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# Variation in thermal tolerance is linked to phosphoglucose isomerase genotype in a montane leaf beetle

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**Summary**

**1.** Sierra Nevada populations of the Willow Beetle *Chrysomela aeneicollis* (Schaeffer) experience extreme elevated and subzero temperatures in nature. In these populations, frequencies of phosphoglucose isomerase (PGI) alleles vary with latitude and altitude and respond to climate change. PGI genotypes differ in expression of a stress-inducible heat shock protein (Hsp70).

**2.** Here, differences in tolerance of elevated and subzero extreme temperatures were compared for field-acclimatized and laboratory-acclimated larvae and adults possessing three common PGI genotypes (PGI 1–1, 1–4 and 4–4). Three indices of thermal tolerance were measured – $CT_{max}$, $LT_{50}$ and Hsp70 expression level.

**3.** Thermal tolerance depended on life stage, prior exposure to sublethal stress and PGI genotype. Larvae were generally less tolerant of thermal extremes than adults. For both adults and larvae, prior exposure to sublethal temperatures increased survival after exposure to subsequent stress. Survival after exposure to thermal extremes was consistently related to PGI genotype (1–1 > 1–4 > 4–4), as were expression levels of Hsp70 (1–1 > 1–4 > 4–4).

**4.** These results suggest that PGI genotypes differ in tolerance of thermal extremes routinely experienced by beetles in nature. A trade-off between thermal tolerance and energy allocation may explain the persistence of the PGI polymorphism.

*Key-words*: Cold tolerance, heat shock protein, heat tolerance, stress response, temperature adaptation

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## Introduction

The existence of enzyme polymorphisms in populations along gradients in environmental temperature provides an excellent opportunity to examine mechanisms of local adaptation (Watt, Cassin & Swan 1983; Watt 1992; Johannesson, Johannesson & Lundgren 1995; Schmidt & Rand 1999; Schmidt & Rand 2001). Relationships between temperature, a protein polymorphism and some aspect of physiological performance or fitness have been detected for a number of organisms (Koehn, Newell & Immermann 1980; Watt *et al*. 1983; DiMichele, Paynter & Powers 1991; Watt 1992; Dahlhoff & Rank 2000). Nevertheless, few studies have evaluated the consequences of enzyme polymorphisms at the biochemical, physiological and ecological levels within natural populations of a single species (Feder & Watt 1993; Mitton 1997). Thermal tolerance describes an organism's ability to maintain functionality at extreme temperatures (Johnston & Bennett 1996), and it represents a physiological character that may link allozyme variation with differences in performance and fitness. Variation among individuals in thermal tolerance may depend on genetic differences in key physiological and biochemical characters (Huey, Partridge & Fowler 1991; Krebs & Feder 1997a; Krebs & Bettencourt 1999). Thermal tolerance is also highly acclimatizable within genetic limits (Dahlhoff *et al*. 1991; Dahlhoff & Somero 1993; Somero, Dahlhoff & Lin 1996). Unfortunately, no studies have yet demonstrated a link between allozyme variation and thermal tolerance.

Here the effects of recent thermal history and genetic variation on indices of thermal tolerance were measured for Eastern Sierra Nevada populations of the Willow Beetle *Chrysomela aeneicollis* (Schaeffer). These beetles are routinely exposed to elevated (30–35 °C) and subzero (minus 2–6 °C) air temperatures (Rank 1994; Rank & Dahlhoff 2002), which cause physiological stress (Dahlhoff & Rank 2000), while beetles are mating, laying eggs and developing to maturity. Previous studies indicate that natural selection for temperature may act on the allozyme locus



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phosphoglucose isomerase (PGI) in this species (Rank & Dahlhoff 2002). PGI frequencies vary along a latitudinal gradient in the Eastern Sierra, with the PGI-1 allele predominating in a northern drainage and the other common allele (PGI-4) predominating in a southern drainage (Rank 1992). PGI genotypes sometimes differ in expression of a stress-inducible 70-kDa heat shock protein (Hsp70) in the field and PGI allozymes have distinct, temperature-dependent kinetic properties (Dahlhoff & Rank 2000). After laboratory exposure to elevated temperatures, PGI genotypes differ with respect to the temperature at which onset of Hsp70 expression occurs (25–30 °C), and the temperature at which peak Hsp70 expression levels (31–36 °C) are reached (Rank & Dahlhoff 2002). PGI 1–1 genotypes are more sensitive to heat than PGI 4–4 genotypes and PGI 1–4 heterozygotes show intermediate levels of heat sensitivity (Dahlhoff & Rank 2000; Rank & Dahlhoff 2002). Increases in the frequency of PGI-1 (and concomitant decreases in the frequency of PGI-4) occurred after a period of relatively cool temperatures in the Sierra Nevada (Rank & Dahlhoff 2002).

