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ALLELE FREQUENCY SHIFTS IN RESPONSE TO CLIMATE CHANGE AND PHYSIOLOGICAL CONSEQUENCES OF ALLOZYME VARIATION IN A MONTANE INSECT

NATHAN E. RANK[1,2,3] AND ELIZABETH P. DAHLHOFF[3,4,5]
[1]*Department of Biology, Sonoma State University, 1801 E. Cotati Avenue, Rohnert Park, California 94928*
[2]*E-mail: rank@sonoma.edu*
[3]*University of California, White Mountain Research Station, Bishop, California 93514*
[4]*Department of Biology and Center for Environmental Studies, Santa Clara University, Santa Clara, California 95053*
[5]*E-mail: edahlhoff@scu.edu*

Abstract.—Rapid changes in climate may impose strong selective pressures on organisms. Evolutionary responses to climate change have been observed in natural populations, yet no example has been documented for a metabolic enzyme locus. Furthermore, few studies have linked physiological responses to stress with allozyme genotypic variation. We quantified changes in allele frequency between 1988 and 1996 at three allozyme loci (isocitrate dehydrogenase, *Idh*; phosphoglucose isomerase, *Pgi*; and phosphoglucomutase, *Pgm*) for the leaf beetle *Chrysomela aeneicollis* in the Bishop Creek region of the Sierra Nevada of California (2900–3300 m). Beetles often experience high daytime (>32°C) and extremely low nighttime (<−5°C) temperatures during summer. Bishop Creek weather station data indicated that conditions were unusually dry before 1988, and that conditions were cool and wet during the years preceding the 1996 collection. We found directional changes in allele frequency at *Pgi* (11% increase in the *Pgi*-1 allele), but not at *Idh* or *Pgm*. We also found that physiological response to thermal extremes depended on *Pgi* genotype. *Pgi* 1–1 individuals induced expression of a 70-kD heat shock protein (HSP) at lower temperatures than 1–4 or 4–4 individuals, and 1–1 individuals expressed higher levels of HSP70 after laboratory exposure to temperatures routinely experienced in nature. Survival after nighttime laboratory exposure to subzero temperatures depended on gender, previous exposure to cold, and *Pgi* genotype. Females expressed higher levels of HSP70 than males after exposure to heat, and recovery by female *Pgi* 1–1 homozygotes after exposure to cold (−5°C) was significantly better than 1–4 or 4–4 genotypes. These data suggest that the cooler climate of the mid-1990s may have caused an increase in frequency of the *Pgi*-1 allele, due to a more robust physiological response to cold by *Pgi* 1–1 and 1–4 genotypes.

Key words.—Climate change, *Chrysomela aeneicollis*, heat shock protein, natural selection, phosphoglucose isomerase, temperature adaptation.

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Understanding the evolutionary significance of protein polymorphisms is one of the most intriguing problems in evolutionary biology (Avise 1994). Some evidence suggests that nonselective processes explain genetic variation at enzyme loci (Kimura 1983); however, it is now clear that natural selection accounts for at least some allozyme variation (Watt et al. 1983; Gillespie 1991; Watt 1992; Mitton 1997; Schmidt and Rand 1999, 2001). Recent, rapid changes in climate offer a tremendous opportunity to evaluate the adaptive significance of enzyme loci (Hoffmann and Blows 1993; Travis and Futuyma 1993; Johnston and Bennett 1996). Genetic variation in many species is related to latitudinal, altitudinal, or tidal gradients in temperature (Johannesson et al. 1995; Feder et al. 1997; Dahlhoff and Rank 2000), which may shift as a result of climate change (Barry et al. 1993; Thomas et al. 2001). Yet, surprisingly little is known about how climate change affects biochemical and physiological characters in natural populations. This is unfortunate, because rapid changes in climate may alter physiological performance and result in reduced survival or fitness. This may lead to local extinction if populations are unable to respond to climate change (Travis and Futuyma 1993; Thomas et al. 2001).

Changes in allozyme frequency have been rarely documented in response to environmental change (Nevo et al. 1983; Johannesson et al. 1990; 1995). However, several studies have shown that heterozygote genotypes are favored by natural selection, resulting in long-term persistence of multiple alleles in a population (Watt et al. 1985; Watt 1992; Mitton 1997; Schmidt and Rand 2001). Other studies suggest that allozyme variation may result from directional selection occurring along latitudinal or tidal clines (Powers and Place 1978; Johannesson et al. 1995; Schmidt and Rand 1999; Dahlhoff and Rank 2000). Evidence for both balancing and directional temperature selection on the glycolytic enzyme phosphoglucose isomerase (*Pgi*) has been found for a wide range of taxa (Corbin 1977; Watt 1977, 1992; Shihab and Heath 1987; Hoffmann 1981b; Johannesson et al. 1995; Katz and Harrison 1997). However, no studies to date have documented a link between changes in allele frequency at *Pgi* and fluctuations in climate.

Organisms cope with extremes in temperature physiologically through induction of heat shock proteins (HSPs), which are up-regulated during periods of cellular stress. HSPs minimize stress-induced aggregation of damaged proteins and they facilitate removal of irreparable proteins (Lindquist 1986). Expression of HSPs enhances thermal tolerance and ability to withstand subsequent stress (Krebs and Feder 1998; Krebs and Bettencourt 1999). Thus, the heat shock response is a key strategy used by ectotherms to survive elevated temperatures in nature (Feder and Hofmann 1999). However, synthesis of HSPs can compete with regular protein synthesis and divert metabolic energy from growth and reproduction. High concentrations of HSPs may also disrupt function of other proteins (Feder et al. 1992; Krebs and Holbrook 2001). Consequently, overproduction of HSPs may incur a fitness cost (Krebs and Feder 1998; Cullum et al. 2001). If HSP

production is affected by an organism's genotype at a polymorphic enzyme locus, for example, *Pgi* investigated here, the relative fitness of allozyme genotypes may depend on the frequency of occurrence of environmental conditions that upregulate HSPs.

Many organisms, including insects, also cope with periods where ambient temperature dips below the freezing point. Exposure to subzero (< 0°C) temperatures can incur substantial fitness costs that equal or exceed those caused by elevated temperature (Denlinger et al. 1991; Lee 1991). HSPs are up-regulated after exposure to subzero temperatures in some insects (Burton et al. 1988; Kelty and Lee 2001). Some HSPs are only up-regulated in response to cold shock (Yocum et al. 1991; Yiangou et al. 1997; Yocum 2001), and these may be in part responsible for rapid acquisition of tolerance to extremely cold temperatures. They may also protect an insect from subsequent heat stress. However, the relationship between heat and cold tolerance has not been examined for an organism experiencing both high and low stressful temperatures in rapid succession in nature. This is unfortunate, because extremes in heat or cold may increase in frequency with global climate change.

Our studies of the Sierra willow beetle, *Chrysomela aeneicollis*, indicate that it represents a valuable model organism to investigate the process of temperature adaptation. Study populations live near the southernmost edge of the species' range (Brown 1956). Beetles experience heterogeneous thermal conditions throughout their lives and are susceptible to both heat and cold stress. Adults overwinter in leaf litter and spend early summer (May–July) exposed on leaves of their host plant. During the day, they mate, feed, and lay eggs. During nighttime torpor, they sit on bare branches or are found curled up inside leaf pockets. Beetles experience large daily fluctuations in body temperature during the summer, from -5°C on cold nights to over 35°C during warm days (Dahlhoff and Rank 2000).

