CYTONUCLEAR INTERACTIONS AMONG METABOLIC ENZYME LOCI MEDIATE REPRODUCTIVE SUCCESS AND LARVAL PERFORMANCE ALONG ENVIRONMENTAL GRADIENTS IN NATURAL POPULATIONS OF A MONTANE LEAF BEETLE

by

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Date

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ABSTRACT

Organisms in natural populations are confronted with significant challenges posed by environmental change. In order for populations to persist, individuals must overcome these challenges; however, the physiological mechanisms that enable survival and reproduction are not well understood. Here, I report the results of a field study that contributes to our understanding of how organisms respond to changing environments. I examined how environmental conditions and an interaction between two metabolic genes influences individual survival and reproduction. I focused on a nuclear gene coding for the glycolytic enzyme *phosphoglucose isomerase (PGI)*, and a mitochondrial gene, *cytochrome oxidase II (COII)*, important for energy production during aerobic metabolism. To investigate this relationship, I studied the Sierra willow leaf beetle *Chrysomela aeneicollis*. In California, beetle populations live at the edge of their species' range in the Eastern Sierra Nevada mountains. Populations are polymorphic at *PGI* and *COII*, and these genes vary along a latitudinal temperature gradient to a greater extent than other genetic markers. I evaluated how local microenvironments where beetles occur differ along elevation gradients, and I also investigated how abiotic stress influences the dynamics of predator-prey interactions. I found that localities varied greatly in abiotic features such as air temperature, sun exposure and soil moisture, and that these site characteristics were associated with elevation. Fecundity of females who laid eggs was greatest for females that possessed the *PGI* genotype and *COII* haplotype most commonly found in the south, but was lowest for those with the southern *PGI* genotype and northern *COII* haplotype. Larval development depended on the interaction between maternal *PGI* genotype and *COII* haplotype, and exhibited the same genotypic pattern observed for fecundity. These results suggest that an interaction is occurring between *PGI* and *COII*. The effect of natural enemies was greatest at low and mid elevation sites, but was lowest at high elevation sites, where beetle egg and larval survival was low regardless of whether they were exposed to enemies. This study may help us predict how individuals with distinct genetic backgrounds adapt to environmental change, and advance our understanding of how organisms persist in challenging environments.

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INTRODUCTION

Organisms in their natural habitats are often confronted with environmental changes that pose challenges for survival and reproduction. These challenges must be overcome for a population to persist and grow under natural conditions. The ability of organisms to survive and reproduce in changing environments depends upon the degree to which they cope with unfavorable conditions (Wilson *et al.* 2006). We could better predict whether a population can persist if we understand how individuals respond metabolically and behaviorally to stressful conditions. The ability to respond to environmental change depends on the natural genetic variation present in the population, and on the conditions to which individuals are exposed. The interaction of environmental and genetic variation may confer tolerance to new conditions and enable novel traits to be expressed, therefore facilitating survival and reproduction (Ghalambor *et al,* 2007; Somero 2010; Hoffmann & Sgrò 2011). While laboratory studies are useful tools to begin to assess the relationship between genes and components of fitness (Hoffmann *et al.* 2003), it is crucial that researchers investigate these processes in nature, where individuals with naturally occurring genetic variation are exposed to environmental conditions under which natural selection operates. It is also important that scientists consider which genes may be particularly susceptible to the forces of selection so that we can deepen our understanding of the mechanisms enabling adaptation.

Metabolic genes are useful genetic markers because they are critical for the conversion of energy that organisms require for life processes (Karl, Schmitt and

Fischer 2008). In addition, metabolic genes can have a profound impact on phenotypic expression of other genes in an organism. For instance, Mitton (1997) describes studies on the common killifish that found that genotype at the glycolytic enzyme, *lactate dehydrogenase (LDH),* directly influences the oxygen saturation of hemoglobin. *LDH* is correlated with ATP concentrations in red blood cells (Powers, Greaney and Place 1979). Depending on *LDH* genotype, the amount of ATP present in red blood cells differs. This has important implications because ATP allosterically modifies hemoglobin and decreases the affinity of hemoglobin for oxygen. Thus, certain genotypes have different oxygen saturation levels that directly influence important traits such as hatching time, developmental rate and adult swimming performance. Another metabolic gene that has been the focus of a great deal of research, *phosphoglucose isomerase (PGI)*, has been linked to adaptive traits in a variety of organisms ranging from bacteria (Dykhuizen and Hartl 1983), to butterflies (Watt 1977; Haag *et al.* 2005) to beetles (Rank and Dahlhoff 2002). For example, a study by Kristjan Niitepõld (2010) explored how different thermal conditions and genotypes at *PGI* affected flight metabolism in *Melitaea cinxia*, the Glanville fritillary butterfly. Increased risk of predation, the necessity of flying for foraging sites and the search for oviposition sites makes flight capacity an important fitness component for these insects. Glycolysis occurs during the anaerobic phase of cellular respiration and is critical for providing energy for metabolically demanding activities such as flight. Certain *PGI* genotypes are associated with increased metabolic rates in response to elevated temperature. The *Melitaea* system provides an example of how an

individual's genotype at a metabolic gene and local environment directly affect its adaptive potential.

While it is important to investigate mechanisms of adaptation at *PGI,* little research has been conducted on possible interactions that *PGI* may have with other critical metabolic genes. Epistasis occurs when an interaction between two genes affects phenotype. One might expect that enzymes involved in anaerobic respiration, such as *PGI,* could closely interact with genes coding for aerobic respiration, including those on the mitochondrion. These genes have long been investigated as neutral genetic markers (Moritz 1994; Rand, Dorfsman and Kann 1994; Allendorf and Seeb 2000). However, a growing number of studies indicate that the mitochondrion may be subject to the process of selection (Rand *et al.* 2001; James and Ballard 2003; Ballard and Whitlock 2004; Dowling *et al.* 2008). For example, Grant, Spies and Canion (2006) found clines in haplotype frequencies in populations of North Pacific and Bering Sea walleye pollock that were correlated with water temperature. The investigators ruled out the possibility that these clines had arisen by random genetic drift, therefore supporting the role of selection as a main driver for the observed clines. In species of *Drosophila*, the ratio of amino acid replacement mutation to synonymous mutation in the mitochondrion is higher than expected under a neutral hypothesis (Ballard and Kreitman 1994). Finally, evidence for selection on the mitochondrion has been highlighted in the medical field as humans with certain mitochondrial haplotypes that differ with respect to susceptibility to Alzheimer's disease (Chagnon *et al* 1999; Lakatos *et al* 2010). As our knowledge regarding the forces of selection on mitochondria

increases, we will also learn more about important epistatic interactions between mitochondrial genes and other genes crucial for metabolism.

When genetically variable individuals from different environments of a species' range meet, a unique opportunity arises to study how environment and genetics influence population persistence. Studies of the willow leaf beetle *Chrysomela aeneicollis*, have found a concordance between *PGI* polymorphisms and the mitochondrial gene, *cytochrome oxidase II (COII)* (Figure 1; Rank *et al.* in prep). These two genes vary similarly along the latitudinal temperature gradient along which beetle populations occur. The concordance between *PGI* and *COII* is striking because other nuclear genetic markers, including seven microsatellite loci and five allozyme loci, do not show high levels of differentiation along the gradient (Rank *et al.* in prep). One hypothesis is that the two loci are under selection; another hypothesis is that the observed concordance may be due to historical migration or genetic drift (Latta, Linhart and Mitton 2001). Selection is the more likely process that explains the concordance seen between *PGI* and *COII,* however this hypothesis has yet to be studied in this system.

In this thesis, I explore how environmental heterogeneity and genetic composition at *PGI* and *COII* influence persistence of *C. aeneicollis* populations living at high elevation in the Sierra Nevada mountains of California. My goal was to investigate the possible epistatic interaction between *PGI* and *COII.* To achieve this, I asked several questions; first, how do localities where beetles occur vary in topography and

microclimate? Second, how does local environment and genetic background affect egg production, hatch rate, larval survival and development rate? Third, how do natural predators affect larval survival? To answer these questions, topographic, geographic and climate variables were quantified among sites to identify differences in site characteristics. Second, female fecundity, the number of days it took eggs to hatch, and development of resulting larvae were measured at sites along elevational gradients within drainages, and between drainages. Third, eggs and larvae were exposed to natural predators to examine interactions between abiotic and biotic stress. Genotypes at three allozyme loci were found and represent maternal beetle genotype; which obviously influences offspring genotype and limits the range of genotypes possible in the offspring. Maternal haplotype for the mitochondrial gene *cytochrome oxidase II* was determined and offspring haplotype was understood to be the same as the mother's due to maternal inheritance of the mitochondrial genome. These metabolic markers were assessed for their influence on life history traits in *C. aeneicollis* because they are proteins critical for metabolism and may be interacting to confer fitness advantages among individuals.

