Effects of temperature on physiology and reproductive success of a montane leaf beetle: implications for persistence of native populations enduring climate change

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\textbf{Running Head:} Effects of temperature on beetle physiology and fecundity

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Abstract

Understanding how climate change impacts natural systems requires investigations of effects of environmental variation on vulnerable species, and documentation of how populations respond to change. The willow beetle *Chrysomela aeneicollis* is ideal for such studies. It lives on the southern edge of its worldwide range in California’s Sierra Nevada. Beetles experience elevated air temperatures during summertime egg-laying and larval development. Exposure to these temperatures causes physiological stress, which may reduce reproductive success and endanger populations. The glycolytic enzyme *phosphoglucose isomerase* (PGI) is a marker of temperature adaptation in *C. aeneicollis*. PGI varies across a latitudinal gradient: allele 1 is common in cool, northern Rock Creek (RC) and allele 4 in warmer, southern Big Pine Creek (BPC). In populations intermediate in geography and climate (Bishop Creek- BC), PGI-4 frequency increases from north to south, such that alleles 1 and 4 are in relatively equal frequency in southern BC. Over the past decade, Sierra beetle populations have colonized high elevations and become extinct at lower elevations where once common. In BC, the magnitude of PGI allele frequency fluctuations among life history stages is related to maximal air temperature, with the frequency of PGI-4 increasing after the hottest part of summer. To identify mechanisms that may cause shifts in PGI allele frequency, we measured metabolic rate and fecundity for beetles collected from BC. Metabolic rate of males and females was measured at 20 and 36°C using flow-through respirometry. To measure laboratory fecundity, mating pairs were acclimated for 4h each afternoon at control (20°C) or mildly elevated temperatures (26, 32°C) and number of eggs laid were counted daily for 24d, after which tissue levels of 70-kD heat shock proteins (Hsp70) were determined. Previous studies had demonstrated differences in Hsp70 expression among PGI genotypes at these temperatures. To measure field fecundity, mating pairs from BC
were transplanted to similar elevations in BPC, BC, and RC and monitored *in situ* for 24d. Metabolic rate was higher for PGI 4-4 genotypes than 1-4 or 1-1 individuals at 36 but not 20°C. In contrast, laboratory fecundity was greatest for females possessing PGI-1, independent of acclimation temperature. At the end of the laboratory fecundity experiment, Hsp70 expression was positively related to fecundity, suggesting minimal reproductive cost of upregulation of Hsps in response to mild heat stress. In the field, fecundity was highest for 1-1 and 1-4 individuals in RC, 4-4 individuals in BPC, and similar for all genotypes in BC. Thus, fecundity in nature was greatest for the genotypes that were most common in each drainage. Taken together, data reported here suggest that hot, dry summers in the Sierra Nevada may result in an increase in frequency of the PGI-4 allele and shifts to higher elevations for *C. aeneicollis* populations.
Introduction

One of the many challenges posed by global climate change is that natural populations will respond unpredictably to an increasingly variable climate. In recent years, ranges of many species have shifted, with local extinction in some areas and colonization of new regions (Hill et al. 2002; Inouye et al. 2000; McLaughlin et al. 2002; Parmesan 2006; Parmesan and Yohe 2003; Sagarin et al. 1999). On the other hand, some species have shown no evident range shift or change in population size (Erasmus et al. 2002; Parmesan et al. 1999), while others have precipitously declined in both range and abundance (McLaughlin et al. 2002). Predicting which populations will be vulnerable to extinction is complicated by the fact that small changes in climate lead to large, unpredictable changes in air temperature, rainfall and persistence of snow (Crozier and Dwyer 2006; Hayhoe et al. 2004; Miller et al. 2003; Pearson and Dawson 2003; Whitham et al. 2006). The ability of a population to persist in the face of environmental change will depend on effects of abiotic stress on individual survival, physiological performance and reproductive output (Chamaille-Jammes et al. 2006; Karlsson and Wiklund 2005; Lester et al. 2007; Musolin 2007; Reed et al. 2007), on phenotypic plasticity (Garland and Kelly 2006; Ghalambor et al. 2007; Nussey et al. 2007), on interactions with other species (Bertness and Ewanchuk 2002; Brooker et al. 2008; Leonard 2000; Petes et al. 2007) and on the population’s genetic composition (Haag et al. 2005; Hanski and Saccheri 2006). Though effects of climate change on natural populations have been extensively investigated, researchers have only recently begun to identify specific features that might allow populations to persist in a changing environment (Balanya et al. 2006; Bradshaw and Holzapfel 2006; Bradshaw et al. 2004; Ellis and Post 2004; Gilman et al. 2006; Harley et al. 2006; Helmuth et al. 2002; McLaughlin et al. 2002; Pearson and Dawson 2003; Svensson et al. 2006; Tran et al. 2007; Umina et al. 2006).
Fluctuations in environmental temperature may become more extreme as a result of climate change (Diffenbaugh et al. 2005; Easterling et al. 2000). This will be especially problematic for small, free living ectotherms, because their body temperature is determined by the environment (Stevenson 1985). Large variations in body temperature may be metabolically costly, due to activation of cellular repair mechanisms after stress exposure (Anestis et al. 2007; Feder and Hofmann 1999; Hofmann and Somero 1995; Li et al. 2007). Activation of heat shock proteins (Hsps) and other repair mechanisms may elevate metabolic rate, due to increased demand for ATP at higher temperatures, or cause metabolic suppression, due to shunting of ATP away from biosynthetic and activity pathways (Hoffmann and Rinas 2001; Lesser and Kruse 2004; Sorensen et al. 2003). This may ultimately lead to reduced performance and reproductive success (Krebs and Feder 1997; Krebs and Holbrook 2001; Loeschcke et al. 1997; Sorensen and Loeschke 2002). Many ectotherms live close to the edge of their physiological tolerance in nature (Angilletta et al. 2006; Feder et al. 2000; Sorensen et al. 2003). Thus, small changes in climate may lead to rapid changes in abundance, in part due to effects of physiological stress. Prior studies have demonstrated effects of temperature variation on physiology, survival and reproductive success of small ectotherms (Boggs and Freeman 2005; Dillon et al. 2007; Hodkinson 2005; Karlsson and Wiklund 2005; McMillan et al. 2005; Rank et al. 2007). A next critical step is to investigate impacts of abiotic stress on features critical for population persistence in a natural setting where effects of climate change are rigorously documented.