In the present study, three different measures of thermal tolerance were used: $LT_{50}$, the temperature at which 50% of animals die from exposure to a thermal extreme; critical thermal maximum ($CT_{max}$), the temperature at which voluntary muscle control is lost; and expression of Hsp70. $LT_{50}$ was used to measure tolerance of a chronic exposure to extreme temperature, and $CT_{max}$ was used to measure tolerance of acute exposure. Hsp70 expression level was measured for individuals after acute exposure. Hsps mediate an organism's response to extreme temperatures. Elevated temperatures induce Hsp expression in most organisms, and Hsps are critical for development of thermal tolerance (Dahlgaard *et al*. 1998; Krebs & Bettencourt 1999; Krebs & Feder 1998; Feder & Hofmann 1999; Sorensen, Dahlgaard & Loeschke 2001). However, Hsp production is energetically costly and may affect survival or other components of fitness (Hoffmann & Parsons 1991; Krebs & Feder 1997b; Feder 1999).

Data from our previous studies suggest that the PGI-1 allele should be evolutionarily favoured in cooler conditions. PGI 1–1 genotypes up-regulate Hsp70 expression to a greater degree after exposure to moderately elevated temperatures and they have a lower threshold temperature for Hsp70 induction than other PGI genotypes (Rank & Dahlhoff 2002). This increased up-regulation of Hsp70 at moderate conditions may enhance tolerance of PGI 1–1 genotypes to subsequent extremes in heat and cold. Conversely, PGI 1–1 genotypes may have a lower thermal tolerance to extreme temperatures, because Hsp70 levels drop off more rapidly for them at high temperatures than they do for other PGI genotypes. In this study, we tested these alternative predictions by measuring tolerance of elevated and subzero temperatures for adults and larvae acclimatized to field conditions and for beetles acclimatized to elevated and subzero temperatures in the laboratory.

For experiments described here, natural populations rather than laboratory lines were used; thus, PGI genotype was not known until after completion of physiological measures. First, $LT_{50}$ for heat and cold of different life stages of field-collected beetles were determined. These values were subsequently used in laboratory treatments, where survival was measured for beetles exposed to $LT_{50}$ heat and cold after several days of exposure to moderate or extreme sublethal temperatures. Information derived from previous studies of the relationship between PGI genotype, temperature and Hsp70 expression (Rank & Dahlhoff 2002) was used to select acclimation temperatures for $CT_{max}$ experiments. $CT_{max}$ was measured for field-acclimatized and laboratory-acclimated adults. Exposure to $CT_{max}$ clearly causes physiological stress to an animal. Unfortunately, the link between $CT_{max}$, which is related to loss of neuromuscular control, and Hsp70 expression, which is related to cellular protein damage, has not been elucidated. We therefore quantified Hsp70 expression level for individuals that had been exposed to $CT_{max}$, to determine whether neuromuscular stress is accompanied by damage to cellular proteins.

## Materials and methods

### STUDY POPULATIONS AND SAMPLE COLLECTION

The Willow Beetle *Chrysomela aeneicollis* is found at high-altitude localities in the Eastern Sierra Nevada. Habitat characteristics and natural history of these beetle populations are described elsewhere (Rank 1992; Rank 1994; Dahlhoff & Rank 2000). For this study, beetles were collected from the area around Bluff Lake, which is located at an elevation of 3200 m in the Green Lake subdrainage of Bishop Creek (37°11′ N, 118°32′ W). This population was selected because the two common PGI alleles (1 and 4) occur in frequencies closest to parity in this locality. Based on previous studies (Rank 1992; Dahlhoff & Rank 2000), the frequency of the least common genotype in Bishop Creek, PGI 4–4, was predicted to be 0·10. Therefore, for experiments involving PGI genotype, sample sizes greater than 60 were used in order to have a minimum of six 4–4 individuals in each treatment combination.

For all experiments, beetles were collected from Bluff Lake in small plastic cups, which were placed in a cooler and returned to the Owens Valley Laboratory of the White Mountain Research Station (WMRS) in Bishop, California. Upon arrival at WMRS, beetles were immediately placed in controlled-temperature incubators (20 °C) and fed on fresh leaves from their favoured host plant, *Salix orestera* Schneider, until measurement of thermal tolerance. Thermal tolerance of field-acclimatized beetles was measured within 12 h of return to WMRS. Thermal tolerance of laboratory-treated individuals was measured after various thermal treatments, which are described in following

sections. Thermal tolerance of three life-history stages was examined: over-wintered adults, larvae and newly emerged adults. It was not possible, owing to time or logistical constraints, to measure thermal tolerance for all life stages in all experiments. Therefore, for each experiment the life stage most likely to be sensitive to that particular thermal stress was selected for analysis.

