals most of the time. Individuals possessing the 'thermostable but inefficient' 4 allele run slower, mate less frequently and have a less robust heat-shock response under moderate conditions. However, after extreme weather events that occasionally occur, these individuals may be favored. Patterns of fecundity and larval survival in nature are consistent with this model (Bruce, 2005; Fearnley, 2003; McMillan et al., 2005).

Another factor that may contribute to differences in running speed among PGI genotypes is the total concentration of the PGI enzyme, which may differ among individuals or genotypes as a consequence of thermal compensation, as it does for metabolic enzymes in other ectotherms (Lesser and Kruse, 2004; Segal and Crawford, 1994). We would expect that if individuals possessing allele 1 were cold acclimatized, they would have higher PGI-specific activities (more active enzyme molecule per gram tissue) than those possessing allele 4, independent of differences in enzyme kinetic properties. Differences in enzyme concentration may result in greater conservation of high running speeds and higher Hsp70 expression levels in individuals possessing the PGI 1 allele after exposure to a single stressful temperature, as more enzyme molecules would be present for Hsp70 to refold, and more functional PGI molecules would be present in muscle cells after that stress. Future studies in this system will test this prediction.

Another possible mechanism for the relationship between PGI genotype and physiological traits is that the PGI locus is linked to a gene that is responsible for the physiological differences observed here (Horacek and Acanova, 2003; Johannesson et al., 1990; Watt, 1994). PGI could be a neutral marker whose frequencies change over time through hitchhiking with other, selected locus or loci (Betancourt and Presgraves, 2002). However, there are several reasons for believing that PGI itself represents the target of selection to temperature. When allozyme polymorphisms were the preferred markers for studies of genetic variation, enzymes were screened in a wide variety of taxa (Avise, 1994). PGI was one of the most commonly observed allozymes that showed evidence of selection (Gillespie, 1991; Mitton, 1997; Riddoch, 1993). This is consistent with predictions about the relationship between locations of enzymes in metabolic pathways and likelihood of a locus being under selection (Carter and Watt, 1988; Dykhuizen and Hartl, 1983; Eanes, 1999; Hochachka and Somero, 2002; Sezgin et al., 2004; Somero, 2004; Watt et al., 1983; Zamer and Hoffmann, 1989). DNA sequence studies of patterns of substitution and conservation also suggest that selection acts more strongly on PGI than on many other genes (Broughton and Harrison, 2003; Filatov and Charlesworth, 1999; Katz and Harrison, 1997; Kawabe et al., 2000; Terauchi et al., 1997; Wheat et al., 2006). Finally, although linkage disequilibrium is clearly a pervasive phenomenon with implications for the evolution of many traits (Freeman and Herron, 2004), recombination rates between most pairs of loci are great enough to prevent significant linkage disequilibrium, even when genes are located on the same chromosome (Dawson et al., 2002). Recombination rates are also generally higher for genes that include a large number of intron sequences (Comeron and Kreitman, 2000), and PGI has a large number of introns (Claes et al., 1994; Terauchi et al., 1997; Thomas et al., 1992). Thus, with increased recombination, the likelihood of linkage disequilibrium between PGI and other genes is greatly reduced (Falconer, 1989).

Conclusions

These results provide crucial evidence that PGI is under temperature selection in *C. aeneicollis* by reporting differences among PGI genotypes in a performance character, running speed, crucial for survival and fitness. Some genotypes run fastest and have highest Hsp70 expression levels after a single temperature stress, others after repeated temperature stresses. Differences in Hsp70 expression levels among PGI genotypes may buffer fitness consequences of differences in locomotor performance among genotypes and facilitate the persistence of the PGI polymorphism in these populations. As some models of climate change predict an increase in the frequency of extreme weather events (Beniston, 2004; Nogaj et al., 2006), organisms with genetic variation that allows them to tolerate extreme climatic variability may be uniquely suited to adapt to rapid climate change and avoid local or total extinction.

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