Montane regions are ideal systems in which to study mechanisms by which organisms cope with a changing environment (Franklin and MacMahon 2000; Hampe and Petit 2005). In mountains, populations experience different environmental conditions over small spatial scales, resulting in distinct climatic regimes within the typical dispersal range of many organisms.
Montane organisms may be especially vulnerable to climate change (Gworek et al. 2007; Hill et al. 2002; Hodkinson 2005; Peterson 2003), because warmer temperatures may result in both increased exposure to extreme cold in early spring due to loss of protective snow cover, and hotter, drier summers (IPCC 2007). Here we describe studies of a model organism for investigating climate change in montane habitats— the willow beetle _Chrysomela aeneicollis_, which lives at high-elevation (2700-3200 meters) in the Eastern Sierra Nevada. Adults emerge from diapause in May or June to feed, mate, and lay eggs. Near the end of July, larvae pupate and shortly thereafter, new adults emerge (Rank 1994; Smiley and Rank 1986). Sierra populations are found at the southern edge of this beetle’s worldwide range, and probably endure greater extremes in environmental temperature during summertime egg-laying and larval development than conspecifics living at higher latitudes (Dahlhoff and Rank 2007; Mardulyn et al. 2008; Rank and Dahlhoff 2002).

In these populations, we have documented differences in expression levels of 70-kD heat shock proteins (Hsp70), a key indicator of physiological stress in nature (Dahlhoff 2004; Dahlhoff and Rank 2007; Feder and Krebs 1997; Sorensen et al. 2003). Hsp70 expression level varies among drainages (BPC > BC > RC), and within a drainage, Hsp70 levels decline with increasing elevation. Hsp70 expression levels vary considerably throughout the day, reaching highest levels in mid-afternoon, when beetle body temperatures routinely exceed 30°C (Dahlhoff and Rank 2000; Rank and Dahlhoff 2002). Cold stress may also be a constraint for _C. aeneicollis_ in montane habitats. Nighttime temperatures may fall below -8°C in June and July, and we have observed mortality after these sub-zero nighttime temperatures on multiple occasions (McMillan et al. 2005; Rank 1994; Smiley and Rank 1986). Upregulation of Hsps during the day may protect against freezing conditions at night (Neargarder et al. 2003; Rank and Dahlhoff 2002).
A unique aspect of Sierra Nevada beetle populations is the presence of a genetic polymorphism that shows evidence of being acted on by temperature selection. The genetic marker for this polymorphism is the allozyme *phosphoglucose isomerase* (PGI). PGI is an enzyme critical for energy metabolism, and shows evidence of being under temperature selection in diverse ectotherms (Reznick and Travis 1996; Riddoch 1993; Watt 1992). In Sierra beetle populations, PGI shows much greater differentiation among drainages than any other polymorphic enzyme locus (Dahlhoff and Rank 2000; Rank 1992). Allele 1 predominates in populations living in the northern drainage Rock Creek (RC), while allele 4 predominates in the southern drainage Big Pine Creek (BPC). Allele frequencies are intermediate in the middle drainage Bishop Creek (BC). This allele frequency gradient at PGI occurs along a temperature gradient. RC is typically coolest, BPC warmest, and BC temperatures are intermediate (Dahlhoff and Rank 2007; Mardulyn et al. 2008; Rank and Dahlhoff 2002).

Our prior work suggests that variation at the PGI locus is consistently related to traits that allow individuals to cope with elevated and sub-zero temperatures in nature. PGI allozymes differ in thermal stability (4-4 > 1-4 > 1-1) and Michaelis-Menten constant (*K*_m fructose-6P), a measure of enzyme binding effectiveness (Dahlhoff and Rank 2000). In addition, catalytic efficiency (indexed by *V*_max/ *K*_m) for the 4-4 allozyme is lower (less efficient) than the 1-1 allozyme at 10°C and 20°C, but higher (more efficient) at higher temperatures (30-40°C; EP Dahlhoff, unpublished data). Peak Hsp70 induction temperature after exposure to elevated temperatures is lower for individuals possessing the less stable form of the enzyme (PGI 1-1; maximal Hsp70 expression at 30°C) than the more stable form PGI 4-4 (maximal Hsp70 expression at 36°C) (Rank and Dahlhoff 2002). In addition, after a single heat or cold “shock”,
PGI 1-1 genotypes are more thermotolerant (measured by LT \(_{50}\) and CT \(_{\text{max}}\)) and run faster than 4-127 individuals; 1-4 heterozygotes are typically intermediate (Neargarder et al. 2003; Rank et al. 2007). However, repeated exposure over consecutive days to extreme heat or cold reverses these patterns (4-1 > 1-4 > 1-1). These data suggest that PGI allele 4 is associated with greater tolerance of repeated thermal stress than allele 1, but that allele 1 is associated with better performance and a more vigorous heat shock response after exposure to a single elevated or tolerable heat or cold stress than allele 4. These data support the hypothesis that PGI alleles 1 and 4 are under selection for temperature adaptation and that these alleles may be associated with other adaptive traits such as performance and fecundity.

