The role of stress proteins in responses of a montane willow leaf beetle to environmental temperature variation

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The heat shock response is a critical mechanism by which organisms buffer effects of variable and unpredictable environmental temperatures. Upregulation of heat shock proteins (Hsps) increases survival after exposure to stressful conditions in nature, although benefits of Hsp expression are often balanced by costs to growth and reproductive success. Hsp-assisted folding of variant polypeptides may prevent development of unfit phenotypes; thus, some differences in Hsp expression among natural populations of ectotherms may be due to interactions between enzyme variants (allozymes) and Hsps. In the Sierra willow leaf beetle *Chrysomela aeneicollis*, which lives in highly variable thermal habitats at the southern edge of their range in the Eastern Sierra Nevada, California, allele frequencies at the enzyme locus *phosphoglucose isomerase* (PGI) vary across a climatic latitudinal gradient. PGI allozymes differ in kinetic properties, and expression of a 70 kDa Hsp differs between populations, along elevation gradients, and among PGI genotypes. Differences in Hsp70 expression among PGI genotypes correspond to differences in thermal tolerance and traits important for reproductive success, such as running speed, survival and fecundity. Thus, differential Hsp expression among genotypes may allow functionally important genetic variation to persist, allowing populations to respond effectively to environmental change.

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1. Introduction

Many organisms live in variable thermal environments, which pose substantial challenges to survival and reproduction. In response to environmental temperature variation, organisms must adapt, disperse to more favourable localities, or face extinction. Understanding mechanisms by which animals respond to environmental variation has taken on new urgency, due to increasing effects of climate change on natural systems. There are now many documented examples of shifts in species' ranges, gene frequencies, changes in mating and migratory behaviour, and local extinctions of critical species in response to climate change (Inouye *et al* 2000; Hill *et al* 2002; Rank and Dahlhoff 2002; Walther *et al* 2002; Barnosky *et al* 2003; Parmesan and Yohe 2003; Balanya *et al* 2006; Harley *et al* 2006). While the mechanisms underlying these responses to changes in natural systems are complex, effects of temperature on the physiology of ectothermic animals has been implicated as a pervasive cause (Clarke 2003; Bradshaw *et al* 2004).

Temperature affects nearly all biological processes, including the structure of proteins and biological membranes and rates of biochemical and physiological reactions (Hazel 1995; Somero 1995; Willmer *et al* 2004). Free-living ectotherms are particularly susceptible to detrimental effects of environmental temperature variation, as their body temperature is determined to a large extent by environmental temperature (Angilletta *et al* 2002; Helmuth 1999; Helmuth 2002). Effective physiological responses to temperature are especially important for ectotherms with highly variable

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body temperatures, such as sessile invertebrates living in the marine rocky intertidal region and terrestrial insects. Many of these species live close to their thermal tolerance limits, such that small changes in environmental conditions may lead to large changes in species distribution and abundance (Sagarin *et al* 1999; Tomanek and Somero 1999; Chown 2001; Denny *et al* 2006). An important unresolved question is whether these species, which are most vulnerable to environmental change, possess sufficient genetic variation to evolve in response to it (Cavicchi *et al* 1995; Hoffmann *et al* 2003; Duffy and Stachowicz 2006; Hill *et al* 2006; Jump *et al* 2006).

Current research investigating effects of temperature on species distribution, abundance, survival and fitness characters have demonstrated that the heat shock response is a critical avenue by which animals tolerate variable environmental temperatures (Feder and Hofmann 1999; Sorensen *et al* 2003; Dahlhoff 2004; Sorensen and Loeschcke 2004; Hofmann 2005; Tomanek 2005). To understand the importance of heat shock protein (Hsp) expression for animals in nature, it is necessary to describe how an animal experiences environmental temperature, measure production of stress-inducible Hsps in response to environmentally relevant temperature variation, and assess the probable physiological, ecological or fitness consequences for Hsp expression in natural populations of ectotherms. Here we first briefly discuss the importance of accurate determination of body temperatures for animals in their natural habitat and describe several classic studies that demonstrate the importance of the heat shock response for animals in nature. We then describe recent results from the Sierra willow beetle *Chrysomela aeneicollis*, in which differences in Hsp expression are related to genetic variation in a metabolic enzyme locus, *phosphoglucose isomerase* (PGI), leading to differences in thermal tolerance, locomotory performance, survival and fecundity among PGI genotypes.