In the Sierra Nevada, *C. aeneicollis* populations are restricted to isolated bog, lake, and stream habitats between 2250 m and 3400 m, with harsh winters and short growing seasons. Snow-covered ridges and regions of dry scrub habitat separate beetle populations in different drainages. Rank (1992) reported evidence for significant genetic divergence among Sierra beetle populations in different drainages. Allele frequencies at *Pgi* vary much more among drainages than other enzymes. *Pgi*-1 predominates in a northern drainage Rock Creek, *Pgi*-4 in a southern drainage Big Pine Creek, and both alleles exist in the intermediate drainage Bishop Creek. Air temperature differs among these drainages. Big Pine Creek is warmer than Bishop Creek, and both drainages are significantly warmer than Rock Creek (Dahlhoff and Rank 2000). Thus, *Pgi*-1 predominates in the coolest drainage, whereas *Pgi*-4 predominates in the warmest one. If *Pgi* allele frequency variation results from adaptation to temperature, *Pgi* frequencies should shift with changes in regional climate.

Climate change models predict that the Sierra Nevada will become warmer, with reduced snow pack and hotter, drier summers in the next 50 years (Jayco and Millar 2001). However, these predicted changes in climate may not result in uniform changes throughout the region, as weather patterns in the Sierra Nevada are highly localized. Furthermore, changes in climate may manifest as an increase in the number of extreme climatic events, in addition to gradual warming or cooling (Easterling et al. 2000). Organisms living in the Sierra Nevada may therefore have to contend with an increase in the frequency of weather events that impose stressful (or lethal) elevated or subzero temperatures at a time in their life cycle when they are not physiologically able to cope with those temperatures.

In this study, we quantified *Pgi* frequency change over time and compared *Pgi* genotypes with respect to physiological response to elevated and subzero thermal extremes experienced by beetles in nature. We addressed three questions. First, how does local climate in Bishop Creek relate to changes in allele frequency at *Pgi* and other allozyme loci? Second, do ambient temperatures of typical beetle habitats reach levels that expose beetles to stressful temperatures? Third, do *Pgi* genotypes differ physiologically in response to exposure to elevated or subzero temperatures typically experienced in nature? This approach will allow us to relate shifts in enzyme genotype frequency to changes in regional climate and to investigate key physiological mechanisms by which animals respond to climate change.

Materials and Methods

Characteristics of Study Populations and Sample Collection

We collected beetles from high-altitude localities in the eastern Sierra Nevada in the Green Lake subdrainage of the South Fork of Bishop Creek (37°18′N, 118°56′W). We focused our work on four previously studied populations that live along an elevation gradient in Bishop Creek (Rank 1992; Dahlhoff and Rank 2000): RV, Rainbow Village, 2878 m; SL, South Lake, 2995 m; PL, Pipeline, 3131 m; and HSC, High Stream Crossing, 3203 m. The dominant shrub at all sites is the willow *Salix orestera*, the favored host plant of *C. aeneicollis*. At RV, willows are found in a moist, open meadow, which is shaded for part of the day by quaking aspen (*Populus tremuloides*) and dries out over the summer. SL has a small creek running through it and is in full sun most of the day. PL is located on a steep hillside alongside a rushing stream and is shaded part of the day. HSC is bisected by a slower portion of the creek and is usually sunny.

Fluctuations in Climate Versus Allele Frequency Change

Annual precipitation.—We obtained daily precipitation values from 1926 to 2001 for the South Lake station (SLK; 2995 m) from the California Data Exchange Center (http://cdec.water.ca.gov/) and summed these to obtain cumulative annual precipitation at the station. The SLK weather station is located equidistant between our RV and SL sites. In all climate analyses, we based annual values on means of data taken from 1 September of the previous year to 31 August in the following year. This water-year approach was used to evaluate habitat conditions for the period when beetles were overwintering and then emerging to mate and lay eggs. To determine whether the coefficient of variation in precipitation changed over time, we calculated 5-year means of precipitation from 1930 to 1999 and conducted linear regression,

with half decade as the independent variable and coefficient of variation in precipitation as the dependent variable.

Air temperature.—We obtained hourly air temperature values from the period when data are available (1989 to 2001) and calculated daily and annual means from these values. Before analysis, we excluded hourly values that had clearly resulted from datalogger malfunction by visual inspection (all of 1999 was excluded based on this assessment). We then excluded days in which fewer than seven hourly measurements, spread evenly across the day, were recorded. On most remaining days (3279 of 3958), 20–24 hourly measurements were included. To confirm that data were accurate, we compared values obtained from SLK logger with two nearby weather stations: Sawmill (SWM; 3109 m) in the Bishop Creek drainage and Rock Creek Lake (RCK, 3048 m) in the Rock Creek drainage. Daily mean temperatures at these two stations were closely correlated to those at the SLK station ($r = 0.965$, $n = 3603$ for SLK vs. SWM; $r = 0.973$, $n = 3557$ for SLK vs. RCK). Unfortunately, data from 1986–1988 were not usable for all three loggers because of logger malfunction for a large proportion (554 of 1095) of days during this period. Thus, temperature data reported here include all data for which we were confident that temperatures were accurately recorded by the logger at the weather station in closest proximity to our field sites. Finally, we determined daily minimum summer air temperatures from 1989 to 2001. Data were analyzed for 90 days after 1 June or for 90 days after a late snowmelt (1995 and 1998). For each year, we obtained minimum daily air temperature and number of nights where air temperature dropped below −5°C.

Allozyme electrophoresis.—We collected adult beetles that had recently emerged from pupae in August 1988 and August 1996 and determined their genotypes at five polymorphic enzyme loci (adenylate kinase, *Ak*; isocitrate dehydrogenase-2, *Idh*; mannose phosphate isomerase, *Mpi*; phosphoglucose isomerase, *Pgi*; and phosphoglucomutase, *Pgm*) using standard methods (Murphy et al. 1990). Data from 1988 were published as part of a study of genetic divergence among drainages in the Eastern Sierra (Rank 1992). In the present study, *Ak* showed little variation (two alleles with one allele occurring at >90% frequency) and *Mpi* bands were not resolvable. We dropped these two loci from our analysis. We also calculated *Pgi* allele frequencies for beetles used for HSP analysis, which were collected from SL and HSC in August 1999 and genotyped for *Pgi* alone. Although we have identified five *Pgi* alleles in Eastern Sierra populations of *C. aeneicollis,* we report only frequencies of *Pgi*-1 and *Pgi*-4 because the combined frequency of other alleles was less than 1%.