MATERIALS AND METHODS

Study system

The Sierra willow beetle, *Chrysomela aeneicollis* (family Chrysomelidae) inhabits a geographic range that extends throughout western North America (Brown 1956). In California, these beetles live at the southern-most edge of their geographic range, in the high mountains of the eastern Sierra Nevada (Smiley & Rank 1986).

Populations of this insect are found along an elevation gradient that extends from 2200 m to 3600 m. Three main creek drainages Rock Creek (RC), Bishop Creek (BC) and Big Pine Creek (BPC) that are the focus of this study are separated by the jagged Sierra crest at high elevations and the arid, high desert landscape of the Owen's Valley at their base (Figure 2). Study drainages differ greatly in their average temperatures. The northern drainage Rock Creek (RC) is the coolest, the southern drainage Big Pine Creek (BPC) is the warmest, and Bishop Creek (BC) is intermediate in location and temperature (Dahlhoff & Rank 2000)(Figure 2). Prior work in the Sierra willow beetle system suggests that *PGI* is under selection in response to thermal variation among population localities. Allele frequencies at the *PGI* locus differ between the three study drainages (Rank 1992b; Dahlhoff & Rank 2000). RC has the highest frequency of allele 1, BPC has the highest frequency of allele 4 and BC shows a roughly equal proportion of alleles 1 and 4 (Rank 1992b). *PGI* allele 1 is more thermolabile, while the *PGI* 4 allele is more thermostable (Dahlhoff and Rank 2000). Studies indicate that *PGI* genotype is associated with performance and fitness differences among individuals (McMillan *et al*. 2005; Rank *et al.* 2007; Dahlhoff *et al.* 2008; Dick *et al*. 2013). The mitochondrial gene, *COII,* expresses unique haplotypes in southern and northern Sierra populations of *C. aeneicollis.* Bishop Creek beetles possess haplotype frequencies that are intermediate between northern and southern populations (Fearnley *et al.* 2003) (Figure 1; adapted from Fearnley 2003).

Beetles are found on willows, primarily the Sierra willow *Salix orestera*, living along creeks and lakes in these drainages (Rank 1992a). They experience a wide range

of air temperatures in summertime, exceeding 35˚C during hot days and sometimes reaching as low as -6˚C on cold nights (Rank 1994; Rank & Dahlhoff 2002; Neargarder *et. al* 2003). Several time-points in beetle life history are important for population persistence during summer: overwintered *C. aeneicollis* adults must emerge from diapause and mate; females must lay eggs, eggs must develop into larvae and proceed through three instar stages before pupating. At the end of the summer season, new adults must enter diapause in the leaf litter at the base of their host plant before the onset of winter. These critical points in the beetle's life cycle are important factors influencing survival of this species.

Characterizing topographic and climatic variation among sites

Quantifiying topographic variables - Elevation, latitude and longitude at each study site were recorded using a hand-held Global Positioning System device (Trimble Navigation Limited, Sunnyvale, CA, USA). Sites were classified into categories based upon their elevation: 'low' sites, 'mid' sites and 'high' sites; Table 1). A USGS 10-meter digital elevation model (National Geographic Database) was used to calculate topographic moisture index (TMI) and solar insolation (SII) value at each site during summer (Table 1). TMI values describe the effects of topography on soil moisture as the natural log of the ratio between upslope drainage area and the slope gradient of a given grid cell (Moore, Grayson, & Ladson, 1991). SII values describe the amount of radiant energy hitting a given location in watt-hours per meter squared. Calculations are based on a hemispherical view shed algorithm that accounts for direct and diffuse insolation using a sun and sky map (Rich 1990; Rich *et al. 1994;* Fu & Rich 2000; Fu &

Rich 2002). All geographic and topographic variables were derived using ArcGIS 10 (Esri, Redlands, CA, USA). We conducted correlation analyses of elevation, TMI, SII value and average temperature at each site using JMP (Version 10.0; SAS Institute, Inc. Cary, North Carolina, USA).

Microclimate measurements - Hobo pendant loggers (Onset Computer Corporation, Bourne, Massachusetts, USA) have been deployed at each site used in this experiment for the last 15 years. Loggers are suspended in inverted white plastic cups in the willow canopy and record daily temperature readings every half-hour. Upon completion of our experiment, logger data was downloaded and daily averages of minimum, maximum and mean temperatures were calculated for the summer experiment period. Two-day averages for minimum, maximum and mean temperature at each site were also computed. Growing degree-day (GDD) values were also computed from these data using Hoboware Pro (Onset Computer Corp.). The minimum daily threshold was set to -12˚C, a temperature that is known to be below the critical minimum lethal temperature (LT_{min50}) for *C. aeneicollis* (Rank & Dahlhoff 2002; Neargarder *et al.* 2003) and well below the lowest minimum temperature observed in nature at these sites. Temperature data for one site, Red Rock Slide (RRS) was interpolated from June 23 through July 7 due to short-term logger failure. To estimate the temperatures at RRS, we first conducted regressions of daily summer logger data at the 10 other sites against the daily logger data we had for RRS. Correlations between Bluff Lake (BL) and RRS were strongest, therefore BL temperatures were used to predict temperature values at RRS. Predicted values were

saved and these values were substituted for the 15 days of missing logger data. To analyze the summer temperature data, we calculated two-day average maximum, minimum and average temperatures and conducted a mixed model analysis (Version 9.2; SAS Institute, Inc. Cary, NC) with site as a random effect nested in elevation category, and day of year and elevation category as fixed effects.

Experimental design

During summer 2011, we conducted a field experiment at 11 sites located along four elevation transects in the Eastern Sierra (Table 1). Experimental localities (hereafter "outplant sites") were chosen that were known to have natural beetle populations occur there within the last 15 years, and have been part of a long-term investigation of beetle abundance. We collected adult beetles from two central populations in Bishop Creek where roughly equal proportions of the *PGI* 1 and 4 allele occur (Rank 1992b; Dahlhoff & Rank 2000; Rank & Dahlhoff 2002), as well as common mitochondrial COII haplotypes (Figure 1; Fearnley 2003). Beetles outplanted to low and medium elevation sites were collected from a mid elevation site in Bishop Creek with an earlier phenology, (37˚ 10’ 37.6” N, 118˚ 33’ 04.5” W; 3200 m), whereas beetles for high elevation experiment sites were collected from a higher elevation site with later phenology, located about 2 kilometers away (37˚ 09’ 06.3” N, 118˚ 33’ 29” W; 3350 m). After collection, adult beetles were returned to the laboratory and immediately sexed by microscopic examination of abdomen. Males and females were held separately until start of experiment. Beetles were held in the lab for 1-3 days

before being placed back in the field; any eggs laid by females during this time were discarded.

Thirty unique *Salix orestera* clones, still in their flowering stage, were identified at each site. Male-female beetle pairs were randomly assigned by removing one of each sex out of cups into which they had been sorted and placed into white mesh bags, following published methods (Dahlhoff *et al*. 2008). Mating pairs were placed at two or three sites in each drainage, which created a replicated environmental gradient among drainages. This study was designed to maximize the probability that each locus genotype and haplotype would be represented at each outplant site. The experiment included a three factorial analysis of variance (ANOVA): *PGI* genotype, mitochondrial *COII* haplotype, and site. Features of the environment, including air temperature, TMI and SII at these sites were used as site characters in these analyses. The elevational gradient was replicated along the four transects.