2. How do we determine the body temperature of ectotherms?

Many studies of ectothermic organisms measure environmental temperature (T_E) as an index of animal body temperature (T_B), since metabolic heat production is negligible and most heat used to do metabolic work comes from the environment. It is critical to accurately predict T_B, as body temperature, not environmental temperature, drives changes in physiological process and affects structure of biochemical molecules. The relationship between T_E and T_B depends on a number of factors (Helmuth 1999; Helmuth 2002; Helmuth *et al* 2005). First, it is strongly influenced by the amount of heat gained and maintained from the environment. This includes visible and infrared radiation from, and to, the sky and the ground, conduction to and from the ground or substratum, heat convected from the animal to the surrounding air, and evaporative water loss (Willmer *et al* 2004). Second, the color and shape of an organism will determine the rate and degree of heat transfer from environment to organism, such that two organisms of different shape or color in an otherwise identical habitat may have very different T_Bs (Forsman *et al* 2002; Hazel 2002; Fitzhenry *et al* 2004). Third, the size of an organism will affect both the rate at which it gains and loses heat and its ultimate thermal capacity. Small animals heat and cool faster, due to higher surface area to volume ratios, and large animals tend to heat to higher temperatures than small animals exposed to the same conditions (Helmuth 2002; Gilchrist and Huey 2003; Kingsolver *et al* 2004; Willmer *et al* 2004). For many animals, and especially for many species of insects, life stages differ in size, shape or color, so that the thermal life of adults may be quite distinct from that of its eggs or larvae, even if they are living in the exact same location (Brown 1956; de Jong *et al* 1996; Kingsolver and Huey 1998; Kingsolver and Wiernasz 1991; Nielsen and Watt 1998; Ellers and Boggs 2004; Price 2006). Fourth, the devices used to measure temperature, such as computerized temperature loggers that have gained prominence of late, are often a different size and shape than the animals they are trying to mimic, sometimes resulting in large errors in estimating T_B from T_E (Helmuth 1999; Helmuth and Hofmann 2001; Fitzhenry *et al* 2004). Finally, many animals have the capacity to regulate body temperature behaviorally, and thus may avoid environmental extremes recorded using stationary loggers (Kingsolver and Huey 1998; Kingsolver *et al* 2004; Willmer *et al* 2004). Therefore, to predict the relationship between T_E and T_B, one must measure T_B directly, or closely match thermal properties of environmental measuring devices with the thermal properties of the organism(s) of interest (Kingsolver 1979; Huey 1991; Helmuth 1999).

3. The heat shock response in nature

The heat shock response is critical for organisms living in a variable environment. Accumulation of denatured or partially unfolded cellular proteins due to exposure to temperature extremes or other physiological stress results in the preferential upregulation of heat-shock proteins (Hsps), which re-fold these proteins into their native, functional state or target them for degradation (Lindquist 1986, Parsell and Lindquist 1993). Upregulation of Hsps may enhance survival after stress exposure by rescuing critical metabolic enzymes from destruction, and Hsps are clearly a key component in the acquisition of thermotolerance (Krebs and Bettencourt 1999; Bettencourt *et al* 2002; Feder *et al* 2002; Garbuz *et al* 2003; Sorensen *et al* 2003). However, stress-inducible Hsp expression consumes a large amount of cellular energy,

and competes with regular metabolic processes; thus, Hsp expression may impose a fitness cost on individuals that regularly experience environmental stress (Krebs and Feder 1997; Loeschcke *et al* 1997; Krebs and Feder 1998; Krebs and Holbrook 2001; Robertson 2004). In addition, Hsp-assisted folding of mutant polypeptides may buffer organisms from developmental abnormalities resulting from exposure to environmental stress (Rutherford and Lindquist 1998; Roberts and Feder 1999; Rutherford 2003). Hsps may therefore be critical for buffering the consequences of genetic variation in an unpredictable habitat.

Differential expression of heat shock proteins may limit the distribution and abundance of ectotherms along steep thermal gradients in nature (Roberts *et al* 1997; Dahlhoff *et al* 2001; Dahlhoff 2004; Hofmann 2004; Hofmann 2005; Sorte and Hofmann 2005). For example, snails in the genus *Tegula* live in wave swept rocky intertidal habitats and experience a wide range of temperatures throughout the day with the ebb and flow of tides, which varies depending on the tidal and latitudinal distribution of each species (Watanabe 1984). *Tegula funebralis* is found in the mid-intertidal zone and experiences higher and more variable body temperatures (determined by thermally-matched snail model loggers) than its low intertidal sister species *T. brunnea* (Tomanek and Somero 1999). Thermal tolerance is greater in *T. funebralis* than *T. brunnea*, and this directly corresponds to the onset of expression of stress-inducible isoforms of Hsp70, the peak of Hsp70 expression, and the subsequent shutdown of Hsp70 expression, which for both species is at temperatures just below the lethal thermal limit, LT_{50}. Furthermore, acclimation to common garden conditions does not eliminate species difference in Hsp induction profiles (Tomanek and Somero 1999, 2002, Tomanek 2002, 2005). An important finding of these studies is that the temperature at which Hsp70 expression was upregulated for each species corresponded to temperatures that it routinely experiences in nature, whereas the lethal thermal limit (where most cellular processes, including Hsp70 synthesis, shuts down) was higher than temperatures normally experienced in nature. Thus, while the heat shock response is correlated to thermal tolerance, it is a much more sensitive and ecologically-relevant indicator of sub-lethal thermal stress, and is thus important in setting limits to distribution of species or populations along environmental temperature gradients.