We have not yet identified the mechanisms that cause the observed relationship between PGI genotype and Hsp70 expression level in \(C. aeneicollis\). One possibility is that Hsp70 molecules interact directly with PGI molecules; e.g. Hsp70 may respond to PGI allozymes that partially unfold at different temperatures. Another possibility is that PGI interacts with factors that regulate expression of Hsp70. PGI may also affect Hsp expression levels indirectly. For example, if PGI allelic variants regulate the production of glucose through glycolysis differently (Eanes 1999), then this may result in differences among genotypes in ATP production. This could in turn affect the enzymatic activity of Hsps, which require ATP to catalyze protein refolding. The fact that PGI genotypes and Hsp70 expression level are correlated with core body temperature (Dahlhoff and Rank 2007; Rank et al. 2007) is consistent with these ideas. However, repeated exposure over consecutive days to extreme heat or cold reverses these patterns (4-1 > 1-4 > 1-1).
and Hsp70 levels after exposure to mild, chronic heat stress typical of conditions in nature, and ascertain if differences among PGI genotypes in Hsp70 expression affect fecundity.

**Materials and Methods**

**Population genetics**

To examine structure of Eastern Sierra Nevada, California (USA) populations of *Chrysomela aeneicollis*, adults were collected in July 1997 from three drainages (Big Pine Creek- BPC, Bishop Creek- BC, and Rock Creek- RC). Genotype frequency data for BPC and RC populations were published previously and are included here for comparison (Dahlhoff and Rank 2000). Six sub-drainages within BC were surveyed across a similar elevation gradient, and mean allele frequency of each sub-drainage calculated. Bishop Creek sub-drainage localities, sample sizes and sampling elevation ranges are shown in Table 1.

To quantify changes in allele frequency over a single summer within BC sub-drainages, beetles were collected from 7 populations in the south fork of Bishop Creek from the Green Lake and Chocolate Lakes sub-drainages, where PGI alleles 1 and 4 are both common. Thirty-five beetles were collected from each site three times during the summer of 2001: over-wintered adults, which had just emerged from winter diapause (7-8 June), 3\textsuperscript{rd}-instar larvae (6-7 August) and newly-emerged adults (8-9 September). After collection, beetles were flash-frozen and stored at -80 °C until genotype analysis.

To measure variation in local climate within Bishop Creek, microhabitat air temperature at each site was measured every 30 min throughout the summer using TidbiT Temperature Data Loggers (Onset Computer Co., Pocaset, MA). Loggers were suspended in white plastic cups and secured onto host willow branches 1.5 m above the ground. Our prior studies demonstrated that this arrangement gave us an excellent measure of the relationship between $T_a$ and beetle $T_b$. 
(Dahlhoff and Rank 2000); the white cup functions as a shield from direct solar radiation in summer (preventing overestimates of $T_a$), and protects the logger housing from damage when loggers are buried in snow each winter. Daily mean maximum, minimum and average temperature was determined for each site from logger data using Boxcar Pro (Version 4.0, Onset Computer Co., Pocasset, MA).

**Abundance**

Adult beetle population abundance was quantified in 1998, 2003 and 2007 for 7-12 sites in each of the three main study drainages along replicate elevation transects 8-12 km in length (2700-3600 m). First surveys were initiated at snowmelt, or in late May, which ever came first. Beetles were never seen before late May, even in years of early snowmelt. Surveys were repeated every 6-10 days throughout June and early July, as long as adults were present. To survey, 2 observers hiked to each locality, identifying exact site using a handheld GPS unit (Geo Explorer, Trimble Corporation, Sunnyvale, CA). Abundance was determined by timed visual survey of approximately 25-100 m$^2$ of willow bog, stream or meadow for 10 person minutes. Each observer selected a location near willow plants and scanned for beetles in the foliage closely, while moving throughout suitable microhabitats. Number of beetles was recorded using a hand-held cell counter. Raw data were converted to scaled counts- 0: none; 1: 1-3; 2: 4-10; 3: 11-40; 4: 41-99; 5: 100-300; 6: > 300. Data reported are abundances observed the week of peak abundance, averaged over all three drainages, each year.

**Effects of temperature on metabolic rate**

Metabolic rate was measured for over-wintered adults collected in June, 2005 at sites between 3100-3200 m in the Chocolate and Green Lakes sub-drainages in BC (Bluff Lake, South Lake Pipeline, Mary Louise Creek and Bull Lake). After collection, sexes were separated to minimize
effects of mating activity on metabolic rate, and held in controlled temperature incubators at
natural diurnal light cycles (14 h day, 20°C; 10 h night 4°C) at White Mountain Research Station
(WMRS) in Bishop, CA for 7 days. Beetles were fed on fresh leaves from their favored host
plant *Salix oreastera* and given moisture by placing a piece of dampened filter paper in Petri dish.
After laboratory acclimation, beetles were transported (in a 4°C cooler) from WMRS to
University of Nevada, Las Vegas for metabolic rate measurements. Beetles were deprived of
food for 4-6 days before measurement. Individuals were randomly assigned to one of two
measurement temperatures (20, 36 °C) and weighed immediately before determination of
metabolic rate.

Metabolic rate (indexed by CO₂ production) and water loss rates were measured using
flow-through respirometry. Beetles were placed in 5-ml glass-aluminum respirometry chambers,
and dry, CO₂-free air was pumped through at 100 ml/min. Carbon dioxide and water vapor of
the air stream were measured with a Licor LI-6262 infrared gas analyzer. Beetle activity was
monitored during respirometry with AD-1 activity detectors (Sable Systems, Las Vegas, Nevada
USA). These use a near-infrared photocell to detect movements. Because metabolic rates and
water-loss rates can increase with activity, these parameters were quantified these during periods
when beetles were quiescent. Thus, measurements reflect standard metabolism at 20 or 36 ºC.
All respirometry data were collected and analyzed using Datacan V software (Sable Systems,
Las Vegas, Nevada USA). After measurement, beetles were flash-frozen and stored at -80°C
until genotype analysis.