Statistical approach

To analyze data that conformed to the assumptions of parametric statics, linear mixed model (LMM) analyses were performed (SAS 9.2). Each model was fit using restricted maximum likelihood. For each global model, a suite of common covariance structures was tested. The lowest Akaike Information Criterion (AIC) was used to select the best covariance structure for the final model fit. The variance components covariance structure produced the lowest AIC score and was used for all linear mixed model analyses. To identify the best model to explain the data, all possible subsets of the global models were analyzed by maximum likelihood using AIC. Models with the

lowest AIC value and most biologically relevant parameters were retained in the final model. The Kenward-Rogers method for calculating degrees of freedom was used for all models. Residuals for each model were visually evaluated for normality. Drainage was initially included as a random term in each of the analyses; however during the model fit process, the drainage covariance parameter estimate was repeatedly estimated to be zero and was removed from all models.

To analyze data from the field experiment, where a large proportion of the data were zeros, Primer v6 (Primer-E Lt, Ivybridge, UK) with the PERMANOVA+ add on was used. PERMANOVA+ does permutation multivariate analysis of variance (PERMANOVA), which relaxes the assumptions of normally distributed data (Anderson, Gorley and Clarke 2008). This was important for the analysis of these data, both of which included important "zero" count data, creating non-normal data distributions which could not be analyzed using parametric statistics. All models run in PERMANOVA were permuted 9999 times and were analyzed with the most robust model that would run.

Measures of adult and larval performance

Female reproductive success- Following installation of adult beetles in the 30 mesh bags at each site, bags were checked every 2-3 d to see if an egg clutch had been laid. Once eggs were laid, we counted numbers of eggs per clutch with a hand-lens and removed adults from the bag. While some females never laid any eggs, some laid multiple clutches throughout the first month of the experiment. After the initial egg clutch was laid, mating pairs were transferred up to 3 more times to new branches on

the same willow. Once a third egg clutch was laid, either the 2nd or 3rd clutch was exposed to predators by removing the mesh bag around the willow branch for use in the predator experiment described below (Figure 3). If a mating pair of beetles never produced an egg clutch, the bagged branch was terminated from the experiment one month after the installation date. Adult beetles were collected at the end of the egg-laying period, flash-frozen and stored at -70°C for genetic analysis. Maximum, minimum and average temperature values were calculated for each site from the installation date until adults were removed from the bags. The number of eggs laid by each female, as well as the zero counts for females that never laid eggs, were imported into PRIMER, square-root transformed, and used to develop a Bray-Curtis similarity matrix. A one-way PERMANOVA examined the effects of site (random factor), *PGI* genotype and *COII* haplotype (fixed factors) on the egg count data. Temperature data were included as covariates in the model after they were imported, normalized and developed into a Euclidean similarity matrix, with site as a factor. LMMs were run on the set of females that were successful in producing offspring after excluding those females that never laid eggs. Each global model contained either site maximum, minimum or average temperature for the first month of the experiment, *PGI* genotype, *COII* haplotype, elevation category and all possible permutations of these factors. The best fitting model included site nested within elevation category listed as a random effect. The square root of the total number of eggs laid by a female was the dependent variable and was examined with average temperature at a site, *PGI* genotype, *COII* haplotype and their interactions.

Quantifying egg hatch rate - In order to quantify the number of days it took for offspring to hatch, clutch oviposition date and hatch date were estimated. Estimated egg clutch oviposition date was calculated by finding the midpoint date between the last observation of zero eggs on a branch and the date it was first observed that eggs had been laid. Estimated hatch dates were calculated by finding the midpoint date between the last observation of only eggs and the date when first instar larvae were first observed. The number of days it took for an egg clutch to hatch was calculated by subtracting the estimated hatch date from the estimated oviposition date. For each site maximum, minimum, and average temperatures were calculated from date of experimental installation until site average hatch date. Time to hatch was calculated using only those egg clutches that were excluded from predators. In preliminary analyses, four different LMMs were calculated, which included *PGI* genotype, *COII* haplotype, elevation category and one of the three site level temperature variables. AIC scores were not improved by including the maximum, minimum or average temperature data as covariates. Thus, the final reduced model included site nested within elevation category and female nested within site and elevation category as random effects. *PGI* genotype, *COII* haplotype, elevation category and the *PGI* by haplotype interaction were the four parameters retained in the final model.

Effect of predators on egg and larval survival - Survival of each egg clutch laid by the females outplanted to the experiment sites were monitored every 2-5 days throughout summer of 2011. Offspring survival was assessed by counting the number of surviving individuals and noting developmental stage (i.e. 16 eggs, thirteen 1st

instars, five 2nd instars, etc.) for the exposed and excluded clutches. If individuals reached pupal or new adult life stage before the end of the experiment (Sept 2011), pupae and new adults were counted and collected. During the weekend of September 4-6th, all remaining branches with larvae that had not pupated were removed. Those larvae were counted, larval stage recorded and collected.

Five critical time points in an egg clutch's lifecycle were identified: 1) the initial number of eggs laid; 2) the proportion of eggs that hatched into first instar larvae; 3) the proportion of firsts that molted to second instars; 4) the proportion of seconds that molted to thirds; and 5) the proportion of thirds that survived to the pre-pupal stage. These stages were used to construct mortality curves, which represent clutch survivorship over the summer. Curves were constructed based upon site elevation classifications and illustrate the difference in survivorship between clutches that were exposed or excluded to predators.

For this analysis, plants were used that had clutch data for one excluded and exposed branch. This pairing of branches resulted in a balanced design by female. Large numbers of offspring did not survive the duration of the experiment, creating many data points with 'zero' counts for the proportion of larvae survived. These data were imported into Primer and a zero-adjusted Bray-Curtis similarity matrix was created. Two random factors, site nested within elevation category and female nested within site and elevation category, were included in this analysis. A full model of treatment, elevation category, treatment interacted with site and treatment

interacted with elevation category was analyzed. To explore the differences in the proportion of larvae survived between the treatment and elevation interaction, pairwise tests were conducted using the square root of the pseudo-F statistic (t-test) with 9999 permutations.

Quantifying larval development rate - Number and life stage of each larva was noted every 3-5 days as described above. For every family excluded from predators, development rate was calculated by assigning beetle life stages a number ranging from 0 to 6 (0 = eggs, 1 = 1^{st} instar, 2 = 2^{nd} instar, etc.). Average life stage found on a particular day was calculated as the mean of these assigned values. The development rate for the clutch was then calculated as the slope of the change in average developmental stage found over the field season (Rank 1994, Rank *et al.* 1998). Observations that occurred when the average instar was below 1 (i.e. eggs had not hatched) and above 4.5 (i.e. when more than half of the larvae had pupated) were excluded from the analysis, so that the slope value reflected the time of larval development. Maximum, minimum and average temperatures were calculated for each family from estimated clutch oviposition date until the bag was terminated from the experiment. The sum of daily growing degrees for each clutch for the same time period was also calculated as described previously. The LMM for development rate was weighted by the number of observations an egg clutch was visited over the summer in SAS. Five global models were tested which included all possible permutations of *PGI* genotype, *COII* haplotype and either maximum, minimum, average or the sum of the growing degree day temperature values. The final reduced,

best fitting model included site nested in elevation category and female nested within site and elevation category as random terms; *PGI* genotype, *COII* haplotype, their interaction and the sum of the GDD values for each egg clutch from the time the branch was installed until the experiment concluded for that particular branch were included as fixed effects.

Genetic analyses

Allozyme genotypes- Adult female beetles collected from the outplant experiment were dissected by cutting where the thorax joins the abdomen. Abdomens were homogenized in water and used for starch gel electrophoresis following published methods (Murphy *et al.* 1990, Rank 1992b). Samples were loaded onto 12.5% potato starch gels (Starch Art Corporation, Smithville, TX) and run at 70 milliamps for approximately 4 hours. Gels were stained with known solutions to produce banding patterns for three enzymes, phosphoglucose isomerase (*PGI*), isocitrate dehydrogenase (IDH) and phosphoglucose mutase (PGM). Each gel was incubated at 38˚C until bands began to appear. Once bands were visible, we scored each individual for the three allozyme genotypes. Samples were rerun if no bands were visible or were questionable in their score.