In many habitats like the rocky intertidal, animals may experience a higher degree of thermal heterogeneity in T_B over small spatial or temporal scales, due to the processes discussed earlier, than over large distances (Helmuth and Hofmann 2001; Helmuth *et al* 2002; Gilman *et al* 2006; Helmuth *et al* 2006; Sagarin and Somero 2006). This heterogeneity in T_B over short spatial scales may lead to heterogeneity in the heat shock response. For example, in recent studies of the rocky intertidal mussel *Mytilus californianus*, Helmuth (2002) used automated temperature loggers that mimicked the shape, color, size and thermal properties of mussels to demonstrate that mussel T_B is more dependent on body size, location along the shore, and aspect of substratum than it was on air or water temperature. As a consequence, Hsp expression patterns for mussels within a mussel bed were complex. Mussels on the flat, horizontal surface of the rock were on average 7ºC warmer than those on vertical slopes less than 50 cm away, and Hsp70 expression was doubled for these horizontal mussels (Helmuth and Hofmann 2001). In contrast, a recent study of Hsp70 expression levels in mussels and in the dogwhelk *Nucella ostrina*, both species measured along their entire geographic range (Vancouver to Baja), were not higher in the southern, presumably warmer habitats than in the north (Sagarin and Somero 2006). Instead, peak values of Hsp70 expression were observed in northern Oregon, where intertidal animals are exposed to air in the afternoon during the warm summer months, and just south of Point Conception, California, where there is a large increase in sea surface temperature and decrease in wave exposure (and thus cooling splash). These examples illustrate the importance of rigorously tracking the response of an organism's T_B to changes in T_E, in part via the heat shock response. Such studies will be critical, given that that a predicted consequence of rapid climate change is that environmental unpredictability and fluctuation will increase, leaving more species vulnerable to stress and extinction (Easterling *et al* 2000; Parmesan and Yohe 2003; Sorensen and Loeschcke 2004; Hofmann 2005; Gilman *et al* 2006; Harley *et al* 2006, Helmuth *et al* 2006).

4. A model organism for studying effects of genetic variation on the heat shock response

The examples described above provide understanding of mechanisms by which heat shock protein expression operates in nature to buffer sometimes complex and unpredictable environmental temperature variation. Additional insights can be gained by using an organism that possesses genetic variation closely associated with the heat shock response. We have made substantial progress in this area in our studies of the Sierra willow leaf beetle *Chrysomela aeneicollis* (Schaeffer), an excellent model organism to study the relationship between thermal exposure, stress protein expression, and natural genetic variation in traits related to temperature adaptation. This beetle is abundant in cool, moist habitats at high latitudes in western North America (Brown 1956), but the range of *C. aeneicollis* extends into the Sierra Nevada mountains of Eastern California, where it occurs at high elevations (2375–3550 m). In the Sierra Nevada, *C. aeneicollis* is found in isolated sub-populations separated by permanent ice and snow at high elevations