*Effects of temperature on laboratory fecundity and Hsp70 expression level*

*Effects of acclimation temperature on fecundity and heat shock protein expression were
determined for females collected in June 2001 from Bluff Lake, located at 3200 m*
in the Green Lake sub-drainage of Bishop Creek. Beetles were transported in coolers to WMRS and sexes were separated. Beetles were acclimated to control conditions in the laboratory (14 h 20°C d, 10 h 4°C n) for 48 h before use. To start the experiment, females were randomly placed in a Petri dish with one male collected from the same site. Prior field measurements of beetle body temperature showed that most individuals experienced temperatures which induce physiological stress between 3-4 hours each day, typically in the afternoon (Dahlhoff and Rank 2000). To mimic field thermal experience, 221 male-female pairs were held at 20 ºC for 4 h in the morning (8:00 AM to 12:00 PM); at either 20, 26 or 32 ºC for 4 h in the afternoon (12:00-4:00 PM)- the “temperature treatment”; at 20 ºC for 6 h in the evening (4:00-10:00 PM); and at 4 ºC for ten hours at night (10:00 PM- 8:00 AM). Willow sprigs were removed during the temperature treatment, to minimize the potential confounding factor of heat-treating willow. Willow sprigs were replaced with freshly-collected plants every 1-2 days. Mating pairs were kept together throughout the experiment, and received the same temperature treatment. Eggs were counted twice daily, immediately before and after temperature treatment. Eggs were removed from the Petri dish and counted using a dissecting microscope. Male mating activity was routinely monitored, and males mated with females periodically throughout the experiment. After 24 days, beetles were weighed, flash-frozen and stored at -80 ºC for biochemical analysis.

**Effects of natural climate on fecundity**

Effects of climate variation on fecundity were measured for females collected in June 2002 from Bishop Creek (Bluff Lake, 3200 meters) and transplanted to similar elevation localities in Big Pine Creek (Falls Site), Rock Creek (Mosquito Flat) and Bishop Creek (Bluff Lake). Females were placed with one male in white tulle mesh bags on willow branches of replicate *Salix orestera* (N = 13 plants per drainage, 6 branches per plant). Egg clutches were removed from
mesh bags twice a day, placed in plastic cups, returned to the laboratory at WMRS, and egg number in each clutch counted using methods described above. As in the laboratory experiment, male mating activity was monitored, and males mated with females throughout the experiment.

At the end of the experiment, all beetles (and mesh bags) were removed from field sites, and beetles weighed, flash-frozen and stored at -80 °C for biochemical analysis.

**Biochemical analyses**

Genotypes at three allozyme loci, *phosphoglucose isomerase* (PGI; E.C. 5.3.1.9), *isocitrate dehydrogenase* (IDH; E.C. 1.1.1.42) and *phosphoglucomutase* (PGM; E.C. 5.4.2.2) were determined by starch gel electrophoreses using established protocols (Murphy et al. 1996; Rank 1992). Expression levels of a 70 kD heat shock protein (Hsp70) were determined for thorax tissue of females in laboratory and field fecundity experiments by Western blot analysis following published methods (Rank et al. 2007).

**Statistical Analysis**

All statistical analyses were performed in JMP IN 5.1 (SAS Institute Inc., Cary, NC). For all experiments, analyses were initially run for all three polymorphic loci scored. However, as has been observed in earlier studies, only PGI genotype shows any significant effects in characters relevant to temperature adaptation. Analyses of other loci are therefore reported elsewhere (Bruce 2005; Fearnley 2003).

*Population structure of the Eastern Sierra Nevada*- We used one-way ANOVA (main effect-sub-drainage) to test for differences in PGI allele frequency among populations sampled in the 6 BC sub-drainages.

*Effects of temperature on PGI allele frequency variation*- We analyzed changes in allele frequency using polytomous logistic regression with PGI frequency (per individual) as
dependent variable, and life stage (over-wintered adult, 3\(^{rd}\)-instar larvae, new adult), BC sub-
drainage (GL or CL), and population nested in sub-drainage as independent variables. Allele
frequency was determined as the frequency of the PGI-1 allele (0 for PGI 4-4 homozygotes, 0.5
for 1-4 heterozygotes, and 1 for 1-1 homozygotes). Polytomous logistic regression was the most
appropriate approach because the dependent variable could only take three values, and could not
be analyzed with parametric statistics using continuous dependent variables. The main
assumption of this logistic regression was that the error variable conforms to a multinomial
distribution, which is considerably less restrictive than the assumption, for continuous variables,
that the error variable is normally distributed (Trexler and Travis 1993).

To determine how the environment relates to PGI frequency change, we first calculated
allele frequency at the population level. We then calculated the selection coefficient ($s$) for the 2
intervals between the 3 collections (over-wintered adults to larvae, larvae to newly emerged
adults) following standard methods for calculating relative fitness and selection coefficients from
longitudinal genotype frequency data (Hartl and Clark 1997). If $s$ was positive, then genotype 1-
1 was favored, if it was negative, then genotype 4-4 was favored. To determine which measure
of temperature predicted the magnitude of the selection coefficient, regression models using
mean minimum, mean average and mean maximum temperatures were compared, and the
variable with the highest $r$ square was selected (detailed in Fearnley (2003).

**Metabolic rate.** Metabolic rates and water loss rates were analyzed using ANCOVA, with PGI
genotype and treatment temperature as main effects and the PGI genotype by temperature
interaction. Body mass was used as a covariate, rather than determining mass-specific metabolic
rate, following recommendations of Packard and Boardman (1999). Five beetles measured at 20
\(^{\circ}\)C had negligible (< 0.02 \(\mu\)l/hr) water loss rates and were removed from analysis. Each beetle
was measured at either 20 or 36°C. \(Q_{10}\) was calculated from resulting mean metabolic rates for each genotype and temperature (Willmer et al. 2004), so no further statistical analyses were possible.