Mitochondrial haplotypes- DNA from adult female beetles was extracted using the head and thorax following the genomic DNA from tissue protocol in the Nucleospin Tissue Kit (E&K Scientific, Santa Clara, CA.). Polymerase chain reaction (PCR) was used to amplify a 650 basepair (bp) region of the mitochondrial gene, cytochrome oxidase II (*COII*) according to the protocol outlined in Table 2. PCR reactions were performed in

an MJ Research Inc. PTC-100 (Harlow Scientific, Arlington, MA.). Successful amplification was verified by running a 1% agarose gel (Amresco, Solon, OH.), including one lane of 100 bp DNA ladder (Promega Corporation, Madison, WI.), blue loading dye and $_{di}H_2O$ to check for appropriate product size. Gels were stained with ethidium bromide (1 µl/20 mL) then photographed using ImageJ (National Institute of Health, rsb.info.nih.gov/ij/). If no band was present or was extremely faint, PCR amplification was repeated using the Phusion polymerase protocol described in Table 2.

Known COII sequence from *C. aeneicollis* was loaded in Sequencher (Gene Codes Corporation, Ann Arbor, MI.) and two restriction enzymes, Alu1 and Sau3AI, were selected to distinguish between the three most common mitochondrial haplotypes found in the Eastern Sierra experiment beetle populations. Using beetle sequences that had been determined in a previous study, three unique haplotype sequences were loaded onto NEBcutter (New England Biolabs Inc., Ipswich, MA.). A custom digest was run using Alu1 and Sau3AI with a 2% agarose gel on each sequence to see predicted cut patterns.

PCR reactions that yielded positive results were digested according to the recipe outline on Table 2. Digestion reactions were performed in an MJ Research Inc. PTC-100 (Harlow Scientific, Arlington, MA.) and run as described on Table 2. Digestion product was run on a 1.5% agarose gel (Amresco, Solon, OH.) using 15 µl digestion product and 7.5 µl of 3X blue loading dye. One lane of 100 bp DNA ladder (Promega Corporation, Madison, WI.) with blue loading dye was also loaded. Gels were run in

0.5% TBE buffer at 85 volts for approximately 1.5 hours, stained in ethidium bromide (1 µl/20 mL) for 30 minutes and rinsed in distilled water for 15 minutes. Three lanes of known (previously identified) *COII* haplotypes were loaded on each gel to ensure we correctly identified haplotypes.

RESULTS

Characterizing topographic and climatic variables among sites - There were large differences in site topography, geography, and climate in the summer of 2011 among experimental sites (Table 1). Not surprisingly, average temperature decreased with increasing elevation; lower elevation sites tended to be warmer than mid- or high-elevation sites (r^2 = -0.6, p = 0.05; Table 1). As sites increase in elevation, they receive significantly more solar radiation (r^2 = 0.6, p = 0.04). Low elevation sites also had lower topographic moisture index (TMI) values than high elevation sites (Table 1). Two-day average mean and maximum temperatures during the summer also show that high sites were significantly cooler than mid- or low-elevation sites (Figure 4). This effect was especially apparent during the early part of summer; after about a month into the season, temperatures converged and then varied in similar ways (Figure 4).

Female reproductive success- We saw considerable variation in fecundity between high and lower elevation sites. At low elevations, 80% of females laid at least one egg clutch, while at mid elevation, 77% of females laid at least one clutch and at high elevation localities, only 69% of females laid any eggs. When we examined all females, including those that laid no eggs, there was no relationship between genotype at *PGI* or *COII* and fecundity (Table 3). However, for females that laid eggs,

the number of eggs oviposited was significantly related to *PGI* genotype and *COII* haplotype (Table 4). Homozygous 4-4 females with the *COII*-3 haplotype laid the greatest number of eggs, and laid 26 % more eggs than homozygous 4-4 females with the northern *COII*-1 haplotype, which laid the fewest number of eggs (Figure 5; Table 4).

Quantifying egg hatch rate- The number of days it took eggs to hatch after being laid tended to be smallest for larvae whose mothers possessed both the 4-4 *PGI* genotype and *COII*-3 haplotype (Figure 6). Clutches whose mothers were 1-4 heterozygotes and had the *COII*-1 haplotype tended to take longer to hatch (Figure 6, Table 5).

Effect of predators on egg and larval survival- Survival of offspring depended strongly on exclusion treatment; larvae exposed to predators had lower survival than those excluded from enemies (Figures 7 and 8; Table 6). There was a significant difference in survival between high- and mid- elevation sites and between high- and low- elevation sites; the effect of predator treatment depended upon elevation (Table 7). While survival was always lower for larvae exposed to predators, this effect was greatest at low- or mid-elevation (Table 7, Figure 8).

Quantifying development rate of larvae- Summer heat accumulation had a significant effect on larval development rate, such that larval development was slower for those larvae that had higher heat unit accumulation values over the summer (Figure 9). Offspring possessing the southern *COII*-3 haplotype developed more quickly

than larvae with the northern *COII*-1 haplotype (Figure 10, Table 8). *PGI* and *COII* jointly affected larval development rate. There was a 16% development rate difference between the fastest developing *PGI* 4-4, *COII*-3 larvae versus the slowest developing *PGI* 4-4, *COII*-1 larvae (Figure 10, Table 8).

DISCUSSION

Genetic diversity and natural environmental variation are key factors influencing population persistence. Understanding how these factors interact and influence individual survival and reproduction is essential. I studied how localities in the Eastern Sierra differ in microhabitat and found that topographic features such as exposure to sunlight and topographic moisture increased with elevation while overall microhabitat temperatures decreased. The relationship between elevation and temperature was most pronounced during early summer. *Chrysomela aeneicollis* egg production and larval survival was low at high elevation, probably because of cold temperatures. At lower elevations, egg production was higher; however these localities experienced greater mortality due to biotic factors (i.e. natural predators). Genetic composition at two critical metabolic enzymes, *PGI* and *COII,* was assessed and found to have important epistatic interactions influencing female fecundity and larval development rate – two traits that are important life history characteristics and crucial for survival and reproduction.

My localities exhibited a broad range of topographic features and local climatic conditions. Elevation was a clear driver of environmental differences in topographic moisture, solar insolation and climate observed among sites; however, drainages did

not differ much with respect to environmental conditions and measurements of fecundity and development. Low elevation sites were warmer than high elevation sites and tended to have lower topographic moisture indices, which suggests drier soils at low elevation. At high elevation localities, more direct and diffuse energy from the sun hit beetle microhabitats. This may be due to the exposed nature of high elevation sites. These localities are near exposed rock outcroppings and are at or above tree line, which have little or no canopy.

In the beginning of the summer season, high elevation sites were significantly cooler than low and mid elevation sites during the day (maximum temperatures occur during the day), but were more similar in temperature at night (minimum temperatures occur at night; Figure 4). This may have been caused by heat dissipating from air into snow that still covered the high mountain slopes. Cool currents of air may have been flowing from high slopes down to high-elevation experiment localities, lowering daytime temperatures. A month into summer, temperatures at all elevations converged and fluctuated similarly. Temperature patterns observed in early summer might explain why many females laid no eggs. Beetles were outplanted to experiment sites during the cold, early portion of the summer. Conditions at high elevation sites may have been so unfavorable that few eggs were laid. It is possible that metabolic resources were allocated towards surviving the cold at these high elevation sites instead of being used for the energetic demands of reproduction.

Local environmental temperature was also influential in shaping larval development. Offspring who experienced more cumulative growing degree-days (GDD) developed slower (Figure 9). This result may seem paradoxical, but may be explained by recognizing a strong correlation between nighttime air temperature and GDD values. The greater the GDD value, the cooler it was at night and those larvae developed more slowly. Perhaps larvae that experienced cooler temperatures at night suppressed their metabolism, therefore slowing rates of development. Larvae in these locations would have experienced longer development periods, and thus accumulated more growing degree-days. Overall, these larvae would have had slower development rates because they were less metabolically efficient at night in producing the energy necessary for development and other life processes than their counterparts that experienced warmer nighttime temperatures. This result might alternatively be explained if larvae were experiencing heat stress during the day causing slower development; however this explanation seems unlikely because the summer was overall very mild in climate.