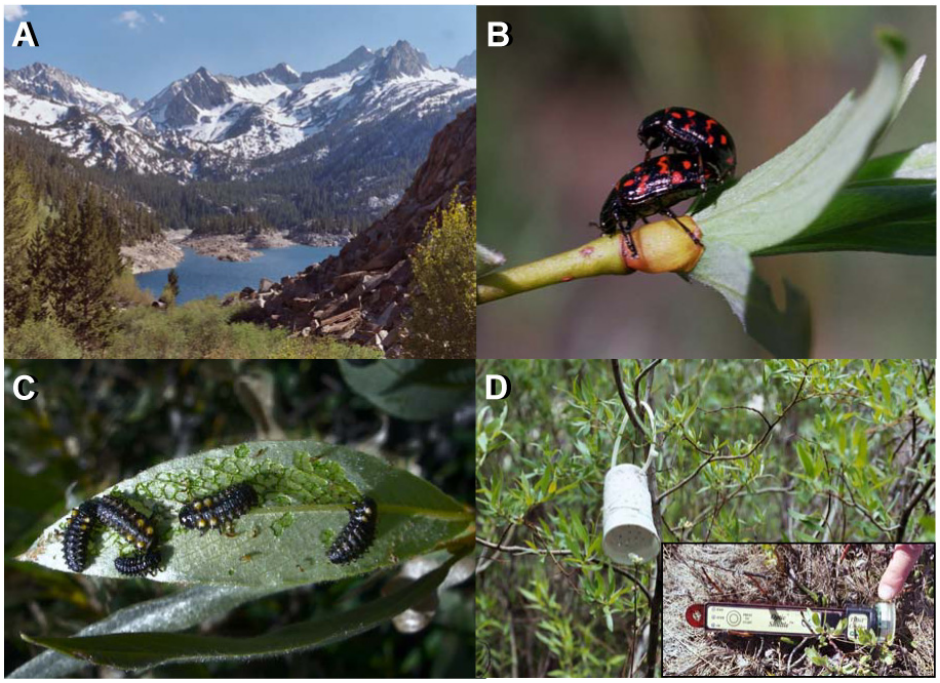

Figure 1. The willow leaf beetle *Chrysomela aeneicollis*. California populations of these beetles live in isolated drainages at high elevations in the Eastern Sierra Nevada (A). Adults emerge at diapause in early June to mate and lay eggs on willow host plants (B). Females lay several clutches of eggs, which hatch into larvae after a few weeks (C). These larvae mature, pupate and develop into new adults before the snows return in September. Measures of environmental temperatures (D) with data loggers that closely match beetle body temperature (inset) demonstrate that adults and larvae experience extreme thermal variation during summer (Figure 3). Photographs: **A, B,** Nathan Rank; **C,** Sonja Otto; **D,** Elizabeth Dahlhoff; Nathan Rank (inset).

(figure 1A), brush and desert scrub at low elevation. Adults (figure 1B) emerge from diapause at snowmelt (typically early June), to feed, mate, and lay eggs on willows along streams and in bogs. During this time, beetles sit and feed, or run throughout the foliage, males in search of mates and females for choice oviposition sites; however, flight is rare. Larvae (figure 1C) are found on the same host plants as the adults, and develop during the warmest months of summer (July and August). Adults from the new generation feed for several weeks before entering diapause in September (Smiley and Rank 1986; Rank 1994). Populations often reach high densities (Rank 1992a), resulting in complete defoliation of their host plants.

Since 1998, we have recorded environmental temperatures in three Eastern Sierra Nevada drainages (figure 2A): Rock Creek (RC), Bishop Creek (BC), and Big Pine Creek (BPC). Temperature dataloggers (figure 1D) were deployed at 6-20 sites along elevation gradients in each drainage that bracketed beetle distribution. Temperatures measured using loggers placed in white vented plastic cups in willow foliage 1.5 m above the soil surface closely matched beetle body temperatures (T_B) measured using fine wire thermocouple. Logger air temperatures (T_L) and beetle T_B's are 4–5°C higher than air temperatures (T_A) during the day. At night, both T_L and T_B are indistinguishable from T_A (Dahlhoff and Rank 2000; Rank and Dahlhoff 2002; McMillan *et al* 2005). We have recorded temperatures using these loggers since 1998.

Analysis of this nearly decade-long temperature record yields some striking patterns. First, there is a strong geographic pattern in thermal variation that persists through time. Average summertime logger (and thus beetle body) temperature is cooler in the northern most drainage (RC) than in the central drainage BC, which is in turn cooler than BPC (figure 2B). Typically, RC has the lowest lows and the lowest highs, and BPC the warmest lows and the highest highs (Dahlhoff and Rank 2000; Rank and Dahlhoff 2002; McMillan *et al* 2005). In all drainages, elevation is negatively related to mean daily temperature (–3.0°C per 1000 m increase in elevation). Not surprisingly, expression of a 70 kD stress-inducible heat shock protein varies among these drainages and elevations for beetles in nature (Dahlhoff and Rank 2000; McMillan *et al* 2005). Beetle T_B (not shown) and Hsp 70 expression level declines with increasing elevation (figure 2C) and Hsp70 expression is highest in the warmest drainage, BPC [(figure 2D; modified from Dahlhoff and Rank (2000)]. Within a site, beetles experience wide fluctuations in T_B during summer, from –5ºC (or lower) on

