

**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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Running Head: Effects of temperature on beetle physiology and fecundity

Keywords: Adaptation, allozyme, insect, Chrysomelidae, phosphoglucose isomerase, PGI

1 **Abstract**

2 The willow beetle *Chrysomela aeneicollis* lives on the edge of its range in the Sierra Nevada,
3 California USA. Beetles commonly experience stressfully high and low temperatures during
4 summer egg-laying and larval development. The glycolytic enzyme locus *phosphoglucose*
5 *isomerase* (PGI) is a marker of temperature adaptation in these populations. Allele frequency at
6 PGI varies across a latitudinal gradient, with PGI allele 1 common in cool northern populations
7 (Rock Creek- RC) and PGI allele 4 in warmer southern ones (Big Pine Creek- BPC).
8 Frequencies of allele 1 and 4 vary greatly among populations in the intermediate region Bishop
9 Creek (BC). Over the past decade, populations have shifted to higher elevations, especially in
10 RC and BPC. In BC, PGI allele frequency fluctuates among life history stages. The magnitude
11 of these fluctuations is related to maximal air temperature, and the frequency of PGI allele 4
12 increased after the hottest time of the summer. We examined effects of temperature on
13 metabolic rate, heat shock protein expression and fecundity. Effects of temperature on metabolic
14 rate varied among PGI genotypes at elevated (36 °C: 4-4 > 1-4 = 1-1) but not moderate (20 °C)
15 temperatures. In the laboratory, egg production was highest for BC females possessing the PGI
16 1 allele (1-1 = 1-4 > 4-4). Hsp70 expression was positively related to fecundity. In nature,
17 differences among PGI genotypes in fecundity depended on transplant drainage. Egg production
18 was highest for 1-1 individuals in RC, 4-4 individuals in BPC, and was similar among genotypes
19 in BC. These data suggest that individuals possessing the PGI 4 allele may be favored when
20 beetles experience extreme stress (e.g. BPC), while those possessing the PGI 1 allele are favored
21 under mild conditions (RC). The data also suggest that the heterozygous populations in Bishop
22 Creek may be more resistant to climate-induced temperature changes by evolving *in situ* rather
23 than shifting upwards in elevation.

24 **Introduction**

25 One of the many challenges posed by global climate change is that natural populations will
26 respond unpredictably to an increasingly variable climate. In recent years, ranges of many
27 species have shifted, with local extinction in some areas and colonization of new regions (Hill et
28 al. 2002; Inouye et al. 2000; McLaughlin et al. 2002; Parmesan 2006; Parmesan and Yohe 2003;
29 Sagarin et al. 1999). On the other hand, other species have shown no apparent response
30 (Erasmus et al. 2002; Parmesan et al. 1999). Predicting which populations will be vulnerable is
31 challenging, since small changes in climate may lead to large, unpredictable changes in air
32 temperature, rainfall and persistence of snow (Hayhoe et al. 2004; Miller et al. 2003; Pearson and
33 Dawson 2003; Whitham et al. 2006). The response of a population to environmental change will
34 depend on its recent evolutionary history, on effects of abiotic conditions on interactions with
35 other species, and on effects of abiotic stress on individual survival, physiological performance,
36 and reproductive output (Chamaille-Jammes et al. 2006; Karlsson and Van Dyck 2005; Lemmon
37 et al. 2007; Lester et al. 2007; Musolin 2007; Nussey et al. 2007). Though effects of climate
38 change on natural populations have been extensively investigated the past two decades, only
39 recently have researchers begun to identify specific features of species or populations that might
40 allow them to persist in a changing environment (Ellis and Post 2004; Gilman et al. 2006; Harley
41 et al. 2006; Helmuth et al. 2002; McLaughlin et al. 2002; Svensson et al. 2006; Tran et al. 2007).

42 Small, free-living ectotherms are especially susceptible to detrimental effects of
43 fluctuations in environmental temperature, because their body temperature is determined to a
44 great extent by the environment. Many small ectotherms live close to their thermal tolerance
45 limits (Angilletta et al. 2006; Feder et al. 2000; Portner et al. 2006; Somero 2004; Sorensen et al.
46 2003; Willmer et al. 2004). Large fluctuations in environmental (and thus body) temperature

47 may ultimately reduce metabolic activity, in part due to costs of repairing cellular damage caused
48 by stress, which may in turn reduce reproductive output and thereby cause declines in population
49 size (Krebs and Feder 1997; Krebs and Holbrook 2001; Loeschke et al. 1997; Portner 2002;
50 Sorensen and Loeschke 2002). Thus, small changes in climate may lead to large and rapid
51 changes in abundance, and ultimately result in local extinction. Prior studies have thoroughly
52 demonstrated effects of temperature on survival, activity and reproductive output of small
53 ectotherms (Boggs and Freeman 2005; Hodkinson 2005; Karlsson and Wiklund 2005; McMillan
54 et al. 2005; Rank et al. 2007). However, few studies have integrated investigations of
55 physiological characters such as metabolic rate with direct impacts of physiological variation on
56 reproductive success, especially in a natural setting (Dillon et al. 2007).

57 In this study, we report the geographic distribution of a genetic polymorphism, which
58 shows evidence of being acted on by natural selection due to variation in environmental
59 temperature. We also document the effect of temperature variation on metabolic rate and
60 reproductive output for individuals that differ genetically with respect to the most common
61 alleles at this locus. The genetic marker for this polymorphism is the allozyme *phosphoglucose*
62 *isomerase*, which varies among Eastern Sierra Nevada, California, populations of the montane
63 willow beetle *Chrysomela aeneicollis*, living at high-elevation (2700-3200 meters) at the
64 southern edge of its worldwide range. In these populations, PGI shows much greater
65 differentiation among drainages than any other polymorphic enzyme locus. Allele 1
66 predominates in populations living in the northern drainage Rock Creek (RC), while allele 4
67 predominates in the southern drainage Big Pine Creek (BPC). Allele frequencies are intermediate
68 in the middle drainage Bishop Creek (BC). This allele frequency gradient at PGI occurs along a
69 temperature gradient. RC is typically coolest, BPC warmest, and BC temperatures are

70 intermediate (Dahlhoff and Rank 2007; Rank 1992; Rank and Dahlhoff 2002).

71 Our prior work on Eastern Sierra Nevada populations of *Chrysomela aeneicollis* suggests
72 that temperature is a potent selective force in nature. In summer, adults emerge from diapause in
73 May or June to feed, mate, and lay eggs. Larvae and adults use the same host plants and larvae
74 are present throughout July. Near the end of July, larvae pupate and hatch out into new adults
75 (Rank 1994; Smiley and Rank 1986). During egg-laying and larval development, summer air
76 temperatures reach 35°C during the daytime, and occasionally fall to -6°C at night. We have
77 observed mortality after subzero nighttime temperatures on multiple occasions and have
78 documented differential expression of 70-kD heat shock proteins (Hsp70) among beetles in
79 different drainages and along elevation gradients (Dahlhoff and Rank 2000; McMillan et al.
80 2005; Rank 1994; Rank and Dahlhoff 2002; Smiley and Rank 1986).