### LT50 OF FIELD-ACCLIMATIZED BEETLES

Larvae (July 2000), newly emerged adults (September 2000), and over-wintered adults (June 2001) were exposed to a range of temperatures near their upper (33–41 °C) and lower (–3 to –7 °C) thermal limits previously determined in preliminary experiments. To determine $LT_{50}$ for each life stage, groups of 20–30 individuals were held at one of 4–5 different temperatures for 4 h. A digital dry bath (Boekel Scientific, Feasterville, PA) was used for heat exposures and a digital hot-cold block incubator (Tropicooler, Boekel Scientific) was used for cold exposures. During temperature treatment, beetles were confined to the bottom of 1·5-ml microcentrifuge tubes with conical screen cages. After heat or cold exposure, beetles were examined under a dissecting scope to determine whether they survived. $LT_{50}$ temperatures were estimated with a logistic regression curve of these data, with temperature as the independent variable and survival (1 = alive, 0 = dead) as the dependent variable. $LT_{50}$ (heat) was determined in this manner for larvae, newly emerged adults, and over-wintered adults, and $LT_{50}$ (cold) was determined for larvae and newly emerged adults. These $LT_{50}$ values were used to select treatment temperatures for subsequent thermal tolerance experiments.

### RECOVERY FROM EXPOSURE TO LT50 AFTER ACCLIMATION TO SUBLETHAL TEMPERATURES

To assay short-term changes in tolerance of thermal extremes, larvae (July 2000), newly emerged adults (September 2000) and over-wintered adults (June 2001) were collected from Bluff Lake and acclimated to moderate or extreme temperatures in the laboratory for two consecutive day–night cycles before being exposed to $LT_{50}$ for 4 h. Prior exposure thermal protocols were as follows. For cold tolerance, larvae and newly emerged adults were held at 20 °C during the day and either a moderate (4 °C) or extreme (–4 °C for newly emerged adults, –3 °C for larvae) low temperature for 4 h at night (1.00–5.00 a.m.). On the third night, both treatment groups (moderate and extreme) were held at $LT_{50}$ (cold) (–6·5 °C for newly emerged adults, –5·6 °C for larvae) from 1.00 to 5.00 a.m. For heat tolerance, over-wintered adults and larvae were held at 4 °C at night, 20 °C from 8.00 a.m. to 1.00 p.m., and either a moderate (20 °C) or elevated (30 °C) temperature from 1.00 to 5.00 p.m. On the third day, both treatment groups (moderate and elevated) were held at $LT_{50}$ (heat) (38·8 °C for over-wintered adults, 36·3 °C for larvae) from 1.00 to 5.00 p.m. After exposure to $LT_{50}$ (cold) or $LT_{50}$ (heat), beetles were placed at 20 °C and recovery was scored as follows: beetle walking or crawling normally (status = 4); grasping forceps but not walking (status = 3); movement of more than one appendage evident but beetle could not grasp forceps (status = 2); movement of one appendage (status = 1); immobile and/or damaged (status = 0). Recovery status was scored 30 min after exposure to $LT_{50}$ (heat) and 16 h after exposure to $LT_{50}$ (cold). Longer recovery times for beetles exposed to cold were required because adequate scoring was not possible until it was clear that immobility was due to death and not torpor.

Recovery status was analysed by ordinal logistic regression. Nominal variables considered in the model included exposure temperature and PGI genotype. With adults, sexes were also compared. Body mass was included as a continuous variable, and the possibility of heterogeneity among slopes was considered by including interactions between body mass and the nominal variables. Models were selected following the procedure described by Christensen (1997). All possible models containing main effects and associated interactions were first considered (no interaction was included if the corresponding main effect was not present) and a modification of the Akaike Information Criterion ($A^*$, proposed by Christensen 1997) was used to evaluate each model. The likelihood ratio test statistic (G2) and AIC value were obtained for each model using SAS 8·01 (SAS 1988), and $A^*$ was calculated for each model as described in Christensen (1997). The model with the lowest $A^*$ value was accepted if it did not exhibit significant lack of fit.

### $CT_{MAX}$ OF FIELD-ACCLIMATIZED BEETLES

In June 2000, over-wintered adult beetles were collected in the afternoon from Bluff Lake and held for one night in the laboratory at 4 °C. At 8.00 a.m. they were moved to 20 °C for 2 h before $CT_{max}$ was measured. Beetles were individually placed in 1·5-ml microcentrifuge tubes filled with small glass beads to a level that was even with the top of a heat block. Each tube was moistened with 200 µl water and equipped with a T-type 36 gauge fine-wire thermocouple attached to a hand-held 2-channel thermometer (HH-82, Omega Co., Stamford, Connecticut). Eight beetles were run at once. To determine critical thermal maximum ($CT_{max}$) of each individual, block temperature was increased at a rate of 0·8 °C $min^{-1}$ using a digital hot–cold block incubator (Tropicooler, Boekel Scientific). The onset of spasms was used as end-point for $CT_{max}$ (Lutterschmidt & Hutchison 1997). This condition is characterized by inability of beetles to control voluntary motor function, combined with an irregular kicking of the legs. Beetles were removed from the temperature

block as soon as onset of spasms was reached, air temperature was recorded, and the tube with beetle was placed on ice until it cooled to room temperature (between 21 and 24 °C). Beetles were held at room temperature for 15 min and weighed. They were then frozen at –80 °C for subsequent Hsp70 analysis and determination of PGI genotype.