_Fecundity experiments._ Results of the laboratory fecundity experiment were analyzed using repeated-measures ANCOVA, with acclimation temperature, genotype, and the interaction term as categorical fixed effects, and total number of eggs laid over every 4 days as response variables (day 4, 8, 12, 16, 20, 24). Body mass, number of days to first egg clutch, and Hsp70 expression level were included as covariates. Two females that did not oviposit during the first 10 days of the experiment were excluded from analysis. Effects of acclimation temperature and PGI genotype on Hsp70 expression level were determined in a separate analysis using ANOVA. Results of field fecundity were analyzed using ANOVA, with transplant drainage and PGI genotype as main effects. Unlike laboratory fecundity, Hsp70 expression level was not a significant covariate in a preliminary ANCOVA, so it was not included in the final analysis.

**Results**

**Geographic variation in PGI allele frequency**

The frequency of PGI allele 1 varies with latitude among drainages (Fig 1; BPC < BC < RC), and the frequency of PGI allele 4 declines as the frequency of allele 1 rises. This north to south increase in frequency of PGI allele 4 is found among the 6 sub-drainages surveyed in Bishop Creek (One-way ANOVA; \(F_{5,35} = 17.0, P < 0.001\)). The frequency of PGI allele 1 in North Bishop Creek is similar to its frequency in Rock Creek, and is greater than its frequency in South Bishop Creek (Fig 1B). Within South Bishop Creek, PGI-1 is most common in the Tyee Lakes, which are in close proximity to North Bishop Creek, and PGI-4 is most common in Green Lake populations, which are closest to Big Pine Creek (Fig 1B).
Allele frequency change among beetle life stages

The frequency of PGI allele 1 in South Bishop Creek populations was 0.67 for over-wintered adults collected early in the summer \((n = 245)\), and it increased by 9.7\% (to 0.74) in the 2\textsuperscript{nd} instar larvae collected 60 days later \((n = 248)\). However, the frequency of PGI allele 1 declined again by 11\% (to 0.66) 30 days later when new adults were collected \((n = 245)\). PGI allele frequencies were significantly different among life history stages \((G = 7.0, \text{df} = 2, P = 0.03)\), as was variation among sub-drainages \((G = 16.2, \text{df} = 5, P = 0.006)\) and populations within a sub-drainage \((G = 6.3, \text{df} = 1, P = 0.012)\). In contrast, there were no changes in allele frequency at IDH-2 or PGM among life history stages, sub-drainage, or population within sub-drainage (Table S1).

The magnitude of directional changes in PGI frequency was related to mean daily maximum temperature (Fig 2). During the first part of summer, the increase in frequency of PGI allele 1 was related to mean maximum air temperature. In contrast, during the second part of summer, a decrease in frequency of allele 1, with a concomitant increase in frequency of allele 4, was related to mean maximum air temperature. Mean maximal air temperatures were significantly higher in the second part of summer, when larvae were developing into new adults and allele 4 was becoming more frequent (matched pairs t-test; \(t = 3.9, \text{df} = 4, P = 0.009)\).

Preliminary regression models showed that site elevation and mean minimum temperature were not related to the selection coefficient.

Temporal and spatial changes in beetle abundance

Populations of *C. aeneicollis* have shifted in elevation range and abundance during the past nine years. In 1998, after a late snowmelt and several years of above average winter precipitation, beetles were most abundant at the lower portion of their elevation range (Table 2). Beetle abundance increased by 55\% between 1998 and 2003 and stayed at these peak abundance levels
from 2003-2006 (data not shown), which coincided with several winters of greater than average
snow pack. However, even during these relatively wet years, summertime air temperatures were
warmer at low elevations and beetle populations declined or disappeared at those sites, while
increasing in abundance at high elevation sites. After the unusually dry winter of 2006-07,
typically wet meadows and pond sites were hot and dry by early June; beetle abundance
decreased by 44% and shifted to higher elevations (Table 2, elevation by year interaction, $F_{2,80} =$
3.8, $P = 0.026$). By the end of the summer in 2007, populations had disappeared entirely
throughout most of their previous elevation range in Big Pine Creek and Rock Creek, but were
persistent at low population size in most localities in Bishop Creek.

**Relationship between PGI genotype and metabolic rate**

Standard metabolic rate depended on measurement temperature and PGI genotype (Fig 3;
temperature: $F_{1,109} = 44.6$, $P < 0.0001$; temperature by PGI genotype interaction: $F_{2,109} = 3.0$, $P <$
0.05). Metabolic rate did not differ among genotypes at a moderate temperature (20 °C), but at
36 °C, near maximal body temperature in nature, metabolic rate of PGI 4-4 individuals was 45%
greater than PGI 1-1 or 1-4 individuals. The $Q_{10}$ of metabolic rate varied two-fold among
genotypes. $Q_{10}$ for PGI 4-4 genotypes was 3.24, compared to 1.54 for PGI 1-4 and 1.66 for PGI
1-1 genotypes. All individuals lost water throughout the experiment, with larger beetles losing
more water ($F_{1,109} = 13.2$, $P < 0.0005$). Water loss rates were greater at 36 °C (1.73 ± 1.7) than
at 20 °C (0.33 ± 0.1) ($F_{1,109} = 119$, $P < 0.0001$) and did not vary among allozyme genotypes.

**Fecundity in the laboratory**

Throughout the 24-day fecundity experiment, PGI 4-4 females laid fewer eggs than PGI 1-1 or
PGI 1-4 females, and the rate of egg production of PGI 4-4 females declined more rapidly than
other PGI genotypes (Fig 4A, Table S2). Egg production was positively related to female body
mass and negatively related to number of days before the first eggs were laid (Table S2, between-subjects factors). The relationships between these variables and egg production varied over the course of the experiment (Table S2, within- by between-subjects factors interaction terms). Female Hsp70 expression levels, which were measured after 24 days of laboratory acclimation and egg laying, were positively correlated with total egg production (Fig 4B).