81 Variation at the PGI locus is consistently related to traits that allow individuals to cope
82 with extremes in temperature. PGI allozymes differ in thermal stability and Michaelis-Menten
83 binding constant, K_m (Dahlhoff and Rank 2000). In addition, catalytic efficiency (indexed by
84 V_{max}/K_m) for the 4-4 allozyme is lower (less efficient) than the 1-1 allozyme at 10°C and 20°C,
85 but higher (more efficient) at higher temperatures (30-40°C) (EP Dahlhoff, unpublished data).
86 Hsp70 expression is typically higher, and induced at lower temperature, for individuals
87 possessing the less stable form of the enzyme (PGI 1-1) than the more stable form PGI 4-4. At
88 moderate temperatures or after a single exposure to stress, PGI 1-1 genotypes are more thermo-
89 tolerant and run faster than 4-4 individuals; 1-4 heterozygotes are typically intermediate
90 (Nearing et al. 2003, Rank et al. 2007). However, repeated exposure to extreme heat or cold
91 reverses these patterns (4-4 > 1-4 > 1-1). These data suggest that PGI allele 4 is associated with
92 greater tolerance of repeated thermal stress than the allele 1, but that allele 1 is associated with

93 better performance and a more rapid response to stress when conditions are not extreme
94 (Dahlhoff and Rank 2007). Differences in Hsp70 expression among PGI genotypes may be due
95 to direct interactions between Hsp70 and PGI (i.e. Hsp70 responds to PGI allozymes that
96 partially unfold at different temperatures) or PGI may interact with factors that regulate
97 expression of Hsp70. A third possibility is that PGI is in linkage disequilibrium with genes that
98 affect Hsp70 expression. In any case, functionally important genetic variation affects the heat
99 shock response, which may allow populations to respond adaptively to environmental change.

100 These prior studies lay the foundations for the present study, in which we address the
101 crucial relationship between temperature physiology, metabolism, reproductive output and
102 population persistence. Here we address the following. How do populations vary at the PGI
103 locus within Bishop Creek, a drainage that lies between Big Pine Creek (where PGI allele 4 is
104 common) and Rock Creek (where PGI allele 1 predominates)? How do PGI frequencies change
105 among life stages within a single generation, and how do these changes relate to environmental
106 temperature? How do effects of temperature on metabolic rate (a key measure of performance)
107 vary among PGI genotypes? How do PGI genotypes vary in reproductive output (fecundity) in
108 the laboratory and in nature, and is Hsp70 expression related to female reproductive output?
109 Finally, how have Eastern Sierra populations of *C. aeneicollis* changed over the past decade, and
110 based on our physiological data, what predictions can we make about future persistence of these
111 populations?

112 **Materials and Methods**

113 Population genetics

114 To examine structure of Eastern Sierra Nevada, California (USA) populations of *Chrysomela*
115 *aeneicollis*, adults were collected in July 1997 from three drainages (Big Pine Creek- BPC,

116 Bishop Creek- BC, and Rock Creek- RC). Genotype frequency data for BPC and RC
117 populations were published previously and are included here for comparison (Fig 1; Dahlhoff
118 and Rank 2000). Bishop Creek sub-drainage localities, sample sizes and sampling elevation
119 ranges are shown in Table 1. Sample size per site ranged for 18-22 individuals at 39 sites and
120 was 10 and 16 beetles at two remaining sites. To quantify changes in allele frequency over a
121 single summer, beetles were collected from 7 populations in the south fork of Bishop Creek
122 where PGI alleles 1 and 4 are both common. Thirty-five beetles were collected from each site
123 three times during the summer of 2001: over-wintered adults, which had just emerged from
124 winter diapause (7-8 June), 3rd-instar larvae (6-7 August) and newly-emerged adults (8-9
125 September). After collection, beetles were flash-frozen and stored at -80 °C until genotype
126 analysis.

127 To relate variation in climate to allele frequency change in BC, microhabitat air
128 temperature at each site was measured every 30 min throughout the summer using TidbiT
129 Temperature Data Loggers (Onset Computer Co., Pocaset, MA) suspended in white plastic cups
130 and secured onto host willow branches 1.5 m above the ground. Daily mean maximum,
131 minimum and average temperature was determined for each site using Boxcar Pro (Version 4.0,
132 Onset Computer Co., Pocaset, MA).

133 Abundance

134 Adult beetle population abundance was quantified in 1998, 2003 and 2007 for 7-12 sites
135 in each of the three main study drainages along replicate elevation transects 8-12 km in length
136 (2700-3600 m). First surveys were initiated at snowmelt, or in late May, which ever came first.
137 Beetles were never seen before late May, even in years of early snowmelt. Surveys were
138 repeated every 6-10 days throughout June and early July, as long as adults were present. To

139 survey, 2 observers hiked to each locality, identifying exact site using a handheld GPS unit (Geo
140 Explorer, Trimble Corporation, Sunnyvale, CA). Abundance was determined by timed visual
141 survey (10 person minutes). Each observer selected a location near willow plants and scanned
142 for beetles in the foliage closely while moving throughout suitable microhabitats. Number of
143 beetles was recorded using a hand-held cell counter. Raw data were converted to scaled counts
144 (see Table 2). Data reported are abundances observed the week of peak abundance (averaged
145 over all three drainages) each year.

146 Effects of temperature on metabolic rate

147 Metabolic rate was measured for over-wintered adults collected at sites between 3100-3200 m in
148 the Chocolate and Green Lakes drainages in BC (Bluff Lake, South Lake Pipeline, Mary Louise
149 Creek and Bull Lake). After collection, sexes were separated to minimize effects of mating
150 activity on metabolic rate, and held in controlled temperature incubators at natural diurnal light
151 cycles (14 h day, 20°C; 10 h night 4°C) at White Mountain Research Station (WMRS) in Bishop,
152 CA for 7 days. Beetles were fed on fresh leaves from their favored host plant *Salix orestera* and
153 given moisture by placing a piece of dampened filter paper in Petri dish. After laboratory
154 acclimation, beetles were transported (in a 4°C cooler) from WMRS to University of Nevada,
155 Las Vegas for metabolic rate measurements. Beetles were deprived of food for 4-6 days before
156 measurement. Individuals were randomly assigned to one of two measurement temperatures (20,
157 36 °C) and weighed immediately before determination of metabolic rate.

158 Metabolic rate (indexed by CO₂ production) and water loss rates were measured using
159 flow-through respirometry. Beetles were placed in 5-ml glass-aluminum respirometry chambers,
160 and dry, CO₂-free air was pumped through at 100 ml/min. Carbon dioxide and water vapor of
161 the air stream were measured with a Licor LI-6262 infrared gas analyzer. Beetle activity was

162 monitored during respirometry with AD-1 activity detectors (Sable Systems, Las Vegas, Nevada
163 USA). These use a near-infrared photocell to detect movements. Because metabolic rates and
164 water-loss rates can increase with activity, these parameters were quantified these during periods
165 when beetles were quiescent. Thus, measurements reflect standard metabolism at 20 or 36 °C.
166 All respirometry data were collected and analyzed using Datacan V software (Sable Systems,
167 Las Vegas, Nevada USA). After measurement, beetles were flash-frozen and stored at -80°C
168 until genotype analysis.