Statistical analysis for this and subsequent experiments was completed using JMP (Version 3·2·6, SAS Institute, Cary, North Carolina). ANCOVA models were built by including all grouping factors (PGI genotype, sex) and interactions. Time of day and time to $CT_{max}$ were included as covariates to adjust for the fact that $CT_{max}$ increases as time of day and time to $CT_{max}$ increase. $CT_{max}$ increased with time of day due to increases in air temperature. In this and subsequent ANCOVA analyses, covariates were included if Type I sum of squares were significant when they were entered into the model (Groeters & Shaw 1992).

### $CT_{max}$ OF LABORATORY-ACCLIMATED BEETLES

To compare PGI genotypes with respect to effects of recent thermal history on acute thermal tolerance, over-wintered adults were collected from Bluff Lake and reared on *Salix orestera* for 8 days at WMRS under three different thermal regimes. All beetles were held at 4 °C at night and at 20 °C from 8.00 a.m. to 1.00 p.m. From 1.00 to 5.00 p.m., beetles were held at 20, 26 or 32 °C. On the ninth day, $CT_{max}$ was measured as described earlier, with the following exceptions. Eight beetles were run in each block, and blocks were assigned randomly with respect to acclimation temperature and sex. To monitor temperature, each tube was equipped with a T-type 36 gauge fine-wire thermocouple, which was attached to a multichannel temperature data recorder (T-1000 thermocouple meter, Sable Systems, Las Vegas, Nevada) connected to a PC. After $CT_{max}$ determination, beetles were weighed, allowed to recover, and then immediately frozen at –80 °C for subsequent Hsp70 analysis and determination of PGI genotype. To analyse results, three grouping factors were included in the ANCOVA: exposure temperature, PGI genotype and sex. Room air temperature and time to $CT_{max}$ were included as covariates. These covariates did not significantly affect Hsp70 expression level and were therefore not included in its analysis.

### ANALYSIS OF PGI GENOTYPE AND HSP70 EXPRESSION

To conduct biochemical analyses, beetles were removed from storage at −80 °C and dissected on dry ice. For adults, thorax tissue was homogenized in 200 µl 40 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid), 70 mM potassium gluconate, 150 mM sorbitol, 5 mM $Mg_2SO_4$ and 5 mM sodium phosphate, pH 7·3 at 20 °C). For larvae, the body wall was homogenized in 200 µl 50 mM TRIS-Cl (tris(hydroxymethyl)-aminomethane; pH 7·4 at 20 °C). From each individual, 50 µl of homogenate was collected for PGI genotype and protein content analysis. The remaining homogenate was suspended in an equal volume of commercially prepared SDS (sodium dodecyl sulphate) sample buffer and stored at –80 °C until Hsp70 analysis. PGI genotypes were determined by starch electrophoresis using published methods (Murphy *et al*. 1990; Rank 1992). Tissue levels of a stress-inducible 72-kDa heat shock protein (Hsp70) were quantified by Western blot analysis using published methods (Rank & Dahlhoff 2002).

## Results

### LT50 OF FIELD-ACCLIMATIZED BEETLES

In nature, tolerance of elevated and subzero extreme temperatures depends on developmental stage. Over-wintered adults had higher tolerance of elevated temperature, as indexed by $LT_{50}$ (heat), than newly emerged adults (Fig. 1a: $LT_{50}$ = 39·2 °C for over-wintered adults, 37·7 °C for newly emerged adults), and both life stages had higher tolerance of elevated temperatures than larvae (Fig. 1a: $LT_{50}$ = 35·7 °C). Interestingly, newly emerged adults were slightly more tolerant of cold temperatures than larvae (Fig. 1b: $LT_{50}$ = −6·6 °C for newly emerged adults, –6·3 °C for larvae).

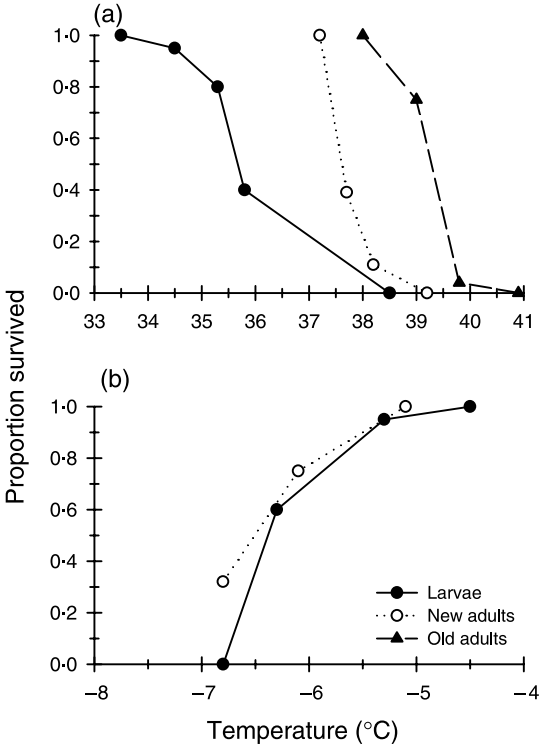


**Fig. 1.** Survival curves for heat (a) and cold (b) exposure of field-acclimatized beetles. Data represent proportion of beetles ($n$ = 20–30 per treatment) that survived a 4-h exposure to each treatment temperature. Ordinal logistic regression of these data was used to calculate $LT_{50}$.