Differences among PGI genotypes in total egg production were most pronounced for females held under control conditions for the entire experiment (Fig 5A). PGI 1-1 individuals held under control conditions each afternoon (20°C) laid more eggs than 1-4 or 4-4 control females. PGI 4-4 females held at 32°C each afternoon tended to lay more eggs than those held at 20 or 26°C, though differences in fecundity were not significant. Female Hsp70 expression levels measured at the end of the experiment were positively related to acclimation temperature \(F_{2,116} = 6.2, P = 0.003\) and tended to be highest at 32°C for 4-4 females (Fig. 5B).

Fecundity in nature

In nature, differences among PGI genotypes (but not other allozyme loci) in egg production among Bishop Creek females depended on the drainage into which those females were transplanted (Fig 6, genotype by drainage interaction \(F_{4,165} = 2.8, P < 0.029\)). In Rock Creek, PGI 1-1 and 1-4 females laid 32% more eggs than PGI 4-4 females, while PGI 4-4 females laid 54% more eggs than the other two genotypes in Big Pine Creek. In Bishop Creek, fecundity was high for all genotypes.

Discussion

Environmental physiologists have long predicted that components of reproductive success depend partly on an organism’s physiological response to temperature. Here and elsewhere we have demonstrated that in *Chrysomela aeneicollis*, variation at the allozyme locus PGI relates to
physiological characters such as Hsp70 expression level, thermal tolerance, running speed and metabolic rate. Observed relationships between PGI genotype and fecundity in the field and laboratory reported here suggest that these physiological responses to temperature result in differential reproductive success among PGI genotypes. The finding that PGI frequencies fluctuate within a single generation shows that differences in reproductive success and survival, both critical components of population persistence, may result in rapid changes in genetic composition of natural populations. Finally, changes in *C. aeneicollis* abundance over 10 years (1998-2007) demonstrate that the range of this native insect has shifted upwards in elevation, concomitant with climate change in the Sierra Nevada. Together with results of the field fecundity experiments, distribution and abundance data also suggest that beetles living in Bishop Creek may be more tolerant of environmental change than those living in Big Pine or Rock Creek. Taken together, these data suggest that *C. aeneicollis* populations possess sufficient genetic variability to respond evolutionarily to a challenging thermal environment, but current conditions are causing local extinction at warmer, drier sites and at lower elevations.

**Geographic variation in PGI frequency** *Chrysomela aeneicollis* populations living in different drainages have distinct responses to seasonal fluctuations in environmental temperature and precipitation. While these unique responses may be the result of acclimatization or adaptation to local conditions, they may also be affected by genetic differences among populations. Findings presented here reveal how geographic variation at PGI is structured among populations within Bishop Creek, which lies between Big Pine and Rock Creek. In general, populations in South Bishop Creek are more genetically similar to populations in Big Pine Creek (high frequency of PGI allele 4), while those in North Bishop Creek are more similar to populations in Rock Creek (high frequency of PGI allele 1). Within South Bishop Creek, PGI allele 4 frequencies are
greatest in populations that are near Big Pine Creek (Green and Chocolate Lakes). One explanation for this pattern is that South Bishop Creek represents a zone of contact between two genetically distinct lineages of *C. aeneicollis*—one from the south and another from the north. Studies of variation at mitochondrial DNA loci (COI and COII) are consistent with this hypothesis (Fearnley 2003; Mardulyn et al. 2008). Bishop Creek populations may ultimately harbor more genetic diversity than other drainages, and may thus be more resilient to impending changes in climate.

Seasonal changes in PGI frequency and relationship to environmental temperature—The current distribution of alleles in South Bishop Creek probably results from a balance between historical migration (discussed above) and natural selection. Previous studies have shown that allele frequencies shifted towards PGI allele 1 in South Bishop Creek after several years of cool, wet conditions (Rank and Dahlhoff 2002). In the present study, the frequency of PGI allele 1 increased during early summer 2001 (over-wintered adults to larvae), whereas PGI allele 4 increased during mid to late summer 2001 (larvae to new adults). These findings suggest that reproductive success of South Bishop Creek adults possessing PGI allele 1 was greater than those possessing allele 4, but that survival of larvae possessing allele 4 was greater than those possessing allele 1. Because each shift was related to maximum air temperature, populations that shifted most towards allele 1 in early summer tended to experience the greatest shift back towards allele 4 subsequently. One possible explanation for this pattern consistent with physiological data (Dahlhoff and Rank 2007; Rank et al. 2007) is that PGI allele 1 was favored during the moderately warm conditions that prevailed when over-wintered adults were reproducing, but that PGI allele 4 was favored when maximum air temperatures were significantly hotter in the latter part of the summer. Studies of other organisms also suggest that
alleles favored at one life-history stage can be disadvantageous at another, in part due to changes in environmental conditions (Johannesson 2003; Johannesson et al. 1995). Thus, fluctuating selection on enzyme loci, such as is apparent in *C. aeneicollis* populations, may occur more often than is generally reported (Mitton 1997; Ward et al. 2004). Future studies are planned that will allow us to determine if fluctuating selection is operating at PGI, and if it is an important component of population persistence in Sierra willow beetle populations.