Analysis of allele frequency variation.—We compared levels of genetic differentiation in 1988 and 1996 by calculating *F*-statistics using GENEPOP 3.1d (Raymond and Rousset 1995). To determine whether F_{ST}-values were significantly different from zero at each locus, we used log-likelihood exact tests of allele frequency heterogeneity (Raymond and Rousset 1995). We used permutation tests of multilocus genotype frequencies provided in the FSTAT program to test for overall significance of F_{ST} (Goudet 1995). To test for changes in allele frequency between 1988 and 1996, we first determined which allele was most common at each locus and then analyzed variation in frequency of that allele. We used analysis of covariance (ANCOVA) with year as a categorical fixed effect, elevation as a covariate, and the year-by-elevation interaction as a test for heterogeneity of slopes.

Physiological Consequences of Temperature Variation

Air temperature variation at Bishop Creek study sites.—We recorded ambient air temperatures at the four sites (RV, PL, SL, HSC) using Stowaway Tidbit dataloggers (Onset Computer Co., Pocaset, MA) every 30 min. Each datalogger was suspended inside a thermally neutral white plastic housing on a willow branch, which avoids overestimation of maximal air temperature by solar irradiance. Previous studies demonstrate that beetle body temperatures are highly correlated with air temperatures measured this way (Dahlhoff and Rank 2000).

To evaluate diurnal variation in air temperature during a period when adult beetles were active at the HSC site, we analyzed temperature data recorded over 62 days in summer 2000 (15 May to 15 July). Final snowmelt occurred at the HSC site on 21 April 2000. To compare the four study sites with respect to air temperature, we calculated mean daily air temperatures from each site over three intervals when no datalogger was covered with snow: summer (1 July to 11 August 2001), fall (2 September to 13 October 2001) and spring (21 April to 1 June 2002). We analyzed site data using ANCOVA, with interval as a grouping factor and site elevation as a covariate.

Effects of Pgi genotype on HSP70 expression.—To determine HSP70 induction profile for field overwintered beetles, we collected 400 adults from the HSC site in June 2000 and reared them on *Salix orestera* for 8 days at White Mountain Research Station (20°C day, 4°C night). On the ninth day, we exposed beetles to one of six temperatures (20, 24, 28, 30, 33, or 36°C) in heater/chiller blocks for 4 h and allowed them to recover for 1 h at 20°C before we flash froze beetles on dry ice. Samples were stored at −70°C until HSP analysis.

To compare HSP70 expression for beetles from different localities, we collected 120 adults that had recently emerged from pupae at the SL and HSC populations in August 1999 and brought them to the laboratory at Santa Clara University. Beetles overwintered under natural light at 4°C in plastic dishes with moist peat moss. In April 2000, we brought beetles out of these cool conditions and fed them *Salix lasiolepis* foliage for 1 week at 20°C day, 4°C night. To induce HSP expression, we exposed beetles to 34°C for 4 h and allowed them to recover at 20°C for 1 h before flash freezing. We stored these samples at −70°C until HSP analysis.

HSP70 quantification.—We quantified expression of a 72-kD HSP by Western blot analyses following methods modified from Dahlhoff and Rank (2000). We homogenized beetle thorax muscle in 200 μl KGB buffer (40 mM HEPES, 70 mM potassium gluconate, 150 mM sorbitol, 5 mM Mg_2SO_4, and 5 mM sodium phosphate, pH 7.3 at 20°C), determined protein content of homogenates using a modified Lowry protein assay (D_c Protein Assay Reagent, Bio-Rad Laboratories, Inc., Hercules, CA), reserved 50 μl of homogenate for *Pgi* genotype analysis and suspended remaining homogenate in an equal volume of commercially-prepared SDS sample buffer (Bio-Rad) to which 5% 2-mercaptoethanol was added. We

heated samples for 5 min at 95°C to insure complete protein denaturation, and stored samples at −70°C until Western blot analysis. We analyzed HSP70 expression for a randomly selected subset of individuals that had been heat shocked and prepared in this fashion after obtaining *Pgi* genotype using starch gel electrophoresis.

We loaded precast polyacrylamide gels (Tris-HCl 10–4% PAGE Ready Gels, Bio-Rad) with equal amounts total protein (40 μg) and electrophoresed samples using methods described previously (Dahlhoff and Rank 2000), with the following exceptions. To locate HSP70, we used 1:1000 mouse monoclonal anti-Hsp70 antibody (SPA-822, StressGen Biotechnologies, Victoria, BC, Canada) in Tris-buffered saline with 0.1% Tween-20 (TBST). This antibody is specific for a stress-inducible isoform of HSP70 in these beetles. After washing blots in TBST, we treated blots with 1:7500 anti-mouse IgG conjugated with peroxidase (Amersham Biosciences, Buckinghamshire, England). The peroxidase was reacted using ECL-Plus (Amersham Biosciences) and the presence of a single, 72-kD band was detected using Kodak (Rochester, NY) X-Omat AR X-ray film. We measured resulting band area and intensity on films using SigmaScanPro (ver. 5.0, SPSS Scientific, Chicago, IL). We compared beetle HSP70 band size and intensities with bands of a serial dilution of pure HSP70 loaded onto each gel (human recombinant; SPP-855, StressGen Biotechnologies). We used this serial dilution to create a standard curve and determined the relative tissue concentration of HSP70 per gram total thorax protein for each beetle by linear regression.

Analysis of HSP data.—We analyzed HSP70 expression in field overwintered beetles by three-way ANOVA with gender, temperature, and *Pgi* genotype as fixed effects and all two-way interactions. We could not include the three-way interaction in the final model because no females from the 24°C treatment possessed the *Pgi* 4–4 genotype. Preliminary analyses without the 24°C treatment found no significant three-way interaction term ($F_{8,35} = 0.35$, $P > 0.94$), so we felt justified in omitting this interaction term from statistical analysis. We analyzed HSP70 expression in laboratory overwintered beetles by two-way analysis of variance with *Pgi* genotype as a fixed effect and the source population and interaction term as random effects.

Determination of LT_{50} to subzero temperatures.—We determined LT_{50}(cold), the temperature at which 50% of beetles died from exposure to subzero temperatures, for beetles from the HSC site. We collected beetles in the afternoon and maintained them at room temperature in the laboratory for 16 h before exposing them for 4 h to cold temperatures. We initiated LT_{50}(cold) measurements by holding beetles at two temperatures ($n = 14$ beetles per temperature) that we expected to lead to 0% and 100% mortality (0°C and −8°C, respectively). We repeated these assays with intermediate temperatures until LT_{50} was reached. During temperature treatment, we kept beetles in 1.5-ml Eppendorf tubes in heater/chiller blocks (A. Daigger and Company, Vernon Hills, IL). After cold treatment, we held beetles for 1 h at 20°C, weighed them, and examined them under a dissecting microscope to determine recovery status. For this experiment, we classified beetles that moved after 1 h as alive (status = 1) and immobile and/or damaged beetles as dead (status = 0). We determined the LT_{50}(cold) using logistic regression analysis. We used these data to select treatment temperatures for subsequent cold-tolerance experiments.

Effects of previous exposure to cold on subsequent cold tolerance.—We collected adult beetles from HSC on 4 July 2000 and held them in the laboratory for two consecutive day-night cycles. We held them at 20°C during the day (0800 to 2000 h) and either 4°C or −3°C for 4 h at night. On the third evening, we exposed all individuals to −5°C for 4 h. We scored recovery 1 h following cold treatment and again 24 h later. For this experiment, we classified beetles that moved normally as fully recovered (status = 2). Beetles that were twitching slightly were classified as partially recovered (status = 1). We considered immobile and/or damaged beetles to be dead (status = 0). We analyzed these data by contingency tables using exact probabilities calculated in SAS (ver. 8.1, SAS Institute, Cary, NC.).