169 Effects of temperature on laboratory fecundity and Hsp70 expression

170 Effects of acclimation temperature on fecundity and heat shock protein expression were
171 determined for females collected in June 2001 from Bluff Lake. Beetles were transported in
172 coolers to WMRS and sexes were separated. Beetles were held in the laboratory as described
173 above for 48 hours before starting experiment to mitigate effects of recent thermal experience in
174 nature. Females were randomly placed in a Petri dish with one male collected from the same
175 site. Male-female pairs were exposed to one of three acclimation temperatures: 20, 26, and 32
176 °C. Each day, 221 pairs were held at 20 °C for 4 h in the morning (8:00 AM to noon), at
177 acclimation temperatures for 4 h in the afternoon (noon-4:00 PM), at 20 °C for 7 h in the evening
178 (4:00-11:00 PM), and at 4 °C for nine hours at night (11:00 PM- 8:00 AM). Willow sprigs were
179 removed during temperature treatments, and pairs were kept together throughout the experiment.
180 To measure fecundity, total number of eggs produced by females was determined daily before
181 and after exposure to treatment temperatures. Egg clutches were removed from the Petri dish
182 each day and eggs counted using a dissecting microscope. Male mating activity was routinely
183 monitored, and males mated with females throughout the experiment. After 24 days, beetles
184 were weighed, flash-frozen and stored at -80 °C for biochemical analysis.

185 Effects of natural climate on fecundity

186 Effects of climate variation on fecundity were measured for females collected in June 2002 from
187 Bishop Creek (Bluff Lake, 3200 meters) and transplanted to similar elevation localities in Big
188 Pine Creek (Falls Site), Rock Creek (Mosquito Flat) and Bishop Creek (Bluff Lake). Females
189 were placed with one male in white tulle mesh bags on willow branches of replicate *Salix*
190 *orestera* (N = 13 plants per drainage, 6 branches per plant). Egg clutches were removed from
191 mesh bags twice a day, placed in plastic cups, returned to the laboratory at WMRS, and egg
192 number in each clutch counted using methods described above. As in the laboratory experiment,
193 male mating activity was monitored. At the end of the experiment, all beetles (and mesh bags)
194 were removed from field sites, beetles weighed, flash-frozen and stored at -80 °C for biochemical
195 analysis.

196 Biochemical analyses

197 Genotypes at three allozyme loci, *phosphoglucose isomerase* (PGI; E.C. 5.3.1.9), *isocitrate*
198 *dehydrogenase* (IDH; E.C. 1.1.1.42) and *phosphoglucomutase* (PGM; E.C. 5.4.2.2) were
199 determined by starch gel electrophoreses using established protocols (Murphy et al. 1996; Rank
200 1992). Expression of a 70 kD heat shock protein (Hsp70) was determined for females in
201 fecundity experiments by Western blot analysis following published methods (Rank et al. 2007).

202 Statistical Analysis

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

208 Population structure of Eastern Sierra Nevada.

209 Effects of temperature on PGI allele frequency variation. We analyzed changes in allele
210 frequency using logistic regression, with PGI frequency (per individual) as dependent variable,
211 and life stage, subdrainage, and population nested in subdrainage as independent variables.
212 Allele frequency was determined as the frequency of the PGI-1 allele (0 for PGI 4-4
213 homozygotes, 0.5 for 1-4 heterozygotes, and 1 for 1-1 homozygotes). To determine how the
214 environment relates to PGI frequency change, we first calculated allele frequency at the
215 population level. We then calculated the selection coefficient (s) for the 2 intervals between the
216 3 collections (over-wintered adults to larvae, larvae to newly emerged adults) following standard
217 methods for calculating relative fitness and selection coefficients from longitudinal genotype
218 frequency data (Hartl and Clark 1997). If s was positive, then genotype 1-1 was favored, if it
219 was negative, then genotype 4-4 was favored. To determine which measure of temperature
220 predicted the magnitude of the selection coefficient, regression models using mean minimum,
221 mean average and mean maximum temperatures were compared, and the variable with the
222 highest r square was selected (detailed in Fearnley 2003).

223 Metabolic rate. Metabolic rates and water loss rates were analyzed using ANCOVA, with PGI
224 genotype and treatment temperature as main effects. Body mass was used as a covariate, rather
225 than determining mass-specific metabolic rate, following recommendations of Packard and
226 Boardman (1999). Five beetles measured at 20 °C had negligible ($< 0.02 \mu\text{l/hr}$) water loss rates
227 and were removed from analysis.

228 Fecundity experiments. Results of the laboratory fecundity experiment were analyzed using
229 repeated-measures ANCOVA, with acclimation temperature, genotype, and the interaction term
230 as categorical fixed effects, and total number of eggs laid over every 4 days as response variables

231 (day 4, 8, 12, 16, 20, 24). Body mass, number of days to first egg clutch, and Hsp70 expression
232 were included as covariates. Two females that did not oviposit during the first 10 days of the
233 experiment were excluded from analysis. Effects of acclimation temperature and PGI genotype
234 on Hsp70 expression were determined in a separate analysis using ANOVA. Results of field
235 fecundity were analyzed using ANOVA, with transplant drainage and PGI genotype as main
236 effects. Unlike laboratory fecundity, Hsp70 expression was not a significant covariate in a
237 preliminary ANCOVA, so it was not included in the final analysis.

238 **Results**

239 Geographic variation in PGI allele frequency

240 The frequency of PGI allele 1 varies with latitude among drainages (Fig 1; BPC < BC < RC),
241 and the frequency of PGI allele 4 declines as the frequency of allele 1 rises. This latitudinal
242 gradient is found within the intermediate drainage Bishop Creek ($F_{7,39} = 28.8$, $P < 0.001$). The
243 frequency of PGI allele 1 in North Bishop Creek is similar to its frequency in Rock Creek, and is
244 greater than its frequency in South Bishop Creek (Fig 1). Within South Bishop Creek, PGI-1 is
245 most common in the Tyee Lakes, which are in close proximity to a pass to North Bishop Creek
246 (Fig 1B).

247 Allele frequency change among beetle life stages

248 The frequency of PGI allele 1 in South Bishop Creek populations was 0.67 for over-wintered
249 adults collected early in the summer ($n = 245$), and it increased by 9.7% (to 0.74) in the 2nd instar
250 larvae collected 60 days later ($n = 248$). However, the frequency of PGI allele 1 declined again
251 by 11% (to 0.66) 30 days later when new adults were collected ($n = 245$). Logistic regression
252 revealed that these PGI allele frequency differences among life stages were statistically
253 significant ($G = 7.0$, $df = 2$, $P = 0.03$), as was variation among sub-drainages ($G = 16.2$, $df = 5$, P

254 = 0.006) and populations within a sub-drainage ($G = 6.3$, $df = 1$, $P = 0.012$). There were no
255 directional changes in allele frequency at IDH-2 or PGM (Table S1).