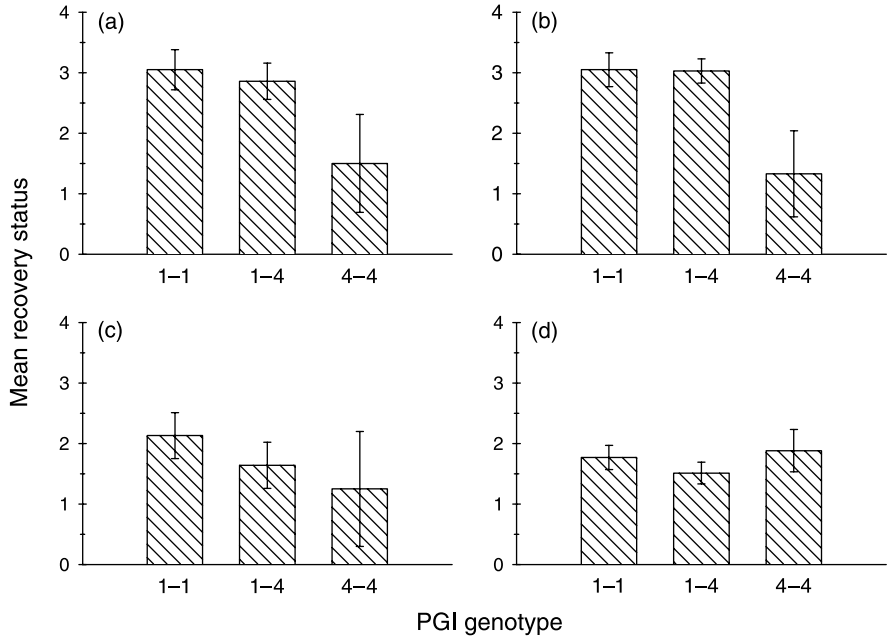


**Fig. 2.** Effect of PGI genotype on recovery from exposure to $LT_{50}$ cold (a, c) and heat (b, d) for larvae (a, b) and adults (c, d) after 2 days prior exposure to typical daytime and night-time temperatures. Temperature treatments and determination of recovery status (0–4) are described in methods. Data shown are least-squares means (± SEM) of each PGI genotype, which were calculated according to the statistical model described in Table 2: (a) 1–1, $n$ = 21; 1–4, $n$ = 28; 4–4, $n$ = 6; (b) 1–1, $n$ = 20; 1–4, $n$ = 29; 4–4, $n$ = 6; (c) 1–1, $n$ = 24; 1–4, $n$ = 22; 4–4, $n$ = 4; (d) 1–1, $n$ = 48; 1–4, $n$ = 59; 4–4, $n$ = 17.

### RECOVERY FROM EXPOSURE TO LT50 AFTER ACCLIMATION TO SUBLETHAL TEMPERATURES

Recovery from exposure to extreme elevated or subzero temperature depends on PGI genotype, prior exposure to a sublethal extreme temperature, and life-history stage. Larvae with the PGI 4–4 genotype recovered less well after exposure to $LT_{50}$ (cold) than PGI 1–1 and 1–4 genotypes (Fig. 2a, Table 1). Larvae that had been exposed to −4 °C for 2 days prior to $LT_{50}$ (cold) exposure recovered better than those that had been exposed to 4 °C, and this effect was most pronounced for PGI 4–4 genotypes (Table 1). In addition, body mass was positively related to recovery, an effect that was most pronounced for individuals with the PGI 1–1 genotype (Table 1).

Heat tolerance in larvae depended on PGI genotype. Larvae with the PGI 4–4 genotype recovered less well after exposure to $LT_{50}$ (heat) than those of other PGI genotypes (Fig. 2b), regardless of previous exposure temperature. However, the effect of PGI genotype on larval heat tolerance depended on recent thermal history. PGI 1–1 genotypes that had been previously exposed to an elevated temperature (30 °C) recovered better than those that had been exposed to the moderate temperature (20 °C). On the other hand, 1–4 and 4–4 genotypes recovered better after previous exposure to 20 °C than those that had been exposed to 30 °C (Table 1). Body mass was positively related to recovery for 4–4 genotypes, but not for the other genotypes. Body mass was related to recovery for individuals held at 20 °C, but not those held at 30 °C (Table 1).

Similar effects of PGI genotype on cold tolerance (but not heat tolerance) were observed for adults as for larvae. Adults with the PGI 4–4 genotype recovered less well from cold exposure than genotypes 1–1 and 1–4 (Fig. 2c, Table 1). Females acclimated to 4 °C recovered better from cold exposure than males acclimated to the same temperature, but there was no sex difference for adults acclimated to −4 °C. In addition, PGI 4–4 females recovered less well after cold exposure than other genotypes, while males of the 1–4 genotype experienced the lowest levels of recovery. Greater body mass was associated with increased recovery in adults with the 1–1 genotype and decreased recovery in adults with the 1–4 and 4–4 genotypes. There was no effect of PGI genotype or acclimation temperature on recovery from exposure to heat in adults (Fig. 2d, Table 1), though females recovered better from exposure to $LT_{50}$ (heat) than males. Body mass was positively associated with recovery in males, but negatively in females (Table 1).