Effects of PGI genotype on cold tolerance.—We collected adult beetles from the HSC (3203 m) population on 4 and 5 July 2000 and kept them in the laboratory for 16 h before exposing them for 4 h to −5°C. We assessed beetle recovery status after 1 h as described in the previous section. We analyzed cold tolerance data using logistic regression with *Pgi* genotype, date, and gender as independent variables and recovery status as the dependent variable. Models were selected following the procedure described by Christenson (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*) was used to evaluate each model. The Akaike information criterion (AIC) is used to compare the explanatory power of a candidate model to other candidate models for a categorical dataset. The likelihood-ratio test statistic (G^2) and AIC value were obtained for each model using SAS version 8.1, and A* was calculated for each model as described in Christenson (1997). The model with the lowest A* value was accepted if it did not exhibit significant lack of fit.

RESULTS

Fluctuations in Climate Versus Allele Frequency Change

Annual variation in precipitation and air temperature.—At the South Lake (SLK) weather station, annual precipitation increased from 1987 to 2001 (Fig. 1A; slope = 1.72 ± 0.9, $r = 0.49$, $n = 14$, $P = 0.078$). Over a longer time scale (1926–2000), there was no significant change in annual precipitation (slope = −0.036 ± 0.09, $r = 0.05$, $n = 65$, $P = 0.69$) and no change in the coefficient of variation in precipitation (slope = 0.040 ± 0.01, $r = 0.14$, $n = 13$, $P = 0.63$). The fourth driest year recorded by this weather station since 1926 was 1987. This dry year immediately preceded the first sample of beetle populations in 1988.

At the SLK weather station, mean air temperature decreased from 1989 to 2001 (Fig. 1B; slope = −0.24 ± 0.09, $r = -0.64$, $n = 12$, $P = 0.025$), while the coefficient of variation in air temperature did not change (slope = 0.023 ± 0.013, $r = 0.48$, $n = 12$, $P = 0.11$). Summer nighttime temperatures dropped below −5°C at least once every year between 1989 and 2001, and they dropped below −10°C

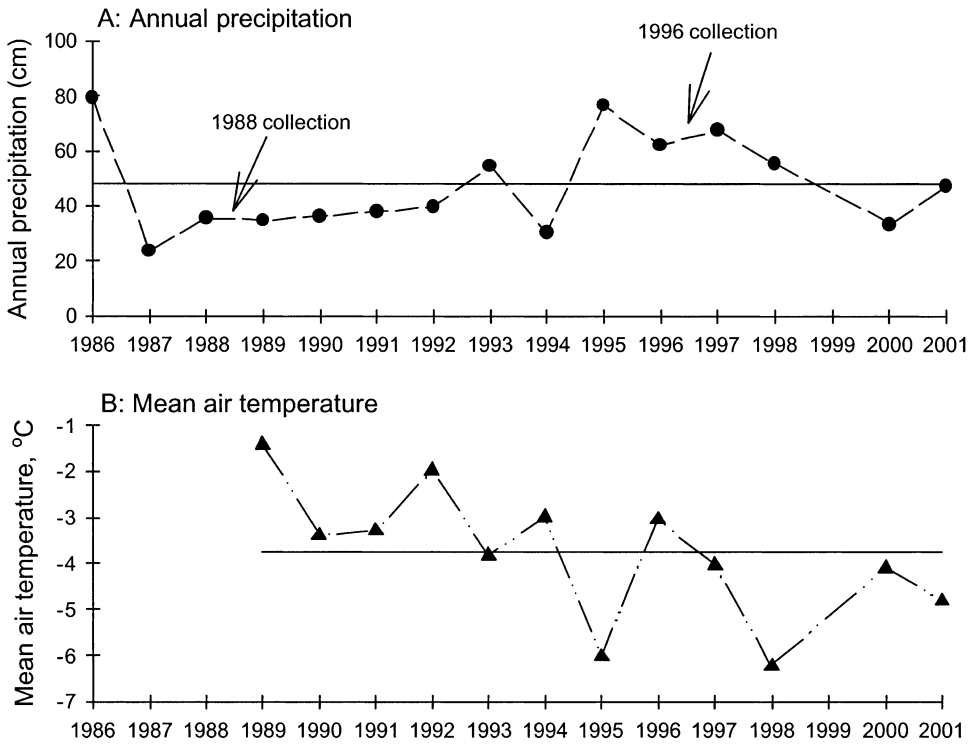

FIG. 1. Annual precipitation (A) and mean air temperature (B) at the South Lake weather station in Bishop Creek. Years reported are from September of previous calendar year to August of year shown. Mean values from 1927 to 1997 (precipitation) and 1989 to 1997 (air temperature) are indicated with solid horizontal lines. Arrows indicate times beetle populations were sampled.

during eight of 12 years sampled. Nighttime temperatures dropped below −5°C at least 10 times during the summers of 1992, 1993, 1997, and 1998. Snowmelt dates were late in 1995 (19 June), one year before the second sample of beetle populations was collected for genetic analysis, and in 1998 (9 June). Finally, air temperature was negatively related to precipitation between 1989 and 2001 (slope = −6.22 ± 2.70, $r = -0.60$, $n = 12$, $P = 0.043$).

Analysis of allele frequency variation.—Beetle populations were significantly genetically differentiated in 1988 and in 1996, yet F_{ST}-values were strikingly greater for each enzyme locus in 1988 than in 1996 (Table 1). In addition, the frequency of *Pgi* allele 1 increased significantly (by 11%) between 1988 and 1996 (Fig. 2, Table 2). We found no such directional changes in allele frequency at *Idh* or *Pgm*. Interestingly, *Pgi*-1 is at higher frequency for low elevation populations in both 1988 and 1996, a trend not observed for the other loci (Fig. 2, Table 2). This elevation trend in *Pgi* frequency contributed toward the higher F_{ST} observed at *Pgi* than at the other loci. However, the altitudinal cline in *Pgi*-1 frequency was less steep by 1996 than it was in 1988 (year-by-elevation interaction, Table 2).

In the fall of 1999, we collected additional beetles from the SL and HSC populations for physiological analysis (see Fig. 5) and determined *Pgi* frequencies in this sample. For SL populations, the frequency of *Pgi*-1 was similar to what we observed in 1996 (0.67, based on 51 individuals). For HSC populations, the frequency of *Pgi*-1 tended to be higher than observed in 1996 (0.68, based on 19 individuals) and was not different from SL *Pgi* frequencies (Pearson $\chi^2 = 0.039$, df = 1, $P > 0.80$).

TABLE 1. Analysis of genetic divergence among Bishop Creek populations of *Chrysomela aeneicollis* in 1988 and 1996. *P*-values were adjusted at the columnwide level using the sequential Bonferroni adjustment (Rice 1989).