256 The magnitude of directional changes in PGI frequency was related to mean daily
257 maximum temperature (Fig 2). During the first part of summer, PGI allele 1 increased most in
258 frequency at sites with high maximum temperature. In contrast, during the second part of
259 summer, PGI allele 1 decreased (and allele 4 increased) most at sites with high maximum
260 temperature. Mean maximal air temperatures were significantly higher in the second part of
261 summer, when larvae were developing into new adults and allele 4 was becoming more frequent
262 (matched pairs t-test; $t = 3.9$, $df = 4$, $P = 0.009$). Preliminary regression models showed that site
263 elevation and mean minimum temperature were not related to the selection coefficient.

264 Temporal and spatial changes in beetle abundance

265 Populations of *C. aeneicollis* shifted in elevation range during the past nine years. In 1998, after
266 a late snowmelt and several years of above average winter precipitation, beetles were most
267 abundant at the lower portion of their elevation range. On average, estimated abundance
268 increased by 55% between 1998 and 2003, was high from 2003-2006, and decreased by 44% by
269 2007 (Table 2). In addition, beetle abundance shifted to higher elevations (Table 2, elevation by
270 year interaction, $F_{2,80} = 3.8$, $P = 0.026$). By 2007, populations had disappeared entirely
271 throughout most of their previous elevation range in Big Pine Creek and Rock Creek, but were
272 persistent (at low population size) in most localities in Bishop Creek.

273 Relationship between PGI genotype and metabolic rate

274 Resting metabolic rate depended on measurement temperature and PGI genotype (Fig 3;
275 temperature: $F_{1,109} = 44.6$, $P < 0.0001$; temperature by PGI genotype interaction: $F_{2,109} = 3.0$, $P <$
276 0.05). Metabolic rate did not differ among genotypes at a moderate temperature (20 °C), but at

277 36 °C, near maximal body temperature in nature, metabolic rate of PGI 4-4 individuals was 45%
278 greater than PGI 1-1 or 1-4 individuals. The Q_{10} of metabolic rate varied two-fold among
279 genotypes. Q_{10} for PGI 4-4 genotypes was 3.24, compared to 1.54 for PGI 1-4 and 1.66 for PGI
280 1-1 genotypes. All individuals lost water throughout the experiment, with larger beetles losing
281 more water ($F_{1,109} = 13.2, P < 0.0005$). Water loss rates were greater at 36 °C (1.73 ± 1.7) than
282 at 20 °C (0.33 ± 0.1) ($F_{1,109} = 119, P < 0.0001$) and did not vary among allozyme genotypes.

283 Female fecundity in the laboratory

284 Throughout the 24 day fecundity experiment, PGI 4-4 females laid fewer eggs than PGI 1-1 or
285 PGI 1-4 females, and the rate of egg production of PGI 4-4 females declined more rapidly than
286 other PGI genotypes (Fig 4A, Table S2). Egg production was positively related to female body
287 mass and Hsp70 expression level (Fig 4B), and negatively related to number of days before the
288 first eggs were laid (Table S2, between-subjects factors). The relationships between these
289 variables and egg production varied over the course of the experiment (Table S2, within- by
290 between-subjects factors interaction terms).

291 At the end of the experiment, differences among PGI genotypes were most pronounced
292 for females kept at 20°C. PGI 1-1 individuals tended to lay more eggs at 20°C than 4-4 females,
293 and PGI 4-4 females tended to lay more eggs at 32°C than at other temperatures (Fig 5A). Hsp70
294 expression levels were positively related to acclimation temperature ($F_{2,116} = 6.2, P = 0.003$) and
295 body mass ($F_{1,116} = 3.7, P = 0.06$), and tended to be highest at 32°C for 4-4 females (Fig. 5B).

296 Female fecundity in nature

297 In nature, differences among PGI genotypes (but not other allozyme loci) in egg production
298 depended on the drainage into which the females were transplanted (Fig 6, genotype by drainage
299 interaction $F_{4,165} = 2.8, P < 0.029$). In Rock Creek, PGI 1-1 and 1-4 females laid 31.5% more

300 eggs than PGI 4-4 females, while PGI 4-4 females laid 53.5% more eggs than the other two
301 genotypes in Big Pine Creek. In Bishop Creek, fecundity was high for all genotypes.

302 **Discussion**

303 Natural populations of *C. aeneicollis* experience substantial fluctuations in environmental
304 temperature that pose significant challenges to survival and reproduction, and they also possess
305 genetic variation in traits that relate to their ability to cope with climatic variation. The current
306 study demonstrates spatial structure in allele frequency at an allozyme locus, PGI, which
307 previous studies have found to be associated with traits that could confer temperature adaptation
308 (Dahlhoff and Rank 2000; McMillan et al. 2005; Nearing et al. 2003; Rank et al. 2007; Rank
309 and Dahlhoff 2002). We have also found that PGI frequencies fluctuate within a single
310 generation, and that the magnitude of these fluctuations is related to maximum temperature.
311 Results presented here show region-wide changes in *C. aeneicollis* abundance over moderate
312 time scales (1998-2007) and suggest that the range of *C. aeneicollis* is shifting to higher
313 elevations, especially in BPC and RC, where PGI is less polymorphic. The relationship between
314 variation at PGI and metabolic rate is consistent with previous studies showing that PGI
315 genotypes differed in functional property and physiological characters such as Hsp70 expression
316 and thermal tolerance (reviewed in Dahlhoff and Rank 2007). Finally, we found that female
317 reproductive rate was related to PGI genotype and environmental conditions experienced by
318 females during egg-laying, and that variation among PGI genotypes in reproductive rate in the
319 field corresponded to natural geographic variation in PGI allele frequency in the Sierra Nevada.

320 Findings presented here reveal how geographic variation at PGI is structured among
321 neighboring populations within the drainage Bishop Creek, which lies between drainages where
322 PGI frequencies are quite distinct, Big Pine and Rock Creek. In general, populations in the

323 South Bishop Creek sub-drainage are genetically more similar to populations in Big Pine Creek,
324 while those in the North Bishop Creek sub-drainage are genetically similar to populations in
325 Rock Creek (Dahlhoff and Rank 2000; Rank 1992). Within the South Bishop Creek
326 subdrainage, populations vary significantly along elevation gradients along creeks. In contrast,
327 populations in the North Bishop Creek subdrainage are less variable, with high PGI 1 allele
328 frequency at all elevations. One explanation for this pattern is that Bishop Creek represents a
329 zone of contact between two genetically distinct lineages of *C. aeneicollis*- one from the south
330 (represented by Big Pine Creek populations), and another from the north (North Bishop Creek
331 and Rock Creek populations). Studies of variation at mitochondrial DNA loci (COI and COII)
332 are consistent with this hypothesis (Fearnley 2003). Beetles that possess the PGI 4 allele also
333 tend to possess mitochondrial haplotypes that are generally found further south, while those
334 possessing the PGI 1 are found in the north. Thus, as a zone of contact between two separated
335 sub-populations of this species, Bishop Creek populations may ultimately harbor more genetic
336 diversity than other drainages, and may be more resilient in the face of environmental
337 unpredictability.