### $CT_{MAX}$ AND HSP EXPRESSION OF FIELD-ACCLIMATIZED BEETLES

Exposure to the critical thermal maximum ($CT_{max}$) causes an acute thermal stress, and physiological responses to that thermal stress depend on PGI genotype. There was no effect of PGI genotype or sex on $CT_{max}$ for field-acclimatized beetles (Fig. 3a). Nevertheless, beetles with the PGI 4–4 genotype expressed significantly less Hsp70

**Table 1.** Results of logistic regression analyses of effect of previous exposure temperature and PGI genotype on recovery of beetles after exposure to $LT_{50}$ temperatures. See text for model selection procedure

| Source | d.f. | *G* | *P* |
|---|---|---|---|
| **Larval cold tolerance** | | | |
| PGI genotype | 2 | 18·325 | < 0·001*** |
| Exposure temperature | 1 | 15·269 | < 0·001*** |
| PGI genotype × Exposure temperature | 2 | 15·897 | < 0·001*** |
| Mass | 1 | 19·865 | < 0·001*** |
| Mass × PGI genotype | 2 | 18·288 | < 0·001*** |
| Mass × PGI genotype × Exposure temperature | 2 | 10·062 | 0·007** |
| **Larval heat tolerance** | | | |
| PGI genotype | 2 | 30·211 | 0·000*** |
| Exposure temperature | 1 | 0·288 | 0·591 |
| PGI genotype × Exposure temperature | 2 | 18·163 | 0·000*** |
| Mass | 1 | 7·976 | 0·005** |
| Mass × PGI genotype | 2 | 25·541 | 0·000*** |
| Mass × Exposure temperature | 1 | 8·160 | 0·004** |
| **Adult cold tolerance** | | | |
| PGI genotype | 2 | 11·142 | 0·004** |
| Exposure temperature | 1 | 1·669 | 0·196 |
| Sex | 1 | 3·031 | 0·082 |
| PGI genotype × Sex | 2 | 6·056 | 0·048* |
| Exposure temperature × Sex | 1 | 5·612 | 0·018* |
| Mass | 1 | 3·517 | 0·061 |
| Mass × PGI | 2 | 11·983 | 0·003** |
| **Adult heat tolerance** | | | |
| Sex | 1 | 28·817 | < 0·001*** |
| Mass | 1 | 0·003 | 0·955 |
| Mass × Sex | 1 | 19·671 | < 0·001*** |

*$P < 0·05$, **$P < 0·01$, ***$P < 0·001$.

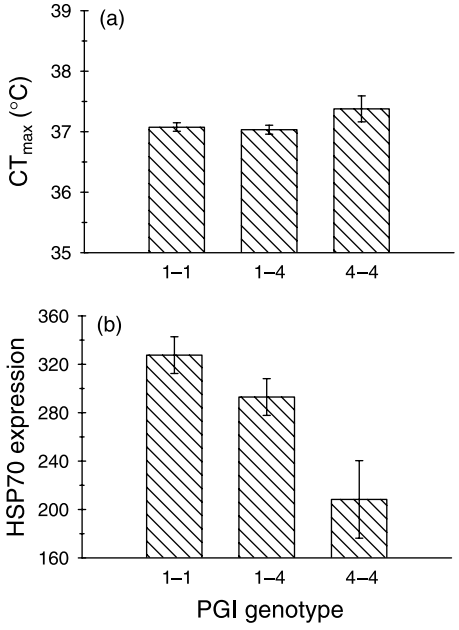


**Fig. 3.** Effect of PGI genotype on $CT_{max}$ for field-acclimatized beetles (a) and expression of a 72-kDa heat shock protein (Hsp70) after determination of $CT_{max}$ (b). Data shown are least-squares means (± SEM) of each PGI genotype ($CT_{max}$: 1–1, $n = 59$; 1–4, $n = 46$; 4–4, $n = 7$; Hsp70: 1–1, $n = 24$; 1–4, $n = 24$; 4–4, $n = 7$). Quantification of Hsp70 expression level described in Rank & Dahlhoff (2002).

than other genotypes after $CT_{max}$ treatment (Fig. 3b, Table 2). There was no direct relationship between $CT_{max}$ and tissue levels of Hsp70 among individuals ($Y = 36·6 + 0·001X$, $r^2 = 0·01$, $F_{1,53} = 0·54$, $P > 0·4$).