Enzyme locus	1988 F_{ST}	1996 F_{ST}
Isocitrate dehydrogenase (*Idh*)	0.059***	0.005*
Phosphoglucose isomerase (*Pgi*)	0.132***	0.032**
Phosphoglucomutase (*Pgm*)	0.021***	0.006*
Mean	0.070***	0.013***

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

Physiological Consequences of Extreme Temperature

Daily variation in air temperature at Bishop Creek.—Large fluctuations in diurnal air temperature occurred at the HSC site in 2000, a fairly typical year for this region (Fig. 1). During most nights, minimum temperature ranged from 1°C to 5°C. However, minimum temperature was at or below the freezing point for 10 nights and it reached −6°C twice during the two-month sampling period (Fig. 3). Maximum daily temperature usually ranged from 18°C to 24°C but exceeded 26°C on 14 days and reached a maximum of 32°C (Fig. 3). Analysis of mean daily air temperature at the four study sites (RV, SL, PL, HSC) revealed that it declined with increasing elevation (ANCOVA $F_{1,7} = 6.2$, $P = 0.04$). Mean temperatures were greater during summer (mean $T_a = 11.8$°C) than fall 2001 (mean $T_a = 8.2$°C) or spring 2002 (mean $T_a = 4.6$°C; ANCOVA $F_{2,7} = 140.8$, $P < 0.0001$).

Effect of Pgi genotype on HSP70 expression.—*Pgi* geno-

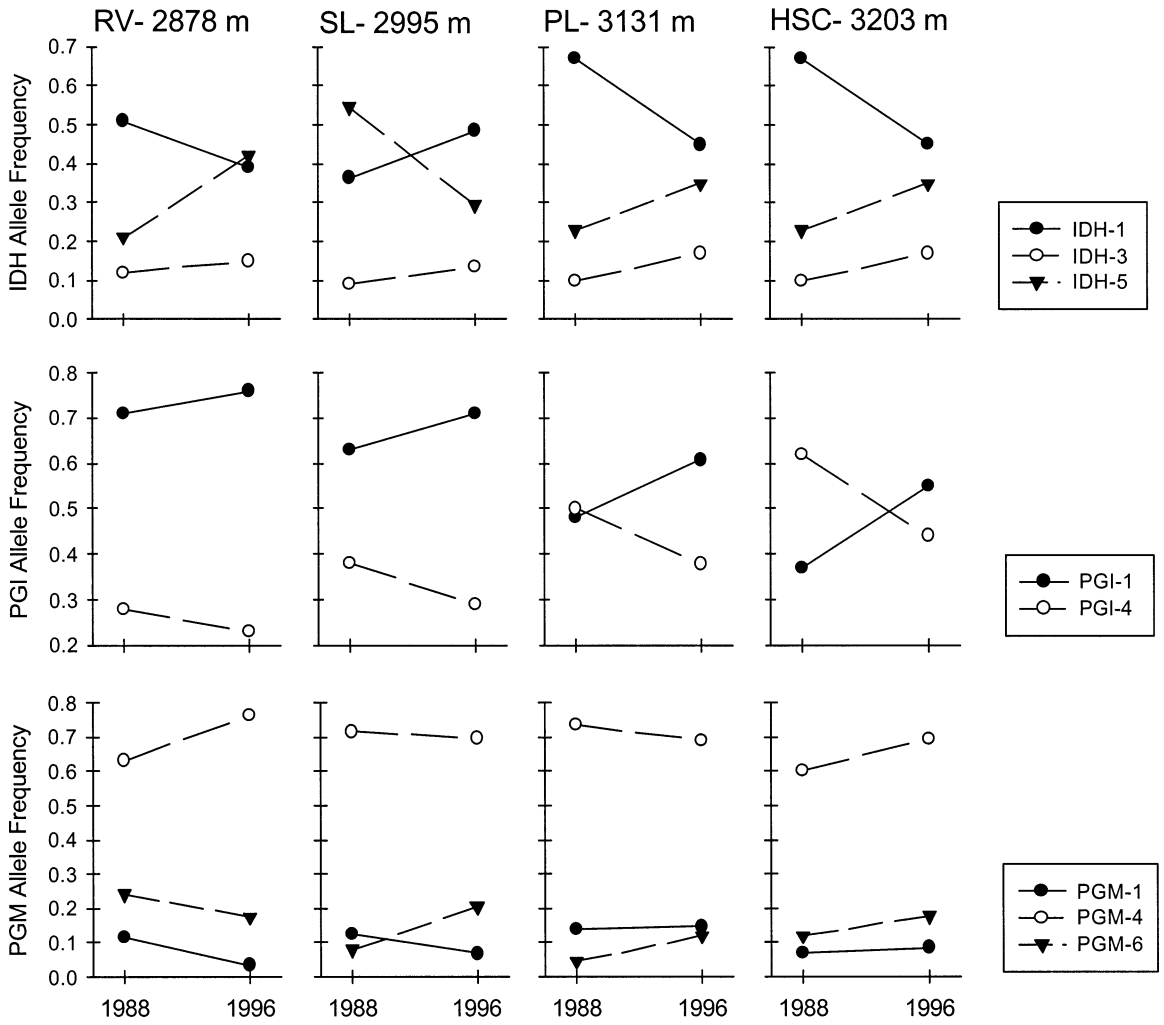

FIG. 2. Allele frequency changes at three allozyme loci in four study populations of *Chrysomela aeneicollis*. Values are shown for alleles with average frequency >0.05 at isocitrate dehydrogenase (*Idh*: upper panels), phosphoglucose isomerase (*Pgi*: middle panels), and phosphoglucomutase (*Pgm*: lower panels). Beetle populations were analyzed at four locales: Rainbow Village (RV), $n_{1988} = 99$, $n_{1996} = 74$; South Lake (SL), $n_{1988} = 44$, $n_{1996} = 66$; Pipeline (PL), $n_{1988} = 157$, $n_{1996} = 136$; High Stream Crossing (HSC), $n_{1988} = 79$, $n_{1996} = 87$.

types differed in the temperature at which HSP70 expression was upregulated in response to heat stress (Fig. 4, genotype-by-temperature term in Table 3). The peak of HSP expression occurred at 30°C for *Pgi* 1–1 genotypes (Fig. 4A), at 33°C for 1–4 genotypes (Fig. 4B), and at 36°C for 4–4 genotypes (Fig. 4C). The intermediate peak for the *Pgi* 1–4 heterozygote suggests incomplete dominance in HSP induction profile. The HSP70 expression profile is steeper for *Pgi* 4–4 individuals than 1–4 or 1–1 individuals, whose profile was flatter at lower treatment temperatures. HSP70 levels were lower at low induction temperatures for 4–4 individuals than 1–4 or 4–4 individuals. Finally, females produced significantly more HSP (9%) than males (Table 3).

HSP expression also depended on *Pgi* genotype for beetles collected from HSC and SL localities and overwintered in the laboratory (Fig. 5, Table 4). The 1–1 and 1–4 genotypes

TABLE 2. ANCOVA of allele frequency change for each enzyme locus in Bishop Creek populations of *Chrysomela aeneicollis* in 1988 and 1996.