**Relationship between metabolic rate and variation at PGI** - The relationship between PGI variation and metabolic rate suggests that individuals possessing the PGI 1 allele are less sensitive to thermal variation (have a lower Q\(_{10}\)) than 4-4 individuals, but that 4-4 individuals have the highest metabolic rates (consistent with enhanced performance) at temperatures near the maximum measured in nature (36°C). This pattern is atypical, as populations or species with high Q\(_{10}\) values for metabolic rate are often most thermally sensitive, or cold-acclimated (Berrigan and Patridge 1997; Chown and Nicolson 2004; Nielsen et al. 1999). However, the findings reported here are not unique, as elevated metabolic rate after acclimation to warm temperatures has been reported in another species of cold-temperate beetle (Terblanche et al. 2005). One possible explanation for patterns in metabolic rate reported here is that genotypes responded differently to experimental starvation and subsequent heat stress. Beetles were held at 20°C and fed *ad libitum* on willow after collection, but were fasted 4-6 d prior to metabolic rate measurements, to reduce confounding effects of feeding variation on metabolic rate (Willmer et al. 2004). Our prior studies, as well as laboratory fecundity data reported here, suggest that 4-4 genotypes are less active (e.g. they run slower, lay fewer eggs) when held at 20°C than individuals possessing allele 1. These data suggest that 4-4 individuals have lower metabolic demands at moderate temperatures; thus, they may have had more energetic reserves to maintain
elevated metabolic rate when challenged with a stressful temperature (36°C) than other genotypes. PGI clearly plays a significant role in insect glucose metabolism (Haag et al. 2005; Staples and Suarez 1997; Watt et al. 1985). Whether functional differences among PGI allozymes reported in earlier studies (Dahlhoff and Rank 2000, 2007) are the ultimate cause of differences in metabolic activity among PGI genotypes in *C. aeneicollis* is not known, but is the focus of current and future studies.

**Laboratory fecundity and the heat shock response** - In the laboratory, fecundity of females held at moderate temperatures (20 and 26°C) was greatest for PGI 1-1 and PGI 1-4 genotypes, and reduced for PGI 4-4 genotypes. This experiment was conducted over the range of acclimation temperatures expected to produce differences among PGI genotypes in Hsp70 expression level, and as predicted, PGI 4-4 individuals had higher Hsp70 levels in thorax at the highest acclimation temperature (32°C). However, there was no overall decline in fecundity with increased temperature, and we found that fecundity was positively related to thorax Hsp70 expression, regardless of acclimation temperature. This was initially surprising, as most studies have suggested that upregulation of the heat shock response ultimately results in a performance or fitness cost (Krebs and Feder 1998; Krebs and Holbrook 2001; Pedersen et al. 2005; Sorensen et al. 2003). However, the Hsp70 expression levels we report here were lower than those for beetles that were subjected to acute stress exposures in nature or in the laboratory. In 1998, rapid snowmelt followed by weeks of hot, sunny weather in the Eastern Sierra Nevada led to *in situ* Hsp70 expression levels 3-4 fold greater than those reported here (Dahlhoff and Rank 2000), as did laboratory measurements of differences in CT$_{max}$ among PGI genotypes (Neargarder et al. 2003). These lower Hsp70 expression levels, along with a positive relationship between thorax Hsp70 expression level and egg production, suggest that we may have observed up-regulation of
non stress-inducible (cognate) forms of Hsp70. If this is the case, it is possible that we detected isoforms of Hsp70 specifically upregulated to protect cellular machinery critical for activities associated with reproduction, as has been reported in other systems (Kellermann et al. 2007; Sambucetti et al. 2005).

Fecundity in nature and implications for population persistence - Differences in fecundity among PGI genotypes in nature corresponded to the natural distribution of PGI alleles in the three main study drainages. This intriguing result provides evidence that the current distribution of allele frequencies results, at least in part, from natural selection. In the warm, southern drainage Big Pine Creek, where PGI allele 4 is most common, 4-4 genotypes had the highest fecundity, while in the cooler northern drainage Rock Creek, fecundity was greater for females possessing PGI allele 1, the most common allele occurring there. Fecundity of all three genotypes was high in Bishop Creek. Most reciprocal transplant studies in terrestrial systems have been conducted using plants (Brady et al. 2005; Espeland and Rice 2007; Sambatti and Rice 2006). These findings suggest that terrestrial animal populations can become rapidly acclimatized to local conditions as well.

These data also have implications for persistence of beetle populations in the Eastern Sierra Nevada. Bishop Creek individuals homozygous for PGI allele 4 lay more eggs where it is warmest, appear to survive best during the warmest part of the growing season, and have higher metabolic rates at 36°C, the highest body temperatures observed in nature. In contrast, individuals homozygous for allele 1 lay the most eggs overall under mild (20, 26°C) or moderately stressful (32°C) laboratory conditions, have highest fecundity in the coolest drainage, appear to survive and reproduce best in the cooler part of the growing season, and have metabolic rates that are less temperature sensitive than PGI 4-4 homozygotes. Heterozygotes are
intermediate in thermal tolerance of metabolism and fecundity. Bishop Creek populations may be able to tolerate a variable climate more readily than other populations, due to a broader thermal tolerance range, indexed by the PGI polymorphism.

Conclusions- These data begin to close gaps in our knowledge about the relationship between population persistence and species distribution and the genetic properties of organisms that live in stressful environments on the edge of their biogeographic range. Our studies show that a genetic polymorphism in a native species is associated with traits that confer temperature adaptation, and that frequencies of the most common alleles at this polymorphic locus fluctuate in the short and medium term. These fluctuations also relate to differences in thermal microclimate. Nevertheless, we still know little about the population-scale consequences of genetic variation at PGI, and this gap hinders our ability to predict how populations will respond to increasingly variable thermal conditions predicted by most current models of climate change. We are continuing to document changes in these populations, to preserve a long-term dataset, gaining insight into effects of climate change on natural populations. Future studies will directly relate population dynamics to the level of physiological stress and genetic composition of C. aeneicollis populations, to address these important questions.
Acknowledgements

We sincerely thank the two anonymous reviewers whose insightful and detailed comments significantly improved this manuscript. We gratefully acknowledge J. Zatorski, P. Kudoo, B. Becker, C. Dick, J. Freeo, T. Goodwin, S. Hurley, M. McCarthy and K. Mulkey for hiking many miles to locate beetle populations. We also thank C. Bayless, B. Butzman, E. Strode, J. Hollister and A. Keil for same, as well as for their countless hours spent measuring fecundity and mating success in the laboratory and field. Thanks to D. Hollis, for his assistance in obtaining genotypes for beetles in the metabolic rate experiment. We thank the director and staff of the White Mountain Research Station for providing laboratory and housing facilities. We are especially grateful to M. Elekonich of University of Nevada, Las Vegas, for allowing A. Gibbs and E. Dahlhoff the use of her laboratory to measure beetle metabolic rates. This research was supported by National Science Foundation award (IBN-RUI-0078464/0078659) to E. Dahlhoff and N. Rank, by a SCU Presidential Research grant to E. Dahlhoff, by an SCU Undergraduate Research Mentorship award, which supported M. McCarthy and C. Dick, by a mini-grant from WMRS for housing support of D. Bruce and S. Fearnley, and a Sonoma State University Undergraduate Research Grant to D. Hollis.
### Table 1. Population genetics sample sizes and sampling localities.