Larval families exposed to predators had far lower survival rates than clutches excluded from predators. Clutches that were exposed to predators had higher rates of mortality across all elevations. Survival of larvae excluded from natural predators was much lower at high elevation sites than at low- or mid- elevation sites (Figures 7 and 8). This suggests that natural predators may have had a greater impact on survival at low elevation sites, while at high elevation larval survivorship was low, regardless of the impact of predators. It appears beetles face a tradeoff depending upon the

elevation they inhabit; at low elevation, they are more susceptible to predation, at high elevation they face increased climate stress (specifically cold). Similar results were found in a study by Smiley and Rank (1986). Predation was a major agent of mortality in low elevation populations of *C. aeneicollis,* whereas cold climate conditions at high elevation had a greater effect on survivorship. The interaction between abiotic and biotic stress has been studied to a great extent in marine and rocky intertidal zones (Menge and Sutherland 1987; Tomanek and Helmuth 2002; Taylor and Schiel 2010; Bulleri *et al.* 2011). Harley (2003) describes how a red alga, *Mazzaella parksii,* planted above its upper zone dies due to sun bleaching, while pressure from grazers influences the lower zone of *Mazzaella*. These studies indicate that complex relationships exist between species at different levels of the food web and that abiotic stress is influential in shaping the relationship between predator-prey interactions. Each individual in an ecosystem faces a tradeoff in stress due to biotic forces (such as that sustained from predation) or stress induced by the environment.

Fecundity for females that laid eggs was tightly correlated with genotype at two metabolic enzyme loci. There was a clear advantage in having the warm adapted *PGI* genotype and southern haplotype combination as this pairing laid the greatest number of eggs in nature. In terms of fecundity, genotypes that had the lowest fitness were females with the warm adapted *PGI* 4-4 genotype and northern *COII*-1 haplotype (Figure 5). Similar results were found for the time it took eggs to hatch. Though not statistically significant, after oviposition eggs also hatched fastest from mothers that possessed the 4-4 genotype and *COII*-3 haplotype.

Maternal *PGI* genotype and *COII* haplotype also interacted to influence offspring development rates. In accordance with patterns observed for fecundity and egg hatch rates, offspring developed fastest from mothers that possessed the warm-adapted, southern *PGI* 4-4 genotype and southern *COII*-3 haplotype and slowest when the southern *PGI* 4-4 genotype was paired with the northern *COII*-1 haplotype. Females who possess genotypes that are paired accordingly to the natural genetic differentiation found along the environmental gradient (i.e. northern cool-adapted *PGI* genotypes matched with the northern *COII* haplotype and vice versa) may be able to more effectively mobilize resources produced through metabolism and better provision offspring.

Prior studies on *C. aeneicollis* also describe differences among *PGI* genotypes in female fecundity and larval development. In an experiment reported by Dahlhoff *et al.* 2008, PGI genotype related to egg production of females collected and outplanted to mid- elevation sites in our three main study drainages along the latitudinal gradient. This experiment found that the ranking of *PGI* genotypes with respect to fecundity differed among drainages, with southern genotypes showing higher fecundity in the southern drainage and northern genotypes laying more eggs in the northern drainage. With respect to larval development, McMillan *et al.* 2005 found that families with high proportions of *PGI*-1 alleles developed more rapidly than families with more *PGI*-4 alleles for larvae that had been outplanted to the three main study drainages, and that this relationship was similar for all three outplant localities. These results are similar to those of the current study in that *PGI* genotype and environment related to the

performance and reproductive success response variables, and yet the genotypic differences were not the same. This could arise because the summer climate varies greatly among years. In addition, the current study takes *COII* haplotype into account which was not available for the earlier studies. Haplotype frequencies for *COII* may have been different during those experiments which could have affected *PGI* genotype performance values.

This experiment is the first to demonstrate that mitochondrial haplotype at *COII* has a direct impact on larval performance in *C. aeneicollis*. Larvae possessing the southern *COII*-3 haplotype developed faster than those offspring that had the northern *COII*-1 haplotype. One explanation for this result is that the mitochondrion is also under temperature selection in the beetle populations. Though studies have illustrated that mtDNA is not selectively neutral (Ballard and Kreitman 1994; Chagnon *et al* 1999; Rand *et al.* 2001; James and Ballard 2003; Ballard and James 2004; Grant, Spies and Canion 2006; Lakatos *et al* 2010), to my knowledge, the results presented in this thesis are one of only two studies that establish a link between mitochondrial genes, PGI, and a performance measure (Wheat *et al.* 2011) and this is the only study to directly demonstrate haplotype differences influence reproductive success of individuals in nature.

Our knowledge of the importance of *PGI* in the willow beetle system has been greatly broadened by this study. First, we now have evidence that a nuclear gene important for glycolysis may be involved in an epistatic interaction with another

metabolic gene. Second, this epistatic interaction may be occurring between *PGI* and a mitochondrial gene that is critical for energy production. Third, I have shown that if epistasis is occurring between *PGI* and *COII,* two important fitness traits, female fecundity and larval development, depend on genetic makeup at these two loci. Indeed, other studies indicate epistatic interactions may occur between *PGI* and other metabolic genes, including other mitochondrial genes (Wheat *et al.* 2011). Studies on the Glanville fritillary butterfly in Finland have studied genetic composition in female butterflies at *PGI* and the mitochondrial gene *succinate dehydrogenase d (SDHD)*, an enzyme important for helping create energy that butterflies use for flight. Certain combinations of *PGI* and *SDHD* exhibit better flight performance, important for females that must fly to host plants to lay eggs. This study, in combination with the results I present here, suggest that further research be directed on natural populations to study the interaction that *PGI* and mitochondrial genes have on traits related to fitness.

The importance of epistasis between *PGI* and *COII* was further suggested in early fall of 2011, just one month after the larval development experiment was terminated. An unseasonably early cold snap occurred in the Eastern Sierras producing the first snow. Beetle larvae that had not yet hatched into new adults were found dead on willows; new adults had crawled down the willows and buried themselves in the snow. Dead larvae and live adults were collected and genotyped at *PGI* and *COII.* Results from these data illustrated that beetles possessing *PGI* 4-4 genotypes and *COII*-3 haplotypes had the greatest survival rates after the early freeze, whereas beetles

with *PGI* 4-4 genotypes and *COII*-1 haplotype had the lowest survival rates (unpublished data). This pattern of *PGI* 4-4 homozygotes with the southern haplotype having better fitness than other genetic combinations is the same that was found for female fecundity and larval development. The 2011 experiment data, as well as the early freeze data, exhibit a strong selective advantage for individuals with genetic backgrounds that lay more eggs, have shorter hatch rates and develop more quickly.

Conclusion - The data presented here demonstrate that environmental variation and genetic variation have a profound impact on an individual's fitness. There are complex interactions that occur between microhabitat, biotic stress and genes that influence how individuals survive and reproduce. We must continue to study organisms living in their natural habitats and assess how environment and genetics enable adaptation, as this will provide insight into the effects that global environmental change will have on species.

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Table 1. Characteristics of experiment sites (Summer 2011). TMI = Topographic moisture index; SII = Solar insolation; WH = Watt hours.

Drainage	*Altitude, meters*	*Average temperature (˚C)*	*TMI*	*SII (WH/m^2)*	*Description*
Big Pine Creek	2773[1]	14.7	3.24	7282	Shaded bog
	2906[2]	12.8	1.47	7237	Steep, open bog
	3220[3]	10.8	4.67	7411	Open bog
South Bishop Creek	2870[1]	12.4	3.51	6968	Shaded bog
	3204[3]	13.4	2.11	7694	Stream side
	3376[3]	11.1	7.82	7569	Open area
North Bishop Creek	2998[2]	12.4	7.23	7443	Open bog
	3115[3]	13.2	2.57	7360	Steep, open bog
Rock Creek	2854[1]	11.9	3.53	7552	Along road-side
	3085[3]	11.6	3.52	7259	Open area
	3317[3]	11.3	4.30	7700	Steep, open bog

Categories: 1 = low (2773 – 2870 m), 2 = medium (2906 – 2998 m), high = (3085 - 3376 m)

Table 2. PCR recipes[1], digestion recipe[2] and cycling and digestion protocols used to amplify and digest a region of mitochondrial *COII*.