	Idh			*Pgi*			*Pgm*		
Source	df	SS	*F*	df	SS	*F*	df	SS	*F*
Year	1	0.006350	0.6	1	0.003825	7.8*	1	0.001465	0.5
Elevation	1	0.007813	0.7	1	0.089934	184.3***	1	0.003281	1.1
Year × elevation		—		1	0.004658	9.6*		—	
Error	5	0.056326		4	0.001952		5	0.014986	

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

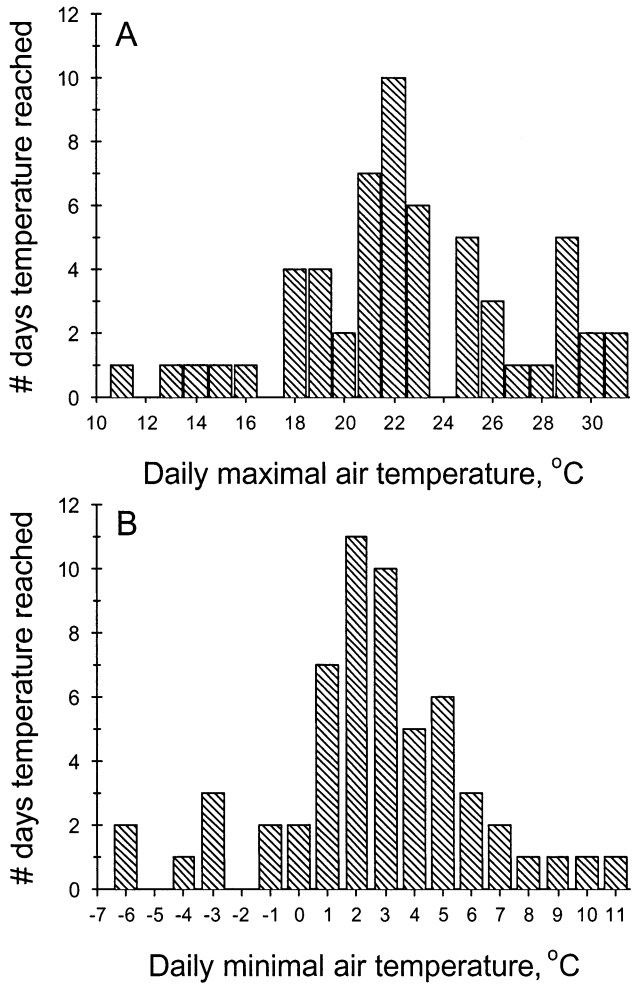

FIG. 3. Daily maximal (A) and minimal (B) air temperatures measured on willows at the HSC locality (3203 m) in Bishop Creek during summer 2000. Data are shown as the number of days the maximal or minimal air temperature reached or exceeded the reported value but not the next highest value (i.e., there were 10 days that maximal air temperature exceeded 22°C but did not exceed 23°C). Air temperature was determined as described in text.

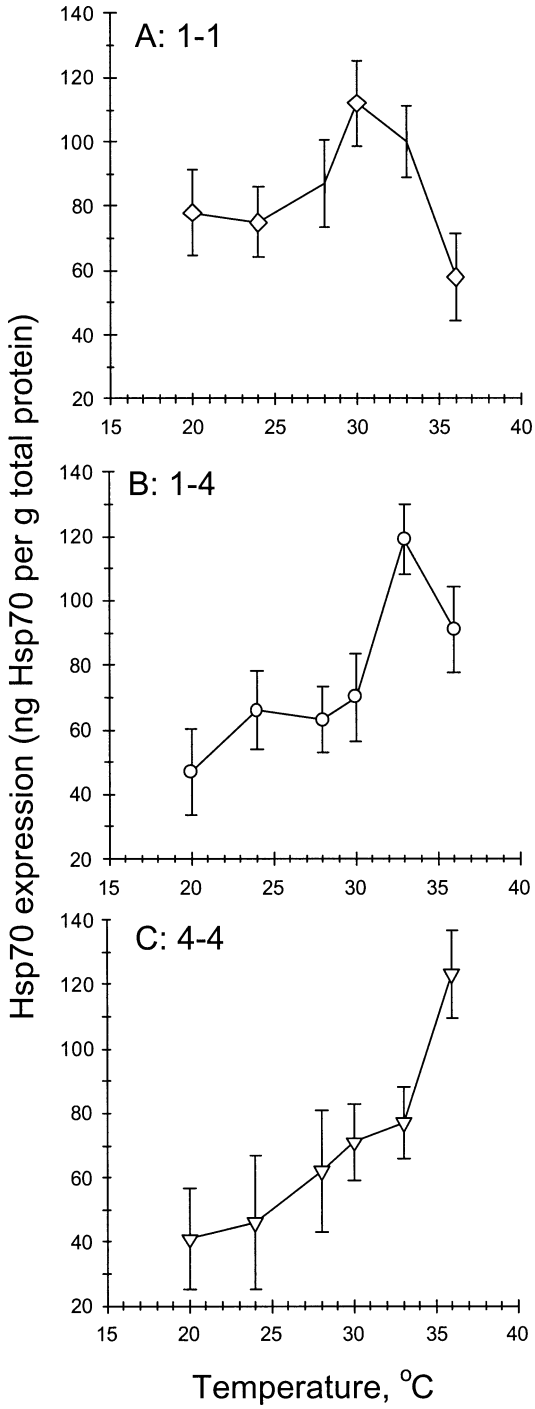

FIG. 4. Effects of *Pgi* genotype on HSP70 induction profiles for beetles that overwintered in nature at the HSC locality in Bishop Creek. HSP70 expression was induced at different temperatures in the laboratory and is reported as nanograms HSP70 per g total thorax muscle protein. Data shown are least-squares means of each *Pgi* genotype at each temperature (A: 1–1, $n = 28$; B: 1–4, $n = 28$; C: 4–4, $n = 21$).

expressed more HSP than 4–4 homozygotes at the single induction temperature (34°C, Fig. 5). SL and HSC populations did not differ in effects of *Pgi* genotype on HSP expression (Table 4).

Determination of LT_{50}(min).—All 84 adults survived and completely recovered from the 4-h nighttime exposure to 0°C, −2°C, and −3°C. Twenty-two of 28 beetles tested at −4°C survived the exposure. Only six of 28 beetles survived exposure to −5°C, and none survived exposure to −6°C. Logistic regression revealed a highly significant effect of temperature on beetle survival ($G = 154$, df = 1, $P < 0.0001$). The LT_{50}(min) predicted from the regression equation is −4.5°C (Fig. 6).

Effect of previous exposure to cold on subsequent cold tolerance.—Beetles recovered from a 4-h exposure to −5°C much more readily if they had been previously exposed to a sublethal cold temperature (−3°C) for two consecutive nights (Table 5). Most beetles previously exposed to a warmer treatment temperature (4°C) died or only partially recovered shortly after exposure to −5°C, whereas most that had been exposed to −3°C survived (Fisher's exact test, $P < 0.0001$). This effect persisted for 24 h (Fisher's exact test $P = 0.0003$).

Effect of Pgi genotype on cold tolerance.—Survival after

TABLE 3. Multiway analysis of variance for effects of gender, temperature, and *Pgi* genotype on HSP70 induction profiles of *Chrysomela aeneicollis* overwintered in the field. Gender, temperature, and genotype were all treated as fixed effects.