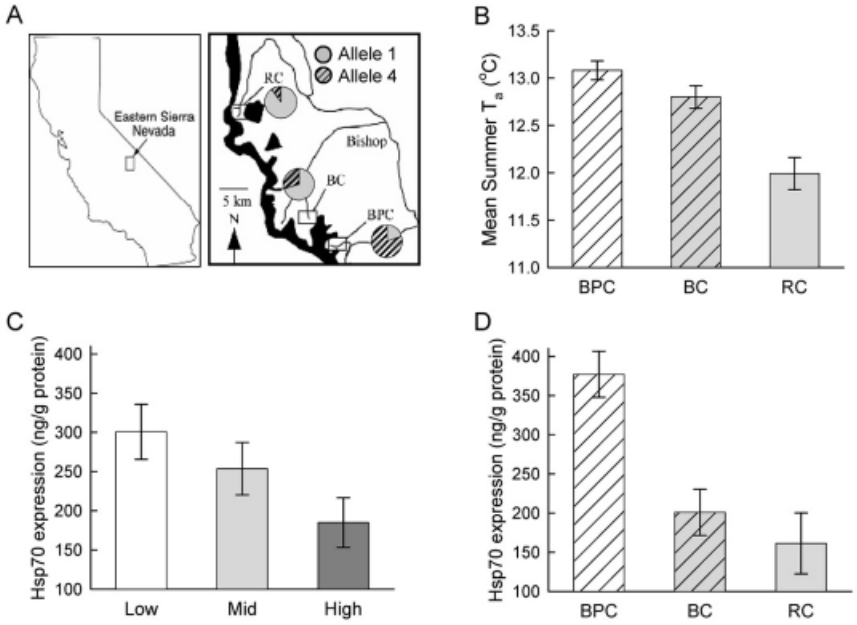

Figure 2. Genotype by environment interaction for heat shock protein (Hsp) expression in nature. (**A**) Genetic variation at the glycolyic enzyme locus *phosphoglucose isomerase* in Rock Creek (RC), Bishop Creek (BC) and Big Pine Creek (BPC); (**B**) Differences in environmental temperature between drainages. Data shown are least squares means (±SE) of average daily summertime air temperatures for 2000-2005, gathered using thermally-matched loggers deployed at five elevations in each drainage. (**C**) Hsp70 expression varies along a natural elevation gradient. Data shown are least squares means (±SE) of Hsp70 for *N*=12 beetles at low (2,750 m), mid (3,080 m) and high (3,270 m) elevation from sites in BPC, BC and RC. D: Differences in Hsp70 expression among drainages. Beetles were collected in early afternoon (1300-1400 h) and flash-frozen on dry ice immediately to preserve natural Hsp70 expression levels. Data shown are least squares means (±SE) of thorax muscle levels of a stress-inducible isoform of Hsp70, determined by Western blot analysis, for N= 36 beetles per drainage; Data modified from Dahlhoff and Rank (2000).

cold nights to over 35ºC during warm days in some lower elevation localities. An example of a trace of temperatures typically experienced by beetles over a summer at a mid-elevation site in BC is shown in figure 3. The consequences of this high degree of thermal heterogeneity on genetic variants in beetle populations and the heat shock response are discussed in detail below. Regional warming observed since 1998 has coincided with local extinction of populations at low elevations, especially in the warmest drainage BPC. In years prior to disappearance, Hsp70 expression levels of beetles living at low elevations in BPC were the highest measured in this species. Thus, environmental variability results in differential heat shock protein expression in native populations of Sierra willow beetles, as it does for other small ectotherms. In addition, high levels of Hsp expression may have negative consequences to fitness when environmental conditions become more stressful due to climate change.

5. Adaptive variation at a glycolytic enzyme locus

One of the unique aspects of the Sierra willow leaf beetle is that natural selection to temperature appears to act on the glycolytic enzyme locus *phosphoglucose isomerase* (PGI). Allele frequency variation at PGI across the biogrographic temperature gradient described above is much greater than for other polymorphic loci (Dahlhoff and Rank 2000, Rank 1992a, Rank and Dahlhoff 2002). Allele 1 (PGI-1) predominates in populations living in the northern drainage RC, and allele 4 (PGI-4) predominates in the southern drainage BPC (figure 2A). PGI allele 1 and 4 frequencies are intermediate in BC. Southern Sierra populations are also distinct from other populations in western North America. The PGI-1 allele predominates in Montana, Colorado, and further north in Sierra Nevada (Mount Dana, near Yosemite Park), whereas PGI-4 is at near fixation in the most southern location we have found these beetles (near Taboose Pass in King's Canyon National Park) (Fearnley 2003).

Annual and seasonal variation in climatic conditions causes shifts in beetle distribution, abundance and allele frequency variation at PGI, but not other polymorphic loci (Fearnley 2003, Rank and Dahlhoff 2002). We sampled allele frequency variation at five allozyme loci in populations in all three drainages in 1988 and 1996. During that time, the frequency of PGI-1 increased by 11% in BC, while PGI-4 decreased. These directional shifts did not occur at other polymorphic enzyme loci, suggesting that changes at PGI