TAQ polymerase PCR recipe:	**Phusion polymerase PCR recipe:**	**Digestion recipe**
• 5µl Colorless GoTaq Flexi Buffer • 1µl dNTP • 0.5µl of both lapCOIIf and lapCOIIr primers • 8µl $MgCl_2$ • 37.2µl $_{dd}H_2O$ • 0.3µl GoTaq Flexi DNA polymerase • 3 µl template DNA	• 10µl Phusion HF buffer • 1µl dNTP • 2.5µl of both lapCOIIf and lapCOIIr primers • 2.5µl $MgCl_2$ • 28µl $_{dd}H_2O$ • 0.5µl Phusion polymerase • 3 µl template DNA	• 1.5µl NEBuffer 1 • 0.5 µl BSA • 0.25µl Alu1 • 0.25µl Sau3AI • 2.5µl $_{dd}H_2O$ • 10µl PCR product
Cycling protocol:	**Cycling protocol:**	**Digestion protocol**
• Initial denaturation - 95˚C, 120 s • 40 cycles: Denature - 95°C, 30 s Anneal - 51°C, 60 s Extend - 72°C, 60 s • Final extension - 72°C, 7 m	• Initial denaturation - 98°C, 30 s • 35 cycles: Denature - 98°C, 5 s Anneal - 59°C, 30 s Extend - 72°C, 30 s • Final extension - 72°C, 7 m	• Incubation - 37˚C, 2.5 h • Deactivation - 65˚C, 22 m

1 - Reagents purchased from Promega Corporation, Madison, WI. with the exception of the primers which were purchased from Eurofins MWG Operon, Huntsville, AL.

2 – Reagents purchased from New England Biolabs Inc., Ipswich, MA.

Table 3. Mixed-model permuted ANOVA results for effects of site, *PGI* and *COII* haplotype on the number of eggs a female laid. *PGI* and haplotype were treated as fixed effects. Site was treated as a random effect.

Source of Variation	df_{num}	df_{den}	MS	F_{pseudo}	P_{perm}
Site	10	220	4885.1	3.9	0.0002
PGI	2	220	1043.8	0.8	0.4
Haplotype	1	21.5	296.97	0.2	0.7
PGI X Haplotype	2	220	1016	0.8	0.5
Site X Haplotype	10	220	1322.4	1.0	0.4
Residual	220		1264.5		

Table 4. Linear mixed model results evaluating the effects of *PGI*, *COII* haplotype and average temperature on female fecundity for those females that laid eggs. Site nested within elevation and site by *COII* haplotype were treated as random effects. *PGI* genotype, *COII* haplotype, their interaction and average temperature were fixed effects.

Fixed effects	df_{num}	df_{den}	*F*	*P*
PGI	2	167.0	1.1	0.34
Haplotype	1	19.8	2.1	0.16
PGI X Haplotype	2	167.0	4.2	0.02
Average temperature	1	8.9	4.4	0.07
Random effects	**Estimate**	**SE**	***Z***	***P***
Site [Elevation]	1.98	1.2	1.7	0.1
Site X Haplotype	0.10	0.5	0.2	0.8

Table 5. Linear mixed-model results evaluating the effects of *PGI* genotype, *COII* haplotype and elevation on egg hatch rate. *PGI* genotype, *COII* haplotype and elevation were treated as fixed effects. Site nested within elevation and female nested within site and elevation were treated as random effects.

Fixed effects	df_{num}	df_{denom}	*F*	*P*
PGI	2	177	1.2	0.31
Haplotype	1	177	2.9	0.09
PGI X Haplotype	2	179	0.3	0.72
Elevation category	2	4.30	3.3	0.14
Random effects	**Estimate**	**SE**	***Z***	***P***
Site [Elevation]	2.6	3.5	0.7	0.47
Female [Site]	1.0×10^{-4}	1.6×10^{-4}	0.7	0.52

Table 6. Mixed-model nested permuted ANOVA results evaluating the effects of treatment, elevation and site on the proportion of larvae survived in the field. Treatment and elevation were treated as fixed effects. Site and female were treated as random effects and were nested in elevation.

Source of Variation	df_{num}	df_{den}	MS	F_{pseudo}	P_{perm}
Treatment	1	7	15532.0	76.0	0.0001
Elevation	2	7	4535.7	4.5	0.02
Site[Elevation]	7	60	897.7	3.4	0.0008
Treatment X Elevation	2	7	871.7	4.4	0.04
Treatment X Site[Elevation]	7	60	180.0	0.7	0.68
Female [Site]	60	60	263.4	1.1	0.35
Residual	60		243.4		

Table 7. Pairwise comparisons of the effect of predator treatment between different elevations. Comparisons made using permutational ANOVA in Primer v6, PERMANOVA+ software.

	Low vs. Medium		**Low vs. High**		**Medium vs. High**	
Treatment	t	P	t	P	t	P
Excluded	1.07	0.3	2.56	0.03	2.60	0.02
Exposed	1.75	0.1	0.91	0.4	2.31	0.03

Table 8. Linear mixed-model results evaluating the effects of *PGI* genotype, *COII* haplotype and sum of GDD on larval development rate. *PGI*, *COII* haplotype and sumGDD were treated as fixed effects. Site nested within elevation and female nested within site were treated as random effects.

Fixed effects	df_{num}	df_{den}	*F*	*P*
PGI	2	89.1	0.7	0.5
Haplotype	1	84.1	7.3	0.008
PGI X Haplotype	2	90.5	2.8	0.06
sum GDD	1	52.8	6.3	0.01
Random effects	**Estimate**	**SE**	***Z***	***P***
Site [Elevation]	5.9×10^{-7}	1.3×10^{-7}	0.04	1.0
Female [Site]	3.1×10^{-5}	6.3×10^{-5}	0.50	0.6

Figure 1 - Latitudinal distribution of phosphoglucose isomerase (*PGI)* allele frequencies and mitochondrial cytochrome oxidase II *(COII)* haplotypes at different localities in Eastern Sierra *Chrysomela aeneicollis* beetle populations. Haplotype frequencies were determined in 2001; *PGI* frequencies shown for 2009. *PGI* allele 1 predominates in the north, *PGI* allele 4 predominates in the south. *COII* haplotypes vary concordantly along the same environmental gradient.