Source of variation	df	SS	*F*
Gender	1	4684.51	7.5**
Temperature	5	16430.12	5.2***
Genotype	2	662.01	0.5
Gender × temperature	5	7826.51	2.5*
Gender × genotype	2	5057.14	4.0*
Temperature × genotype	10	19361.09	3.1**
Error	53	33298.24	

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

TABLE 4. Two-way analysis of variance for effects of locality and *Pgi* genotype on HSP70 expression of *Chrysomela aeneicollis* overwintered in the laboratory. Locality was treated as a random effect and genotype was treated as a fixed effect.

Source of variation	df	SS	*F*
Locality	1	18.03	0.05
Genotype	2	14043.92	42.32*
Locality × genotype	2	331.83	0.1
Error	30	49566.31	

* $P < 0.05$.

exposure to subzero temperatures also depended on field collection date, gender, and *Pgi* genotype (Tables 6, 7). Most females with the 1–1 and 1–4 genotypes survived the cold exposure, whereas half of the 4–4 homozygous females died. However, most males of all three genotypes survived the cold exposure (Table 6). In addition, recovery status depended on collection date. Of beetles collected after a relatively warm night (minimum temperature = 2.81°C) 16% recovered fully, whereas 52% of beetles recovered from cold stress fully when collected after a night when temperatures had fallen below freezing (minimum temperature = −0.16°C; Table 7).

DISCUSSION

The results reported here suggest that differences among *Pgi* genotypes in thermal physiology are responsible for *Pgi* allele frequency change in nature. We found significant changes in *Pgi* frequency after several years of cool, wet conditions in the mid-1990s, which were quite different from the hot, dry drought conditions common throughout California in the 1980s. *Pgi*-1 increased in frequency at all study localities in Bishop Creek between 1988 and 1996 and appeared to continue to increase in frequency at one locality (HSC) through 1999. No such directional changes between 1988 and 1996 occurred for two other enzyme loci (*Idh*, *Pgm*), suggesting that natural selection favored the *Pgi*-1 allele during this period.

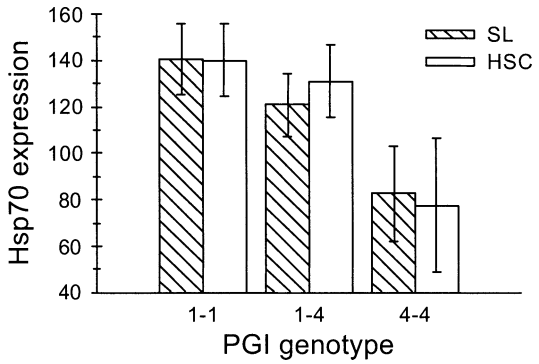

FIG. 5. Effects of *Pgi* genotype on HSP70 expression for laboratory overwintered beetles collected from two sites in Bishop Creek: South Lake (SL: striped bars) and High Stream Crossing (HSC: open bars). Amount of HSP70 is reported as nanograms HSP70 per g total thorax muscle protein. Data shown are least-squares means ± SE (1–1, $n = 14$; 1–4, $n = 16$; 4–4, $n = 7$). *Pgi* genotype frequencies of these samples are given in the text.

Daytime and nighttime thermal conditions in nature frequently reach levels that cause different physiological responses to heat and cold among *Pgi* genotypes. *Pgi* 1–1 genotypes had a lower HSP induction temperature than 1–4 genotypes, which in turn had a lower HSP induction temperature than 4–4 genotypes. Though previous exposure to cold was important to surviving a cold stress, for females survival of extreme cold temperatures was highest for individuals possessing the *Pgi*-1 allele. Thus, the increase in frequency of *Pgi*-1 associated with recent cooler climatic conditions in the eastern Sierra Nevada may have been mediated by a more robust physiological response to cold by individuals possessing the *Pgi*-1 allele.

Fluctuations in Climate Versus Allele Frequency Change

The directional shifts in *Pgi* allele frequency observed here suggest that the *Pgi*-1 allele was selectively favored during

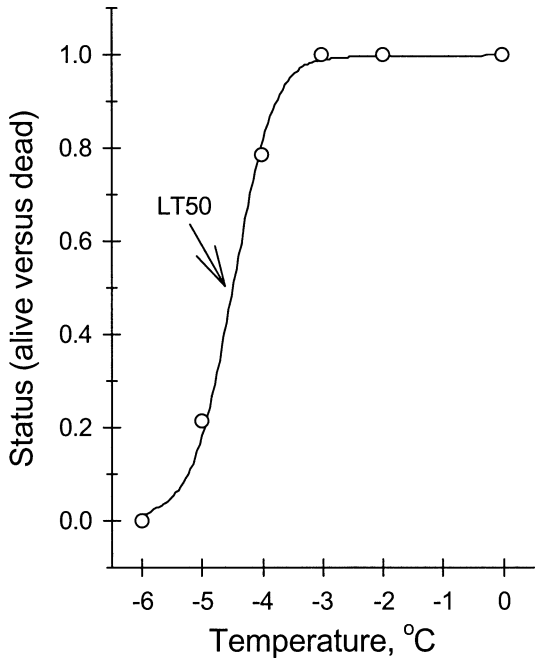

FIG. 6. Determination of the LT_{50} for beetles collected from the HSC locality of Bishop Creek and exposed to subzero nighttime temperatures in the laboratory. Data shown are mean recovery status (1 = alive, 0 = dead) for $n = 28$ individuals per treatment temperature. Arrow indicates LT_{50} (−4.5°C), calculated by fitting a logistic regression to data. Curve indicates predicted values and symbols represent actual values.

TABLE 5. Effects of previous exposure to low temperature (4 h at either −3°C or 4°C) on the ability to survive cold-shock at −5°C. Data shown are percent fully recovered, partially recovered, and dead male and female beetles scored 1 h after 4 h cold-shock and 24 h later. Percentages are taken on row totals.

Time treatment	*N*	Recovered	Partially recovered	Dead
1 h				
−3°C	28	86%	14%	0%
4°C	28	25%	64%	11%
24 h				
−3°C	28	43%	18%	39%
4°C	28	7%	4%	89%

TABLE 6. Effects of *Pgi* genotype on the ability to survive cold-shock. Data shown are percent fully recovered, partially recovered, and dead male and female beetles of each *Pgi* genotype scored 1 h after 4 h cold-shock. Percentages are taken on row totals.

Gender genotype	*N*	Recovered	Partially recovered	Dead
Female				
1-1	29	31%	45%	24%
1-4	37	43%	24%	32%
4-4	10	20%	30%	50%
Male				
1-1	32	25%	47%	28%
1-4	22	18%	50%	32%
4-4	7	43%	29%	29%

cooler climatic conditions. It is not clear from the present data whether this shift toward *Pgi*-1 was due to gradual cooling in the South Lake region in the mid-1990s, a few very cold periods (especially in 1993 and 1995), or a combination of effects. However, these results are consistent with the known broad geographic distribution of *Pgi* alleles in the eastern Sierra Nevada, as *Pgi*-1 is most common in the coolest drainage Rock Creek and least common in the warmest drainage Big Pine Creek (Rank 1992; Dahlhoff and Rank 2000). This correspondence between among-drainage differences in ambient temperature and allele frequency was not found for four other allozyme loci (*Ak, Idh, Mpi, Pgm*). In general, when a single locus shows a pattern of variation that is highly discordant with other loci, it is likely that natural selection acts on that locus (Slatkin 1987).