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*These data published previously (Dahlhoff and Rank 2000).
Table 2. Persistence and abundance of Eastern Sierra beetle populations. Data are relative abundance values of populations surveyed at peak early summer adult abundance in 1998, 2003 and 2007. Size of population determined by standardized visual survey of 25-100 m² areas of 7-12 populations per drainage and is indicated on the following scale: 0: none; 1: 1-3; 2: 4-10; 3: 11-40; 4: 41-99; 5: 100-300; 6: > 300

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Literature Cited


Nielsen, M.G., G.W. Elmes and V.E. Kipyatkov. 1999. Respiratory Q_{10} varies between populations of two species of Myrmica ants according to the latitude of their sites. Journal of Insect Physiology 45: 559-564.


Figure Legends

Fig 1. A- Location of Eastern Sierra Nevada, California populations of the willow leaf beetle *Chrysomela aeneicollis*, including detailed location of Bishop Creek sub-populations (far right panel). B- Allele frequency variation at the glycolytic enzyme locus *phosphoglucone isomerase* (PGI) along a north-south latitudinal gradient. Data shown are least squares means (± SE) of PGI allele 1 frequencies for 2-8 populations per site; BC sub-populations are shown as asterisks on far right panel. RC- open bar, Northern BC- wide striped bars, Southern BC- fine striped bars, BPC- filled bar. Sample sizes, elevation ranges and abbreviations for BC sub-drainages given in Table 1.

Fig 2. Effects of environmental temperature on PGI selection coefficient (s) in Bishop Creek. Data are selection coefficients for the frequency of PGI allele 1 between over-wintered adults and larvae (A) and larvae and new adults (B) at sites in GL (triangles) and CL (circles) regressed against mean maximal air temperature at each site when each life stage was present (A: $y = 0.20x - 4.39$, $R^2 = 0.64$, $F = 9.0$, $P = 0.03$; B: $y = -0.15x + 3.57$, $R^2 = 0.77$, $F = 17.0$, $P = 0.01$). A positive number indicates a positive selection coefficient for PGI allele 1.

Fig 3. Differences among PGI genotypes in effect of temperature on metabolic rate. Data are least squares means (± SE) of standard metabolic rate (indexed by rate of CO$_2$ production) for PGI 1-1 (open bars), 1-4 (striped bars), and 4-4 (filled bars). Subsequent figures follow same fill pattern for PGI genotypes. Beetles collected from GL and CL in BC (20ºC: $n = 23, 30, 3$; 36ºC: $n = 31, 24, 7$). Additional statistical analyses reported in text.

Fig 4. Differences among PGI genotypes in number of eggs produced by Bishop Creek females in the laboratory (A) and relationship between Hsp70 expression level at end of experiment and fecundity (B). Data shown for Panel A are means of total number of eggs laid.
per day for PGI 1-1 ($n = 63$), 1-4 ($n = 57$), and 4-4 ($n = 14$) females. Statistical analysis shown in Table S2.

**Fig 5. Effects of PGI genotype and acclimation temperature on egg production and Hsp70 expression level for Bishop Creek females.** Data are least square means ($\pm$SE) of total number of eggs produced (A) and Hsp70 expression level (B) after 24 days of laboratory acclimation to different temperatures. Sample sizes are as follows (1-1, 1-4, 4-4): 20 °C, $n = 19, 18, 4$; 26 °C, $n = 15, 18, 5$; 32 °C, $n = 25, 17, 5$. Additional statistical analysis described in text.

**Fig 6. Differences among PGI genotypes in fecundity of Bishop Creek females transplanted to drainages differing in local climate.** Data are least square means ($\pm$SE) of total number of eggs produced in nature. Sample sizes are as follows (1-1, 1-4, 4-4): RC, $n = 34, 18, 9$; BC, $n = 21, 34, 6$; BPC, $n = 25, 23, 4$. Additional statistical analysis described in text.
Table S1. Genotype frequencies for three loci (IDH-1, PGI-1, PGM-4) for three life stages sampled in Chocolate and Green Lake sub-populations in the Bishop Creek drainage.

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Table S2. Multivariate analysis of covariance showing effect of laboratory acclimation temperature (20, 26 or 32 °C) and PGI genotype on fecundity of BC females. Acclimation temperature and PGI genotype were treated as main effects, with body mass, days to first clutch, and Hsp70 expression level of each female as covariates.

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<td>1.1</td>
</tr>
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<td>T* M</td>
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<td>9.7**</td>
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<td>18.5***</td>
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<tr>
<td>Error</td>
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***P < 0.0001, **P < 0.01, *P = 0.05
Figure 1

A

B

Mean PGI allele 1 frequency

Site

RC  PP  BL  LS  TL  CL  GL  BPC
Figure 2

(A) Positive correlation between selection coefficient and mean maximum temperature.

(B) Negative correlation between selection coefficient and mean maximum temperature.
Figure 3

![Graph showing resting metabolic rate (ml CO2/h) at different temperatures (20°C and 36°C) for three groups (1-1, 1-4, 4-4). The graph includes error bars indicating variability.]
Figure 5

(A) Fecundity (total number of eggs) across different acclimation temperatures and treatments. 
(B) Hsp70 expression level (ng/g) across different acclimation temperatures and treatments.

Acclimation temperature, °C