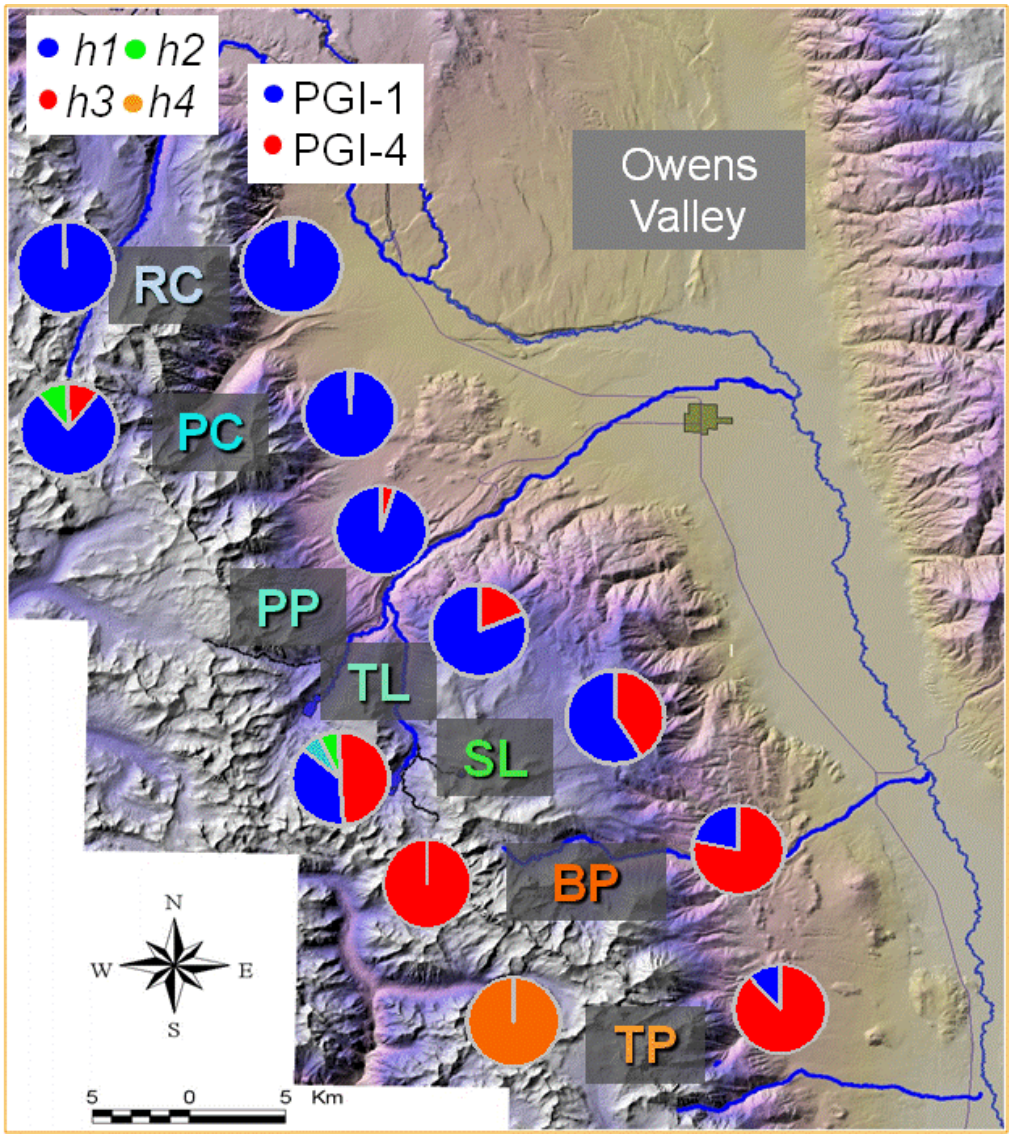

Figure 2 – Map of California showing study populations. (*A)* Experiment sites located in the Eastern Sierra Nevada mountains. (*B*) Three study drainages: Rock Creek (RC), Bishop Creek (BC), and Big Pine Creek (BPC).

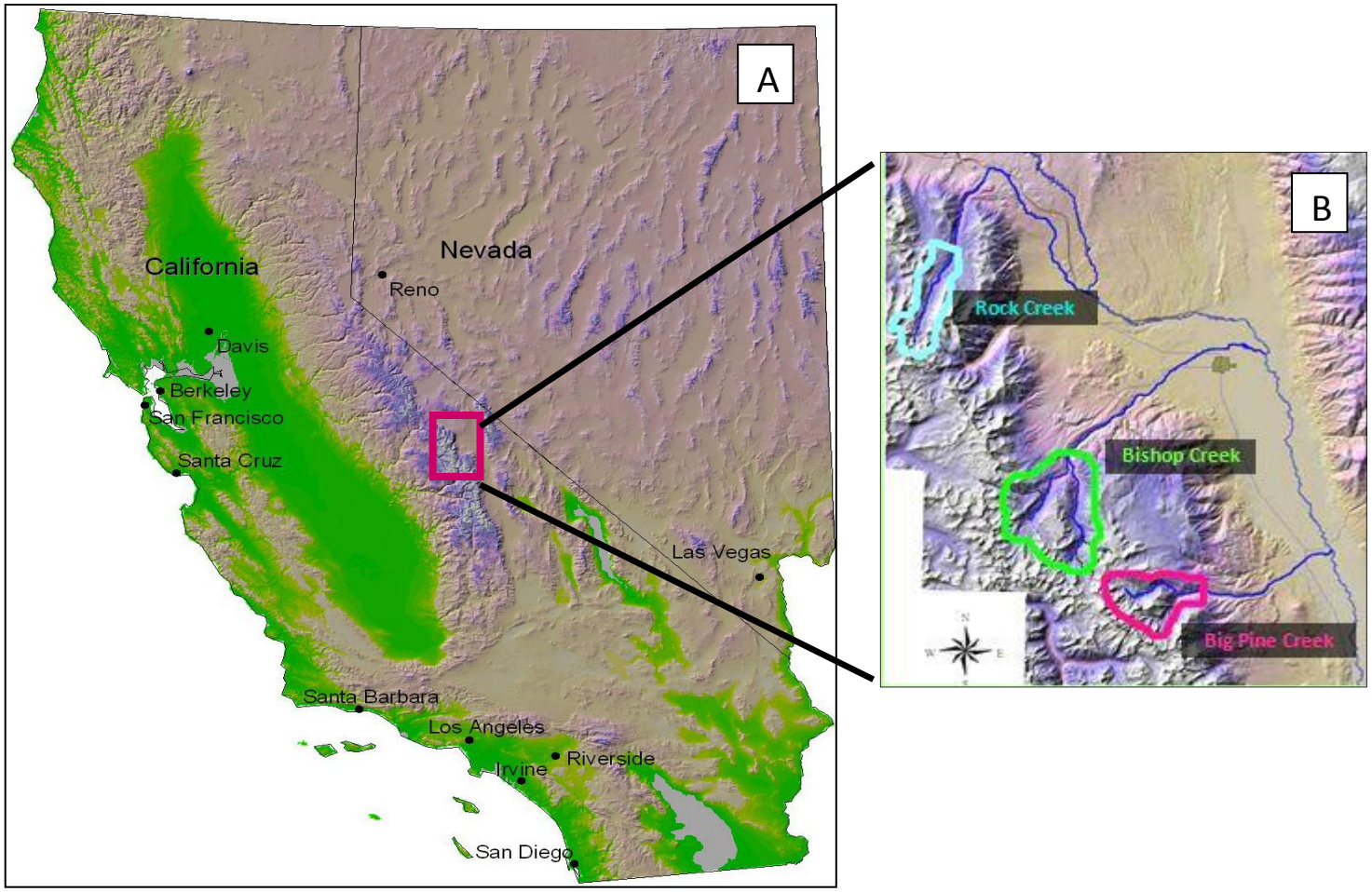

Figure 3 - Experimental set-up. *A*) Experimental branch with larvae excluded from predators and (*B*), beetle family exposed to predators (pictured here, a syrphid fly larvae crawling toward 1st instar beetle larvae)

(*A*)

(*B*)

Figure 4. Maximum, mean and average temperatures at low, mid- and high elevation sites during summer 2011. Number of sites analyzed were n=3 (low elevation), n=2 (mid elevation), n=6 (high elevation); statistical analysis as described in text.