338 The current distribution of alleles in Bishop Creek may result from a balance between
339 migration and selection. In the present study, we show that the frequency of PGI allele 1
340 increased during early summer (over-wintered adults to larvae), whereas PGI allele 4 increased
341 during mid to late summer (larvae to new adults). These findings suggest that reproductive
342 success of BC adults possessing PGI allele 1 was greater than those possessing allele 4 (as we
343 observe under mild conditions in the laboratory), but that the survival of larvae possessing allele
344 4 was greater than those possessing allele 1, as reported in earlier studies, (e.g. McMillan et al.
345 2005). The relationship between environmental temperature and the magnitude of the change at

346 PGI suggests that the shifts at both life stages occurred as an adaptive response to high
347 temperature. Studies in other organisms also suggest that alleles favored at one stage can be
348 disadvantageous at another one (Johannesson 2003; Johannesson et al. 1995). In some cases,
349 this kind of selection can lead to long-term “over-dominance”, where the episodic selection
350 against either homozygote results in higher fitness of individuals carrying the heterozygote
351 (Mitton 1997). Our previous studies suggested in a wet year (1996), which was predated by a
352 series of dry years (1988-1995), directional selection favored individuals possessing PGI allele 1.

353 The data presented here, like our earlier work and those of others investigating natural
354 selection at PGI (Dykhuizen and Hartl 1983; Hanski and Saccheri 2006; Katz and Harrison 1997;
355 Watt 1992; Wheat et al. 2006), suggest that in willow beetle populations, selection is operating
356 on PGI (rather than loci physically linked to PGI). However, results of performance and fitness
357 data presented here and elsewhere show little evidence of over-dominance, but instead suggest
358 shifting directional selection, which depends on recent climatic conditions. Furthermore, for
359 nearly all physiological and fitness characters measured, PGI 1-4 heterozygotes show
360 intermediate values, rather than being most thermotolerant or having greatest fitness (Dahlhoff
361 and Rank 2007). Persistence of beetles in a Sierra Nevada environment might be greatest in
362 regions like Bishop Creek where PGI alleles 1 and 4 co-occur. In regions with low PGI
363 polymorphism (e.g. BPC and RC), strong selection resulting from climate extremes may result in
364 local extinction. Our data on fluctuations in beetle abundance in all three drainages is consistent
365 with this hypothesis, because BC populations appear to fluctuate less in distribution and
366 abundance than populations in the other two drainages (Table 2).

367 The relationship between PGI variation and metabolic rate suggests that individuals
368 possessing the PGI 1 allele are less sensitive to thermal variation (have a lower Q_{10}) than 4-4

369 individuals, but that 4-4 individuals have the highest metabolic rates (consistent with enhanced
370 performance) at temperatures near the maximum measured in nature (36°C). This pattern is
371 unusual, as populations or species with high Q_{10} values for metabolic rate are typically most
372 thermally sensitive (Nielsen et al. 1999; Terblanche and Chown 2007). However, differences in
373 thermal tolerance among PGI genotypes may be mediated by the heat shock response. Prior
374 studies have revealed that PGI 1-1 and 1-4 genotypes express higher levels of heat shock
375 proteins (Hsp70) at moderately elevated temperatures than PGI 4-4 homozygotes, and that they
376 survive single episodes of stressful temperature exposure better than PGI 4-4 homozygotes
377 (Nearing et al. 2003; Rank and Dahlhoff 2002). In contrast, PGI 4-4 individuals express
378 higher levels of Hsps at extreme temperatures and upregulation Hsp70 expression at higher
379 temperatures than PGI 1-1 or PGI 1-4 genotypes, and they also survive and recover more
380 effectively from repeated exposure to stressful temperatures (Rank et al. 2007). Together, these
381 data suggest that the temperature “set point” for 4-4 genotypes may be near the most extreme
382 temperatures beetles experience in nature (Dahlhoff and Rank 2007).

383 The fecundity data reported here show that individuals possessing allele 1 have the
384 greatest reproductive success under mild climatic conditions. Fecundity of females held in the
385 laboratory at 20°C was greatest for PGI 1-1 and PGI 1-4 genotypes, and reduced for PGI 4-4
386 genotypes. This result is consistent with previous findings comparing larval growth among PGI
387 genotypes under mild conditions in the laboratory (McMillan et al. 2005) and with the
388 hypothesis described above. One probable mechanism underlying these results may involve the
389 heat shock response. There is a positive correlation between fecundity and Hsp70 expression,
390 independent of acclimation temperature. This is surprising, as most studies have suggested that
391 upregulation of the heat shock response ultimately results in a performance or fitness cost (Feder

392 et al. 1996; Krebs and Feder 1998; Pedersen et al. 2005; Sorensen and Loeschcke 2004).
393 However, the data reported here suggest that Hsp70 expression may protect females from
394 reduction in fecundity caused by thermal stress. This hypothesis is supported by the fact that
395 individuals possessing PGI allele 1, which upregulate Hsp70 expression at lower temperatures,
396 have the highest fecundity. Individuals possessing allele 4 do not upregulate Hsp70 expression
397 until much higher temperatures, and thus reproductive output may not be maintained at moderate
398 temperatures. The metabolic rate, fecundity and Hsp70 expression results all suggest a possible
399 mechanism for observed shifts in PGI allele frequency observed in Bishop Creek. PGI allele 1
400 increased in frequency between over-wintered adults and larvae (high fecundity and robust heat
401 shock response of females possessing allele 1 during exposure to mild early summer climate),
402 but 4 allele increases in frequency later in the summer (better survival and robust heat shock
403 response for larvae possessing PGI allele 4 during exposure to repeatedly hot, extreme
404 temperatures later in summer).

405 Differences among PGI genotypes in reproductive output in the field depended on the
406 drainage to which BC beetles were transplanted. In the warm southern drainage BPC, PGI allele
407 4 was associated with increased fecundity, while in the cooler northern drainage RC,
408 reproductive output was greater for individuals possessing PGI allele 1. Fecundity of all three
409 genotypes was high in Bishop Creek. There is therefore a general correspondence between the
410 geographic distribution of PGI genotypes across the three drainages and the relative ranking of
411 each genotype in the field fecundity experiment. As conditions change, we would expect these
412 rankings to change. Nevertheless, one expected outcome of long term differences among female
413 PGI genotypes in fecundity would be that different alleles would predominate in different
414 drainages, as we observed.

415 These results suggest that suites of characteristics which adapt the *C.aeneicollis*
416 populations to their respective regions, but suggest that BC populations, which may have the
417 highest genetic diversity, will survive best in an unpredictable climate. Individuals homozygous
418 for the PGI allele 4 lay more eggs at warmer sites (BPC, BC), survive best during the warmest
419 part of the growing season, upregulate Hsp70 only after repeated heat stress, and have a higher
420 metabolic rate than other genotypes at 36°C, near the highest body temperatures observed in
421 nature. However, populations in which allele 4 predominates (e.g. BPC) have shifted upwards in
422 elevation by about 300 m in elevation in the past decade, suggesting that there is a limit to their
423 tolerance of thermal extremes. Individuals homozygous for allele 1 lay more eggs than 4-4
424 genotypes under mild conditions in the laboratory and field, survive best in the cooler part of the
425 growing season, rapidly upregulate Hsp70 expression in response to exposure to a single thermal
426 stress (hot or cold), and effects of temperature on metabolic rate is less sensitive to temperature
427 (i.e. lower Q_{10}) than PGI 4-4 homozygotes. Populations in which allele 1 predominates are
428 found in the cooler, Rock Creek and northern Bishop Creek drainages, and have also shifted
429 upwards in elevation. The populations most highly polymorphic at PGI are found in South
430 Bishop Creek. These populations appear to have a mix of both southern and northern traits, and
431 are potentially able to adapt to increased unpredictability in climate variability by evolving *in*
432 *situ*, rather than moving upwards in elevation. The upward shift in BC populations is less
433 pronounced than in the nearly monomorphic populations to the north and south. We predict that
434 in these BC populations, we will observe increase in frequency of PGI allele 4 if summertime air
435 temperatures continue to rise, and sites continue to experience repeated high daytime and
436 extreme low nighttime temperatures in early spring due to loss of snow pack. If conditions
437 return to being mild, with wet springs and abundant winter snow pack, we expect a shift towards

438 the 1 allele, as was observed during the last cool, wet period.