We found that the frequency of the *Pgi*-1 allele declined with increasing elevation in 1988 and 1996. Because temperature also declines with elevation, this result appears at first to be inconsistent with the selection hypothesis, because one would expect that *Pgi*-1 frequencies should be greater at cool, high elevations than at warm, low ones. We suspect that the observed cline in *Pgi* frequency may have initially resulted from migration into Bishop Creek from the neighboring drainage to the south, Baker Creek. Baker Creek lies between Bishop Creek and Big Pine Creek and a migration corridor exists between Baker Creek and high-elevation localities in Bishop Creek (Grunion Plateau, 3300 m; see map in Rank 1992). *Pgi*-1 frequencies for beetles in Baker Creek (0.43, based on 41 individuals) are considerably lower than those found in Bishop Creek (0.64, based on 363 individuals). A mass northward migration from Baker to Bishop Creek may have occurred at some point in the recent past, causing the present elevation cline in *Pgi* allele frequencies in Bishop Creek. Related species of leaf beetles are known to occasionally migrate en masse to distant localities (Palmén 1944).

While *Pgi* allele frequencies observed in our Bishop Creek study populations may have been influenced by migration, the fact that the directional change in *Pgi*-1 frequency was greater in high-elevation populations than in low-elevation populations supports the hypothesis that natural selection favored the *Pgi*-1 allele between 1988 and 1996. The frequencies of *Pgi*-1 and *Pgi*-4 were close to parity in the high-elevation populations. Assuming that selection coefficients are similar in each population, single-locus selection theory predicts greater directional change in allele frequency when initial frequencies are relatively equal than when frequencies are biased toward one allele (Hartl and Clark 1989). In addition, we suspect that the selection coefficients favoring the *Pgi*-1 allele may have been even greater at high elevations than at low ones, because those populations experienced the coldest nighttime temperatures.

Physiological Consequences of Extreme Temperature

Previous studies on *C. aeneicollis* and other organisms propose that *Pgi* is likely a key enzyme for temperature adaptation. Population genetic studies have revealed unusual patterns of variation at *Pgi* in a wide range of organisms, including sea anemones (Hoffmann 1981a,b), mollusks (Byers 1983; Lavie and Nevo 1986; Johannesson et al. 1990), insects (Watt 1983; Zera 1987a,b; Katz 1997; Katz and Harrison 1997), isopods (Edwards and Heath 1983; Shihab and Heath 1987; McCluskey et al. 1993), plants (Gottlieb and Greve 1981; Filatov and Charlesworth 1999), and bacteria (Dykhuizen and Hartl 1983). Temperature affects *Pgi* allozyme functional properties such as Michaelis-Menten binding constant, K_m, and thermal stability in a diverse array of species (Hoffmann 1981a; Watt 1983; Mitton 1997). In *C. aeneicollis*, the 1–1 homozygote exhibits a lower K_m-value and thermal stability compared to the 1–4 heterozygote or 4–4 homozygote, suggesting that the *Pgi*-1 allele may function better than the *Pgi*-4 allele at low temperature (Dahlhoff and Rank 2000). Watt and colleagues have proposed that *Pgi*'s central role in glycolysis makes it especially critical for temperature adaptation (Kingsolver and Watt 1983; Watt 1983). Our results show that PGI variation also affects physiological processes such as HSP production and cold tolerance, which may be associated with differences in enzyme functional property that we described previously (Dahlhoff and Rank 2000). Al-

TABLE 7. Results of logistic regression analysis of effects of gender, collection date, and *Pgi* genotype on cold tolerance of *Chrysomela aeneicollis* adults. See text for model fitting procedure. Log-likelihood values (*G*) were used in significance testing.

Source of variation	df	*G*
Gender	1	0.075
Date	1	11.829***
Gender × genotype	2	6.229*
Gender × date	1	0.829
Gender × genotype × date	2	6.185*

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

though these physiological differences among *Pgi* genotypes may result directly from *Pgi*'s role in glycolysis (and metabolic performance), they may also represent a previously undiscovered mechanism for natural selection to act on *Pgi*.

In the present study, we report a pervasive relationship between *Pgi* genotype and HSP production. The HSP induction experiment revealed that the 1–1 genotype induces HSP production and reaches a peak at lower temperatures than the other genotypes. In contrast, the experiment with laboratory overwintered beetles revealed that differences among *Pgi* genotypes did not vary among source populations. These results are consistent with our previous report of an effect of *Pgi* genotype on HSP production for field-collected beetles (Dahlhoff and Rank 2000). However, they also indicate that differences among genotypes depend strongly on exposure temperature, recent thermal history, and gender. Of note are the observations that the effects of *Pgi* genotype on cold tolerance (1–1, 1–4 > 4–4) are only significant for female beetles and that HSP70 expression is higher in females than males. Elevated levels of HSP70 in females may help protect eggs from thermal stress or may simply be a consequence of higher rates of new protein synthesis in egg-producing individuals. In either case, the energetic load of egg production may make females more vulnerable than males to differences in cost of HSP70 expression among *Pgi* genotypes.

Our results suggest that a relationship may exist between HSP induction and ability to tolerate stressfully cold temperatures. Because *Pgi* genotype may affect both HSP expression and cold tolerance, adaptation to cold may occur via the observed changes in HSP induction profile. Other studies indicate that HSPs can indeed play a role in protecting insects from extreme cold while insects are metabolically active (Burton et al. 1988; Kelty and Lee 2001). The montane beetle described in the present study may represent an ideal model organism to test the hypothesis that ecologically relevant warm temperatures affect an ectotherm's ability to cope with ecologically relevant extremes in cold. The relationship between HSP production and cold tolerance may also explain the shifts toward the *Pgi*-1 allele during the cool period between 1988 and 1996.

Conclusions

Few studies have discovered directional shifts in allele frequency at allozyme loci (Johannesson et al. 1995; Schmidt and Rand 1999, 2001) or have proposed a mechanism underlying allele frequency change (Burton and Feldman 1982, 1983). Our results suggest that elevated HSP production by beetles possessing the *Pgi*-1 allele may enhance cold tolerance, and this may explain the increased frequency of the *Pgi*-1 allele during cool periods. The consequences of global warming on Sierra Nevada climate are as yet undetermined. While conditions in Bishop Creek were cooler in the 1990s, many climate models predict an increase in both temperature and precipitation in the Sierra Nevada the next several decades (Jayco and Millar 2001). Furthermore, other models predict an increase in the frequency of extreme weather events (Easterling et al. 2000). Therefore, Sierra populations of *C. aeneicollis* may experience both gradual directional shifts in climate, as well as an increase in frequency of exposure to elevated and subzero stressful temperatures. It remains to be seen whether existing genetic variation will confer on these beetles an ability to respond to predicted changes in climate or if climate change will cause them to become extinct in the Sierra Nevada.

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