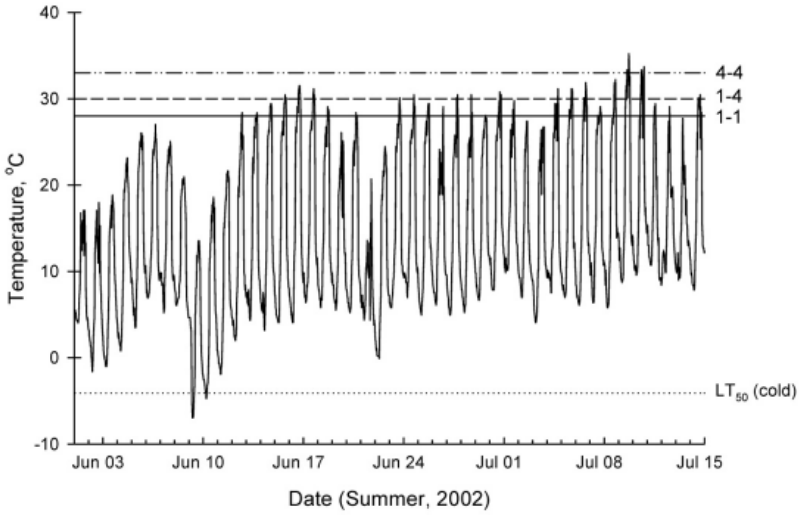

Figure 3. Environmental temperature variation routinely experienced in nature results in differential expression of Hsp70 among PGI genotypes at elevated temperatures, and occasional mortality after nighttime freezing. Temperature data shown were measured using a thermally-matched logger deployed at mid-elevation (3,130 m) in Bishop Creek, where both PGI alleles 1 and 4 are common, during summer 2002. Lines indicate temperatures of up-regulation of stress-inducible Hsp70 for PGI 1-1, 1-4 and 4-4 adult beetles, and LT_{50} cold, the temperature at which 50% of adults recently emerged from diapause die from cold exposure. Hsp70 expression and cold tolerance data modified from Rank and Dahlhoff (2002) and Neargarder *et al* (2003).

resulted from natural selection favoring the PGI-1 allele. In 1996, Eastern Sierra populations had recently re-colonized lower elevations after widespread declines during a dry period in late 1980's. The increased frequency of the PGI-1 allele may have resulted from cooler, wetter conditions that occurred during summers of 1995–96 before the second sampling period (Rank and Dahlhoff 2002). We have also observed rapid shifts in PGI allele frequency over short time scales. In a single summer (2001), PGI-1 increased in frequency by 5.6% early in the season (over-wintered adults to larvae), but decreased by 5.9% later in summer (larvae to new adults). The magnitude of the increase was related to daily maximum air temperature. Genotypic differences in fecundity or adult survival may have caused the initial increase in PGI-1 frequency, while differential larval or pupal survival may have caused its decrease. These data suggest that the relative fitness of PGI genotypes depends on life stage (Fearnley 2003). A possible outcome of such changes in relative fitness would be a net fitness advantage for heterozygotes (Mitton 1997), which could result in balancing selection and promote long-term maintenance of the PGI polymorphism.

6. The importance of genetic variation at PGI for the heat shock response

One of the most surprising findings of this work has been that Hsp70 induction temperature and total expression level varies among PGI genotypes (Dahlhoff and Rank 2000; Rank and Dahlhoff 2002; Neargarder *et al* 2003; McMillan *et al* 2005; Rank *et al* 2007). Differences in induction temperature among PGI genotypes occur at environmentally relevant temperatures (figure 3). PGI 1-1 individuals start to upregulate Hsp70 at 28ºC, a temperature routinely experienced while beetles are mating and larvae are developing, whereas PGI 4-4 individuals upregulate Hsp expression at temperatures rarely experienced in that habitat (33ºC). PGI 1-4 heterozygotes are intermediate. While we have not observed direct mortality due to elevated temperatures in nature, our own recent work (discussed below), along with other studies of ectotherms, have demonstrated that over-expression of Hsps may reduce performance or reproductive success (Patton and Krebs 2001; Roberts *et al* 2003; Sorensen and Loeschcke 2004; Folk and Gilchrist 2005). Differences in Hsp70 expression among PGI genotypes may be important for cold tolerance as well, as Hsps have been demonstrated to enhance cold hardiness in other insects (Chown 2001; Kelty and Lee 1999; Yiangou *et al* 1997; Yocum 2001). Though it may seem counterintuitive that a montane insect suffers cold mortality in summer, we have observed this phenomenon on several occasions (Rank 1994; McMillan *et al* 2005). Larval mortality in nature is related to minimum nighttime temperature, and larval survival is significantly lower in the coldest drainage RC than in BC or BPC. Survival after cold exposure differs among PGI genotypes (1-1>1-4>4-4) in adults and larvae (Rank and Dahlhoff 2002; Neargarder *et al* 2003). Because beetles experience exposure to cold

each night after several hours of exposure to extreme high temperatures, upregulation of Hsps during the day (which differs among PGI genotypes) may afford protection from cold in some individuals the following night. Thus, cold, like heat, is probably a significant selective force in these populations.

7. Adaptive significance of the PGI polymorphism to temperature adaptation in beetles

We have used this intriguing PGI polymorphism to study the consequences of naturally occurring genetic variation in a species adapted to physically challenging environments (figure 4). Allozyme loci provide excellent models to study this problem at multiple levels of biological organization. Mitton (1997) and Feder and Watt (1993) advocated a multi-tier approach to determining the adaptive significance of allozyme loci which includes investigations of (i) functional and biochemical properties of allozymes, (ii) physiological and performance consequences of differences in functional properties; (iii) the link between physiology, performance and components of reproductive success and (iv) a probable mechanism by which the polymorphism is maintained by selection in the population or interest. In this final section of the paper, we briefly describe our application of this approach to demonstrate a probable mechanism by which the PGI polymorphism is maintained in these habitats, and the relevance of the heat shock response at each level of organization.