### $CT_{MAX}$ AND HSP70 EXPRESSION OF LABORATORY-ACCLIMATED BEETLES

Laboratory acclimation to different thermal conditions revealed differences among PGI genotypes in $CT_{max}$ and the heat shock response. Laboratory-acclimated PGI 1–1 adults had a higher $CT_{max}$ than PGI 1–4 or 4–4 adults (Table 3, Fig. 4a). Exposure temperature did not affect $CT_{max}$, and there was no interaction between exposure temperature and PGI genotype. As expected, PGI genotypes also differed in Hsp70 expression level (Table 3, Fig. 4b). The difference among PGI genotypes depended on sex, but not on exposure temperature (Table 3). Female 1–1 individuals expressed more Hsp70 than male 1–1 individuals. Males of the other two genotypes produced more Hsp70 than females (Fig. 4b, Table 3). Thus, HSP production in females differed among PGI genotypes in the same way as in previous studies (1–1 > 1–4 > 4–4), but maximal Hsp70 expression level in males occurred in the 1–4 heterozygote (Fig. 4b).

**Table 2.** ANOVA results of $CT_{max}$ and Hsp70 expression level in field-acclimatized, over-wintered adults. Analysis included PGI genotypes 1–1, 1–4 and 4–4

| | $CT_{max}$ | | | Hsp70 expression level | | |
|---|---|---|---|---|---|---|
| Source | d.f. | SS | *F* | d.f. | SS | *F* |
| PGI genotype | 2 | 0·5633 | 1·2 | 2 | 64 105 | 5·9** |
| Sex | 1 | 0·0105 | 0·0 | 1 | 938 | 0·2 |
| PGI genotype × Sex | 2 | 0·7483 | 1·6 | 2 | 15 345 | 1·4 |
| Time of day | 1 | 17·7723 | 73·9*** | – | – | – |
| Time to $CT_{max}$ | 1 | 51·0792 | 212·3*** | – | – | – |
| Error | 94 | 22·6152 | | 48 | 262 520 | |

$^{**}P < 0{\cdot}005$; $^{***}P < 0{\cdot}001$.

**Table 3.** ANCOVA results of the effect of previous exposure temperature and PGI genotype on $CT_{max}$ of and Hsp70 expression level in laboratory-acclimated adult beetles

| | $CT_{max}$ | | | Hsp70 expression level | | |
|---|---|---|---|---|---|---|
| Source | d.f. | SS | *F* | d.f. | SS | *F* |
| PGI genotype | 2 | 36·5783 | 6·1** | 2 | 17 587 | 3·7* |
| Exposure temperature | 2 | 0·6986 | 0·1 | 2 | 3 239 | 0·7 |
| Sex | 1 | 0·3885 | 0·1 | 1 | 0·1 | 0·0 |
| PGI × Exposure temperature | 4 | 17·2849 | 1·4 | 4 | 7 695 | 0·8 |
| PGI × Sex | 2 | 2·4513 | 0·4 | 2 | 15 118 | 3·2* |
| Exposure temperature × Sex | 2 | 1·4438 | 0·2 | 2 | 121 | 0·0 |
| PGI × Exposure temperature × Sex | 4 | 9·2733 | 0·8 | 4 | 11 828 | 1·3 |
| Air temperature | 1 | 39·5957 | 13·3*** | – | – | – |
| Time to $CT_{max}$ | 1 | 43·7098 | 14·7*** | – | – | – |
| Error | 189 | 563·527 | | 53 | 124 661 | |

$^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}005$; $^{***}P < 0{\cdot}001$.

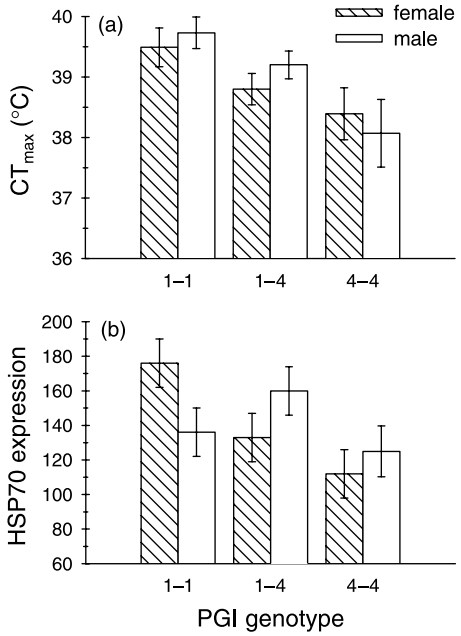


**Fig. 4.** Effects of sex and PGI genotype on $CT_{max}$ (a) and Hsp70 expression level after determination of $CT_{max}$ (b) after 8 days of laboratory acclimation. Data shown are least-squares means (± SEM) of each PGI genotype ($CT_{max}$: 1–1, = 31f, 48 m; 1–4, *n* = 48f, 58 m; 4–4, *n* = 18f, 15 m; Hsp70: *n* = 12 for all except 4–4: *n* = 12f, 11 m).