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

452 **Acknowledgements**

453 We gratefully acknowledge J. Zatorski, P. Kudoo, S. Hurley, T. Goodwin, B. Becker, K.
454 Mulkey, J. Freeto, and S. Anthony for hiking many miles to locate beetle populations. We also
455 thank C. Bayless, B. Butzman, E. Strode, J. Hollister and A. Keil for same, as well as for their
456 countless hours spent measuring fecundity and mating success in the laboratory and field. D.
457 Hollis helped obtain the genotypes for beetles in the metabolic rate experiment. We thank the
458 director and staff of the White Mountain Research Station for providing laboratory and housing
459 facilities. We are especially grateful to M. Elekonich of University of Nevada, Las Vegas, for
460 allowing A. Gibbs and E. Dahlhoff the use of her laboratory to measure beetle metabolic rates.
461 This research was supported by National Science Foundation award (IBN-RUI-
462 0078464/0078659) to E. Dahlhoff and N. Rank, by a SCU Presidential Research grant to E.
463 Dahlhoff, by a mini-grant from WMRS for housing support of D. Bruce and S. Fearnley, and a
464 Sonoma State University Undergraduate Research Grant to D. Hollis.

Table 1. Population genetics sample sizes and sampling localities.

Basin	Sub-drainage	Sites	N	Elevation (m)
Rock Creek (RC)*		3	153	2705-3317
North Bishop Creek	Paiute Pass (PP)	9	166	2564-3276
	Blue Lake (BL)	3	48	3127-3164
	Lake Sabrina (LS)	7	138	2875-3274
South Bishop Creek	Tyee Lakes (TL)	2	66	3134-3227
	Green Lake (GL)	10	288	2760-3286
	Chocolate Lakes (CL)	10	192	2983-3230
Big Pine Creek (BPC)*		3	60	2960-3332

*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 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.

Big Pine Creek

YEAR	2700-2800	2850-2930	2996-3100	3130-3200	3220-3300	3310-3450	>3500
1998	●	●	●	●	●	○	○
2003	○	●	●	●	●	●	○
2007	○	○	○	●	●	●	●

South Bishop Creek

YEAR	2700-2800	2850-2930	2996-3100	3130-3200	3220-3300	3310-3450	>3500
1998	●	●	●	●	●	●	○
2003	○	●	●	●	●	●	○
2007	○	●	●	●	●	●	○

Rock Creek

YEAR	2700-2800	2850-2930	2996-3100	3130-3200	3220-3300	3310-3450	>3500
1998	●	●	●	●	○	○	○
2003	●	●	●	●	●	○	○
2007	○	○	●	●	●	●	○



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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 *phosphoglucose isomerase* (PGI) along a north-south latitudinal gradient. Data shown are least squares means (\pm SE) of PGI allele 1 frequencies for 2-8 populations per locality. RC- open bar, BC- 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: $y = -0.15x + 3.57$, $R^2 = 0.77$, $F = 17.0$, $P = 0.01$).

Fig 3. Differences among PGI genotypes in effect of temperature on metabolic rate. Data are least squares means (\pm SE) of metabolic rate (indexed by rate of CO₂ 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 (20°C: $n = 23, 30, 3$; 36°C: $n = 31, 24, 7$). Additional statistical analyses reported in text.

Fig 4. Differences among PGI genotypes in cumulative number of eggs produced by Bishop Creek females in the laboratory (A) and leverage plot from ANOVA model showing relationship between Hsp70 expression and cumulative 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 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.

Fig 1

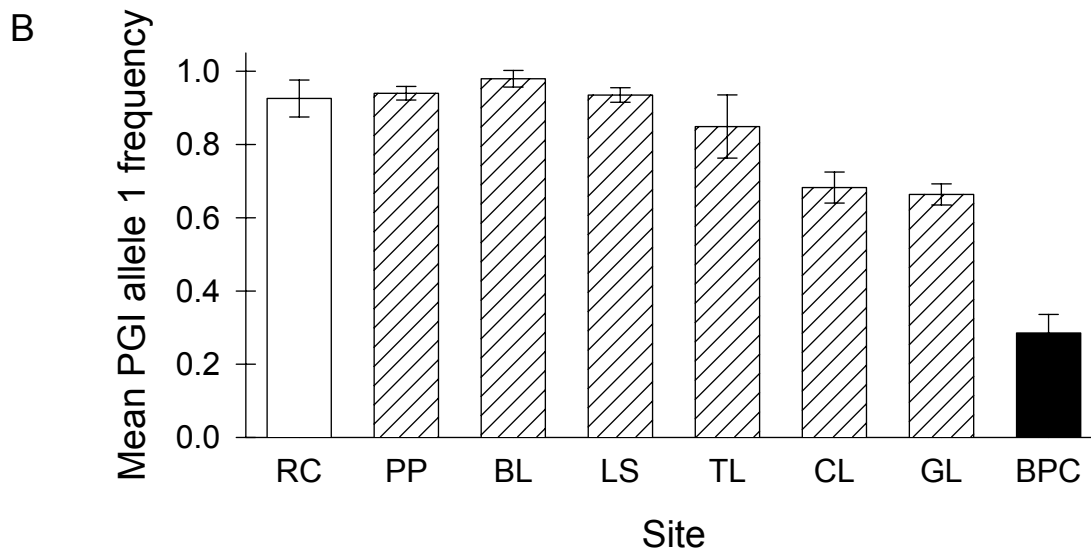
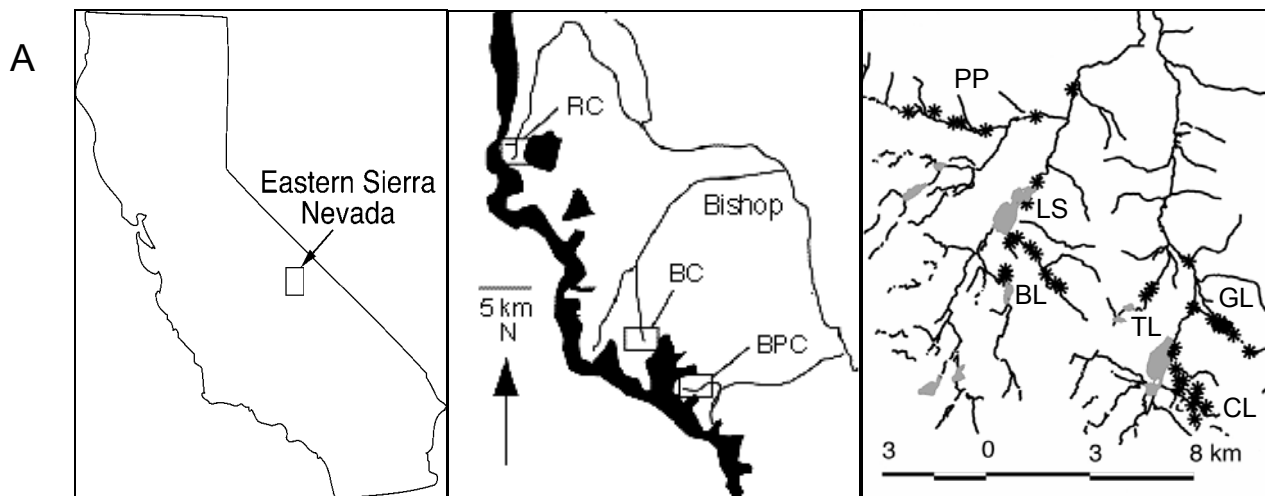