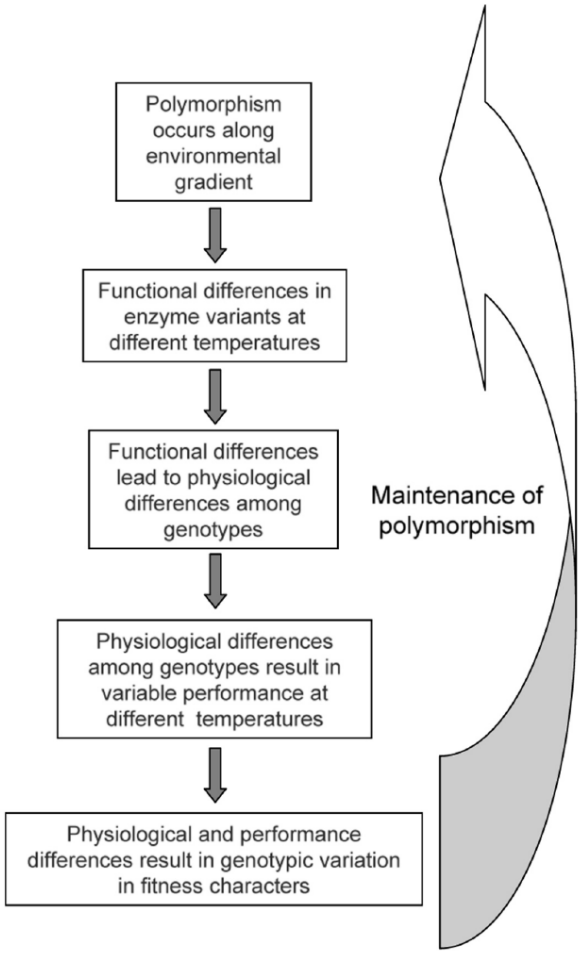

Figure 4. Willow beetles as a model for studying the adaptive significance of genetic variation in natural populations. Variation in Hsp expression along environmental temperature gradients and among *phosphoglucose isomerase* genotypes results in differential thermal tolerance among genotypes. Differences in Hsp expression are also linked to effects of temperature on running speed among genotypes, and to fitness characters like larval growth, survival and female fecundity. These interactions may ultimately cause the persistence of the PGI polymorphism in these populations.

7.1 *Functional differences among PGI allozymes.*

Our studies of PGI kinetics in *C. aeneicollis* have shown that there are small differences among PGI allozymes in the Michaelis-Menten binding constant, (K_m) and enzyme thermal stability (4-4 > 1-4 > 1-1). The K_m (which varies inversely with binding affinity) for the PGI allozyme common in RC (PGI 1-1) is greater at all measurement temperatures than PGI 4-4, the allozyme common in BPC (PGI 4-4; Dahlhoff and Rank 2000). PGI 1-4 heterozygotes showed intermediate K_m, especially at high measurement temperature. In addition, catalytic efficiency, indexed by V_{max}/K_m, is higher for 1-1 than 4-4 allozymes at moderate temperature (E P Dahlhoff, unpublished data). These data suggest that PGI allele 4 (a slow migrating allele) is more thermostable, and thus less efficient at moderate temperatures, than allele 1 (a fast-migrating allele). These results are consistent with other functional studies of PGI allozymes in ectotherms (Watt 1977, 1983 Hoffmann 1981a, 1981b). Homologous charge substitutions across multiple taxa could be responsible for the observation that PGI 'fast' alleles tend to make thermolabile allozymes, 'slow' alleles thermostable ones (Riddoch 1993; Wheat *et al* 2006).

Variation in thermostability among PGI alleles could be one mechanism driving differential expression of Hsps among PGI genotypes. After moderate heat stress (in the laboratory or in nature), individuals possessing the thermolabile PGI 1-1 allozyme have higher Hsp70 expression levels in thorax tissue than those possessing the more thermostable 4-4 allozyme. This may be in response to higher cellular levels of partially unfolded PGI, the concentration of which will depend on PGI genotype (1-1 > 1-4 > 4-4). While PGI is one of many cytosolic proteins that Hsp70 will chaperone, PGI is typically one of the most abundant glycolytic enzymes in the cell (Maughan *et al* 2005). In addition, recent studies of

the role of the heat shock response in targeting cancer cells for destruction by the mammalian immune system have demonstrated that Hsps act as "chaperokinins" to facilitate the presentation of tumor antigens to immune system cells (Asea 2005). One of the most common antigen sequences found on tumor cells is homologous with PGI (Yanagawa *et al* 2004; Funasaka *et al* 2005). While we have no evidence that there is a preferential response of Hsp70 to PGI over other glycolytic enzymes in willow beetles, these data are suggestive of a more general causal connection between Hsp70 and PGI.