## Discussion

Three major conclusions can be drawn from these results. First, life stages of *C. aeneicollis* differed substantially in ability to tolerate extremes of heat, but differed less in cold tolerance. Over-wintered adults were the most heat-tolerant life stage, consistent with the fact that they are present during the early summer (May–June), when the warmest daytime air temperatures typically occur (Rank & Dahlhoff 2002). Larvae tended to be less tolerant of temperature extremes than adults. Second, PGI genotypes differed in thermal tolerance. In general, 4–4 genotypes were less tolerant of chronic exposure to thermal extremes than 1–1 and 1–4 genotypes. Third, after exposure to $CT_{max}$, Hsp70 expression for all genotypes was near maximal levels (85–95%) observed for this species (Dahlhoff & Rank 2000; Rank & Dahlhoff 2002), suggesting that beetles were at or near their thermal tolerance limits when exposed to $CT_{max}$. PGI 4–4 genotypes expressed lower levels of Hsp70 than 1–1 and 1–4 genotypes after exposure to $CT_{max}$. The fact that PGI 4–4 genotypes generally express lower levels of Hsp70 under all conditions may have left them vulnerable to acute experimental thermal stress.

Data presented in this study suggest that PGI genotypes differ in tolerance to both heat and cold and that

PGI variation is linked to the heat shock response. The effect of PGI genotype on thermal tolerance and Hsp70 expression level depends on previous temperature exposure and life stage. Previous studies of other insects have shown that though Hsp expression enhances thermal tolerance, it displaces regular protein synthesis, and at high concentrations Hsps may disrupt cellular metabolism (Feder *et al*. 1992; Dahlgaard *et al*. 1998; Kelty & Lee 2001; Krebs & Holbrook 2001; Yocum 2001). These results therefore suggest that differences may exist in energetic expenditure among PGI genotypes after exposure to thermal stress, mediated in part by the heat shock response, and this may be one of the mechanisms by which selection favours different genotypes in different environments.

This appears to be the first study to document differences in thermal tolerance among enzyme genotypes. Thermal tolerance is clearly a complex trait, which is influenced by genetic variation, the thermal history of the organism, and probably by genotype by environment interactions at multiple loci. However, the link reported here between PGI genotype and thermal tolerance is particularly intriguing, because it is consistent with results from other studies that indicate that PGI plays an important role in temperature adaptation. PGI is one of several enzymes regulating flow of glucose through glycolysis and is therefore important in cellular energetics. It appears to be under temperature selection in a number of organisms (Watt 1977, 1983; Hoffmann 1983; Zera 1987; Katz & Harrison 1997). Furthermore, results of other studies on *C. aeneicollis* suggest that PGI genotypes, but not genotypes at other polymorphic enzyme loci, differ in thermal physiology (N. E. Rank and E. P. Dahlhoff, personal observation).

These data are also some of the first to suggest that PGI genotype is associated with cold tolerance. The ability to tolerate both elevated and subzero temperatures in succession may be mediated through the heat shock response. Previous exposure to cold has been shown to cause up-regulation of Hsp70 in other chrysomelids (Yocum 2001). While many ectothermic insects, especially those found in the Arctic, are quite tolerant of cold in winter, they typically lose their ability to tolerate thermal extremes in summer and rarely experience elevated summertime temperatures (Ring & Tesar 1981; Danks, Kukal & Ring 1994). In contrast, Sierra Nevada populations of *C. aeneicollis* (a species usually found at high latitudes) sometimes experience temperatures from −4 °C to 30 °C within a 24-h period (Dahlhoff & Rank 2000; Rank & Dahlhoff 2002). Because PGI 4–4 genotypes are less tolerant of cold than 1–1 or 1–4 genotypes, it is possible that Hsps, which are up-regulated to a greater extent in PGI 1–1 genotypes than others in response to daytime exposure to heat, protect cellular proteins (especially cytosolic proteins like PGI) from cold stress at night.

While these data clearly demonstrate significant differences among PGI genotypes in thermal tolerance, it is not yet clear which aspects of the thermal environment are most critical for temperature adaptation. Survival of adults and larvae after exposure to both elevated and subzero temperatures was greatest for PGI 1–1 genotypes under most measurement conditions, and Hsp70 expression levels were always lowest for 4–4 genotypes. It is therefore possible that 4–4 genotypes are more vulnerable to acute thermal extremes, especially night-time freezing. On the other hand, lower Hsp70 expression levels in 4–4 genotypes could result from down-regulation of the stress response (and other metabolic processes) in response to acclimatization or adaptation to higher environmental temperatures. Such acclimatization is often found in other insects (Sorensen *et al*. 1999; Sorensen *et al*. 2001). In this view, PGI 1–1 and 1–4 genotypes would perform best under most conditions, whereas 4–4 genotypes would be favoured in warmer habitats. Differences among PGI genotypes in thermal stability and kinetic properties are consistent with this prediction (Dahlhoff & Rank 2000). The PGI 4–4 allele is more thermally stable than enzymes from 1 to 1 or 1–4 genotypes, and may therefore need less Hsp protection under typical environmental conditions (Dahlhoff & Rank 2000). The observed distribution of PGI alleles in the Eastern Sierra Nevada is also consistent with this hypothesis (Rank 1992). Further research into the adaptive significance of the PGI polymorphism should allow us to distinguish between these alternative hypotheses.

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