Fig 2

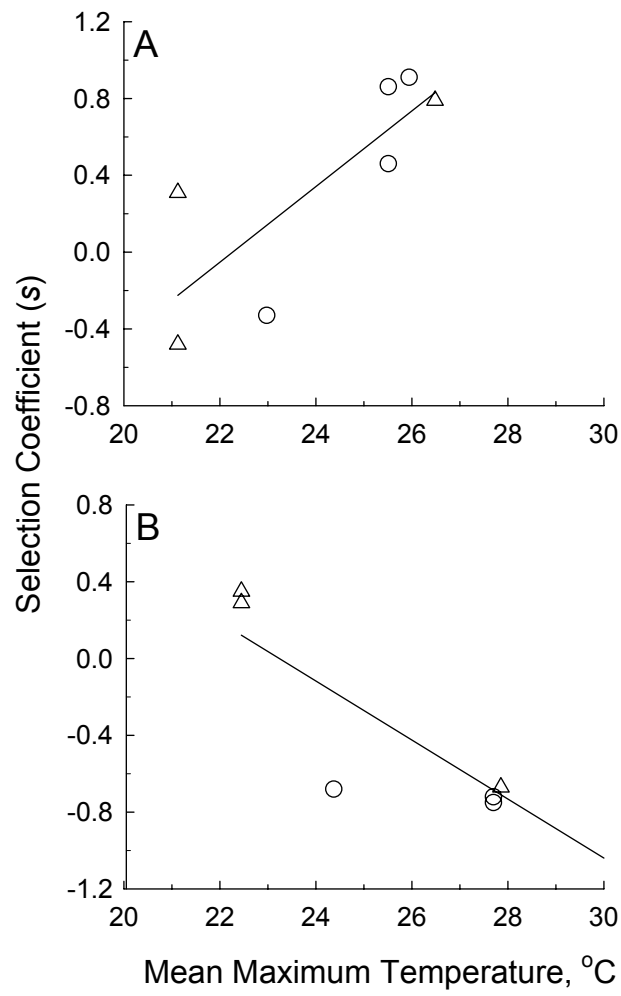


Fig 3

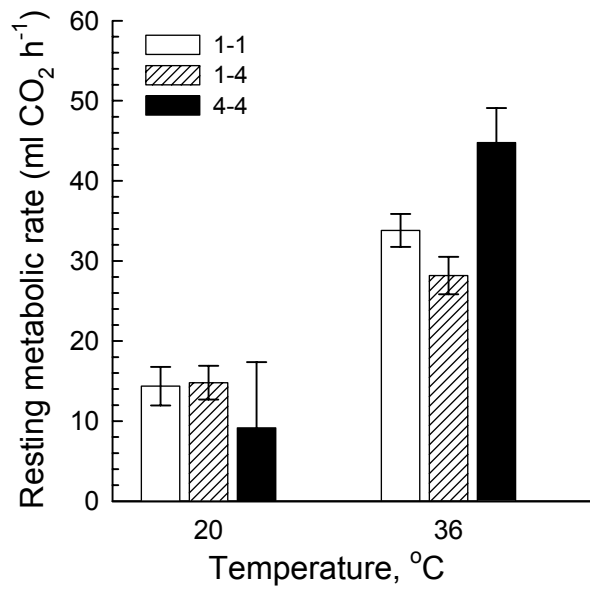


Fig 4

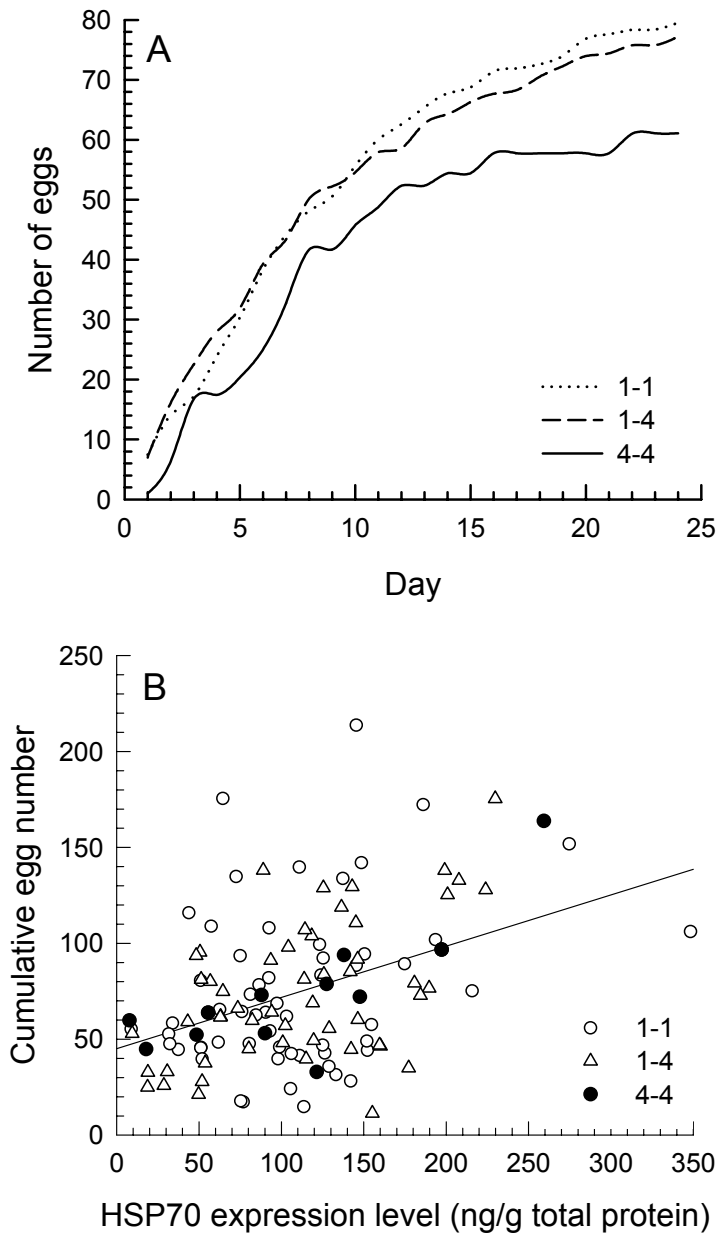


Fig 5

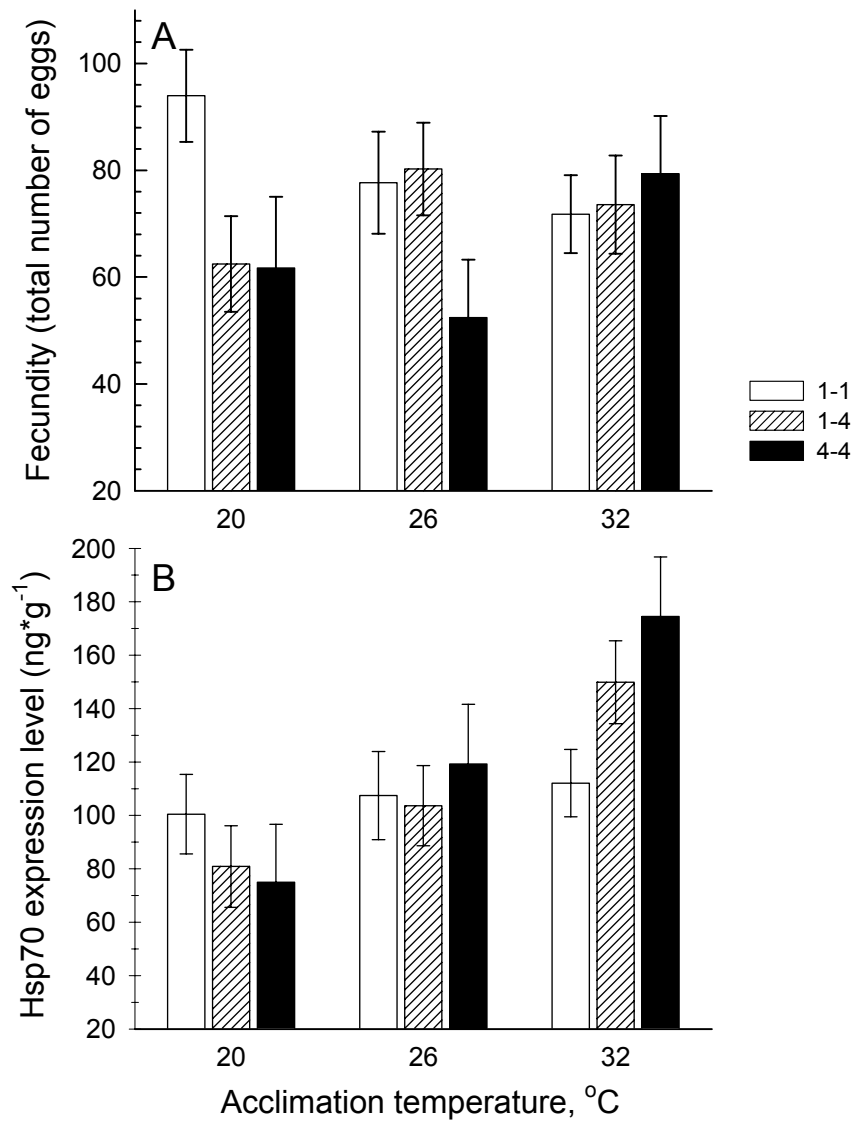


Fig 6

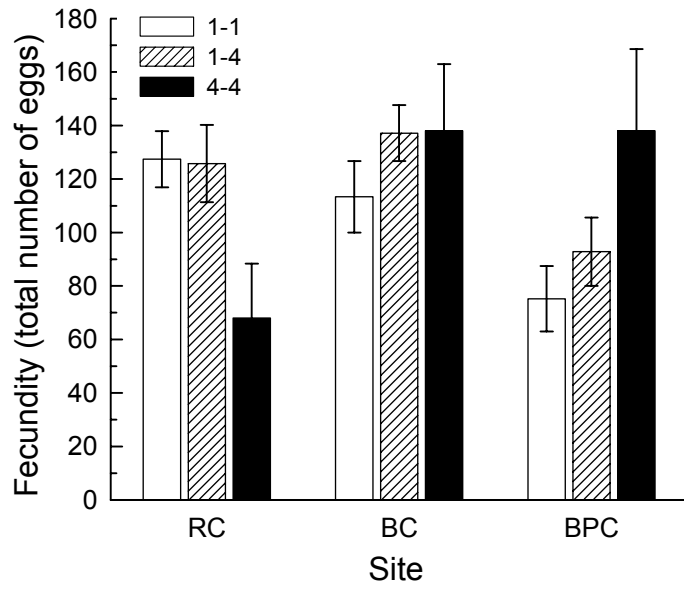


Table S1. Genotype frequencies for three loci (IDH-1, PGI-1, PGM-4) for three life stages sampled in Chocolate and Green Lake sub-populations in the Bishop Creek drainage.

<i>Locus</i>	<i>Genotype</i>	<i>overwintered adults</i>	<i>2nd instar larvae</i>	<i>New adults</i>
PGI		<i>N</i> = 245	<i>N</i> = 244	<i>N</i> = 245
	1-1	0.449	0.553	0.441
	1-2	0.004	0.016	0.012
	1-3	0.000	0.004	---
	1-4	0.445	0.352	0.424
	2-4	---	---	0.020
	4-4	0.102	0.070	0.102
IDH		<i>N</i> = 248	<i>N</i> = 240	<i>N</i> = 245
	1-1	0.218	0.229	0.180
	1-2	0.012	0.004	0.012
	1-3	---	---	0.004
	1-4	0.125	0.083	0.110
	1-5	0.347	0.350	0.294
	2-2	---	---	0.012