7.2 *Differences in thermal tolerance among PGI genotypes*

Functional differences among PGI allozymes suggest that onset of a physiological response to elevated temperature should occur at lower temperatures for 1-1 individuals than for 1-4 or 4-4 individuals. Results for the physiological traits of Hsp70 expression and thermal tolerance are consistent with this prediction (Neargarder *et al* 2003, Rank and Dahlhoff 2002). We quantified thermal tolerance using critical thermal maximum (CT_{max}), the temperature at which an individual exposed to slowly increasing temperatures loses neuromuscular control, and LT_{50}, the temperature at which 50% of beetles did not survive a 4 hr exposure. Mean CT_{max} was 39.2°C for adults and LT_{50} for heat ranged from 35.7 to 39.2°C. For exposure to cold, LT_{50} ranged from –6.3 to –6.6°C. Larvae were less tolerant of thermal extremes than adults. Previous exposure to acutely cold or warm temperatures enhanced beetles' ability to survive subsequent exposure to extreme temperatures. Most importantly, CT_{max} values and survival after exposure to LT_{50} (heat and cold) were consistently related to PGI genotype for adults and larvae (1-1 > 1-4 > 4-4). Beetles with greatest tolerance of extreme heat or cold generally expressed higher levels of Hsp70, results consistent with other studies of thermotolerance in ectotherms (Feder *et al* 1996; Krebs 1999; Tomanek and Somero 1999). In addition, field data suggest that upregulation of Hsp70 after exposure to daytime high temperatures may increase tolerance of subsequent nighttime cold (McMillan *et al* 2005; Rank *et al* 2007).

7.3 *Differences in physiology and performance among PGI genotypes*

To investigate mechanisms by which physiological differences among PGI genotypes affect performance characters, especially after exposure to repeated thermal stress, we measured larval growth, metabolic rate, and adult and larval running speed. In all experiments, we collected beetles from a population where all three genotypes were relatively abundant, thus starting with naturally bred and reared individuals whose phenotype is shaped by interactions between genotype and natural environment. We found that differences among PGI genotypes existed for all three characters, but that the rank order of genotypes depended on treatment temperature (McMillan *et al* 2005; Rank *et al* 2007). For example, PGI 4-4 larvae grew faster than other genotypes when repeatedly exposed to a moderately elevated temperature (27°C), but not at lower temperatures. PGI genotypes also differed in effects of temperature on running speed. Adults possessing the PGI 1 allele ran faster than PGI 4-4 homozygotes in nature and after one exposure to extreme temperature, but repeated exposure to extreme temperature in adults, or a single extreme exposure for larvae, resulted in greater running speed in 4-4 homozygotes. In these beetles, Hsp70 expression depended on recent thermal history and on PGI genotype. However, the ranking of each PGI genotype with respect to Hsp70 expression depended on whether the animal had been exposed to a single stress or to repeated bouts of extreme temperature. Thus, phenotypic expression of genetic differences at PGI depended on recent environmental conditions experienced by the beetle, suggesting phenotypic plasticity in performance characters and the stress response.

7.4 *Fecundity differs among PGI genotypes*

We compared female PGI genotypes with respect to fecundity in the laboratory and field (Bruce 2005). In the laboratory under mild conditions (20ºC), PGI 1-1 females produced more eggs than PGI 4-4 females. However, at elevated temperatures (32ºC), PGI 1-1 female fecundity declined, while number of eggs laid by PGI 4-4 females increased. After several weeks' exposure to routine stress, Hsp70 expression levels tended to be higher in 4-4 than 1-1 genotypes, suggesting that PGI genotypes vary in protection for protein synthesis critical for egg production. At field sites in RC, PGI 1-1 individuals produced more eggs than PGI 4-4 individuals. However, in BPC, 4-4 individuals produced more eggs than 1-1 individuals, corresponding to the naturally occurring distribution of PGI alleles (figure 2A, map). These data are especially powerful since beetles used in field and laboratory experiments were from a single source population in BC where PGI allele frequencies are intermediate. Differences in fecundity, like differences in locomotor performance and thermal tolerance, may be responsible for the observed geographic divergence in PGI allele frequency.

8. Conclusions

In this paper, we have described environmental and phenotypic factors that influence expression of stress

proteins that buffer organisms from environmental change, and described model systems where the relationship between environment and stress protein expression have been well characterized. We have also shown that a model organism that possesses genetic variation needed to evolve in response to environmental change can be used to develop a more profound understanding of the adaptive significance of the heat shock response in nature. We hope that these studies provide new momentum to further research on the potential for organisms to evolve in response to environmental change, and that they also illustrate the limits of adaptive evolution to compensate for human-caused global warming.

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