	2-3	0.004	---	---
	2-4	0.008	0.008	0.004
	2-5	0.028	0.021	0.033
	4-4	0.008	0.029	0.029
	4-5	0.101	0.104	0.114
	5-5	0.149	0.171	0.208
PGM		<i>N</i> = 184	<i>N</i> = 174	<i>N</i> = 184
	1-1	0.005	0.006	0.011
	1-4	0.163	0.126	0.163
	1-5	0.038	0.040	0.027
	1-6	0.005	0.006	---
	2-2	0.005	---	---
	2-4	0.054	0.080	0.027
	2-5	0.005	0.006	---
	4-4	0.696	0.690	0.707
	5-5	0.011	0.040	0.049
	4-6	0.011	0.006	0.016

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.

Source	df	F
<i>Between-subjects factors</i>		
PGI Genotype (G)	2	2.9*
Acclimation temperature (A)	2	0.3
A X T	4	1.1
Mass, g (M)	1	7.3**
Hsp70 expression level (H)	1	18.5***
Days to first clutch (D)	1	8.0**
<i>Within-subjects factors</i>		
Time	1.8	17.4***
T * G	3.6	1.1
T * A * G	3.6	0.1
T * A * G	7.2	1.3
T* M	1.8	9.7**
T X H	1.8	18.5***
T X D	1.8	13.4***
Error	114	

*** P < 0.0001, ** P < 0.01, * P = 0.05