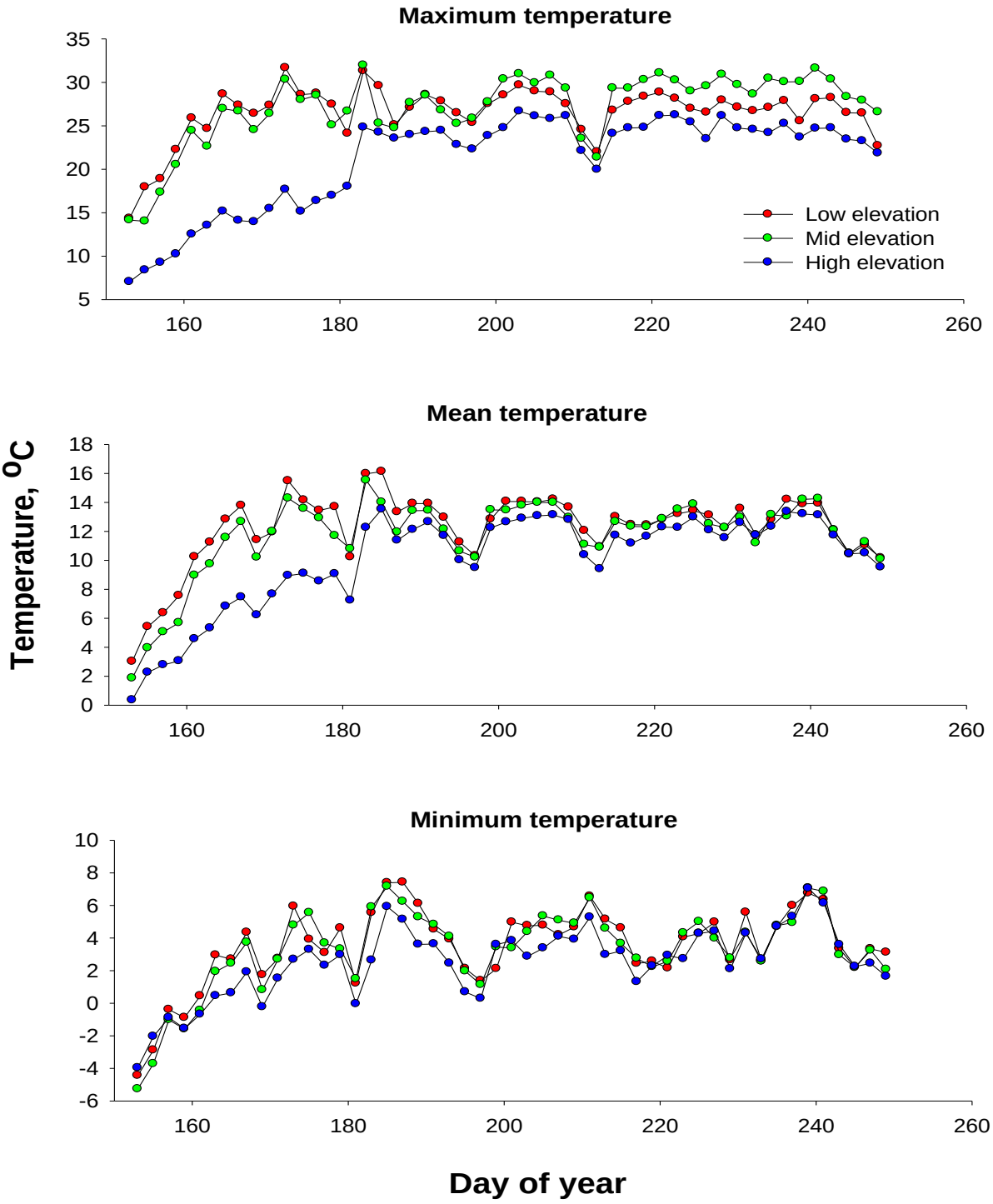

Figure 5 - Effect of maternal *PGI* genotype and *COII* haplotype on the number of eggs laid by experimental females. Open bars, mt-*COII*-3 haplotypes; crosshatched bars, mt-*COII*-1 haplotypes. Data shown are least square means (±SE) of each *PGI* and *COII* combination (1-1, *COII*-1*,* n=37*;* 1-1, *COII-3*, n=31; 1-4, *COII*-1, n=47; 1-4, *COII*-3, n=46; 4-4, *COII*-1, n=13; 4-4, *COII*-3, n=8).

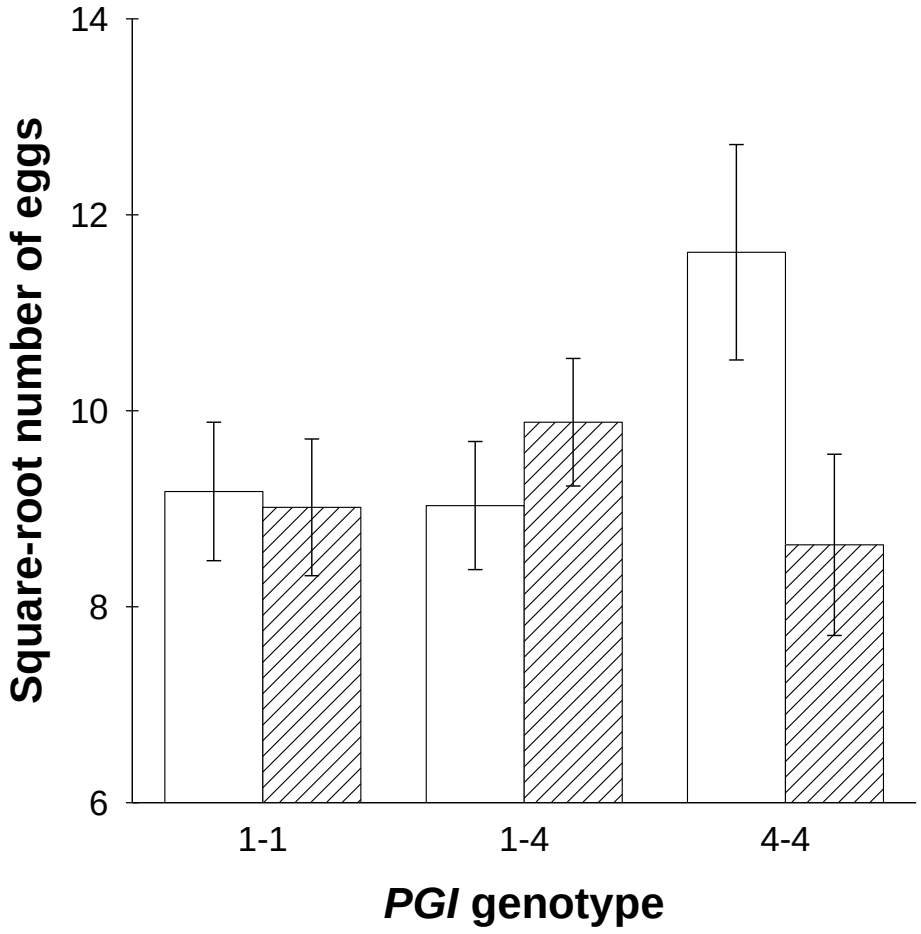

Figure 6 - Effect of maternal *PGI* genotype and *COII* haplotype on the number of days it took eggs to hatch. Open bars, mt-*COII*-3 haplotypes; crosshatched bars, mt-*COII*-1 haplotypes. Data shown are least square means (±SE) of each *PGI* and *COII* combination (1-1, *COII*-1*,* n=33*;* 1-1, *COII-3*, n=29; 1-4, *COII*-1, n=57; 1-4, *COII*-3, n=47; 4-4, *COII*-1, n=12; 4-4, *COII*-3, n=12).

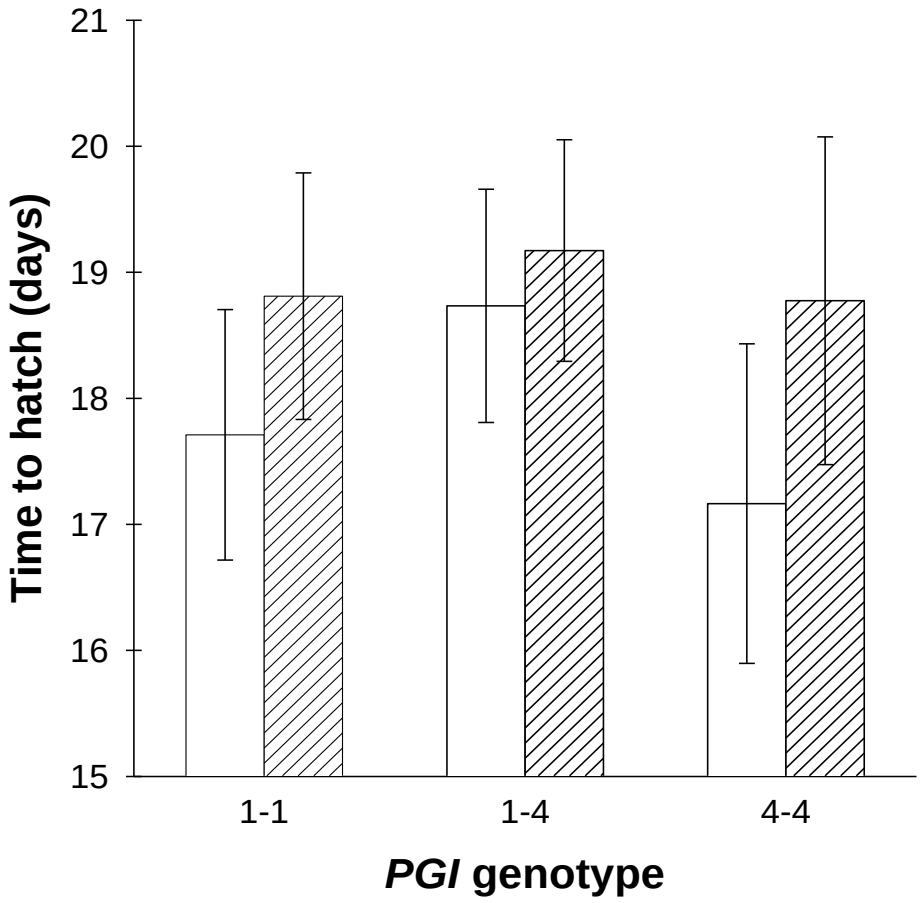

Figure 7 – Survival of *C. aeneicollis* larvae at different life stages excluded from or exposed to natural predators at three different elevations. Data show the proportion of larvae from egg oviposition through the third instar that survived during the experiment.

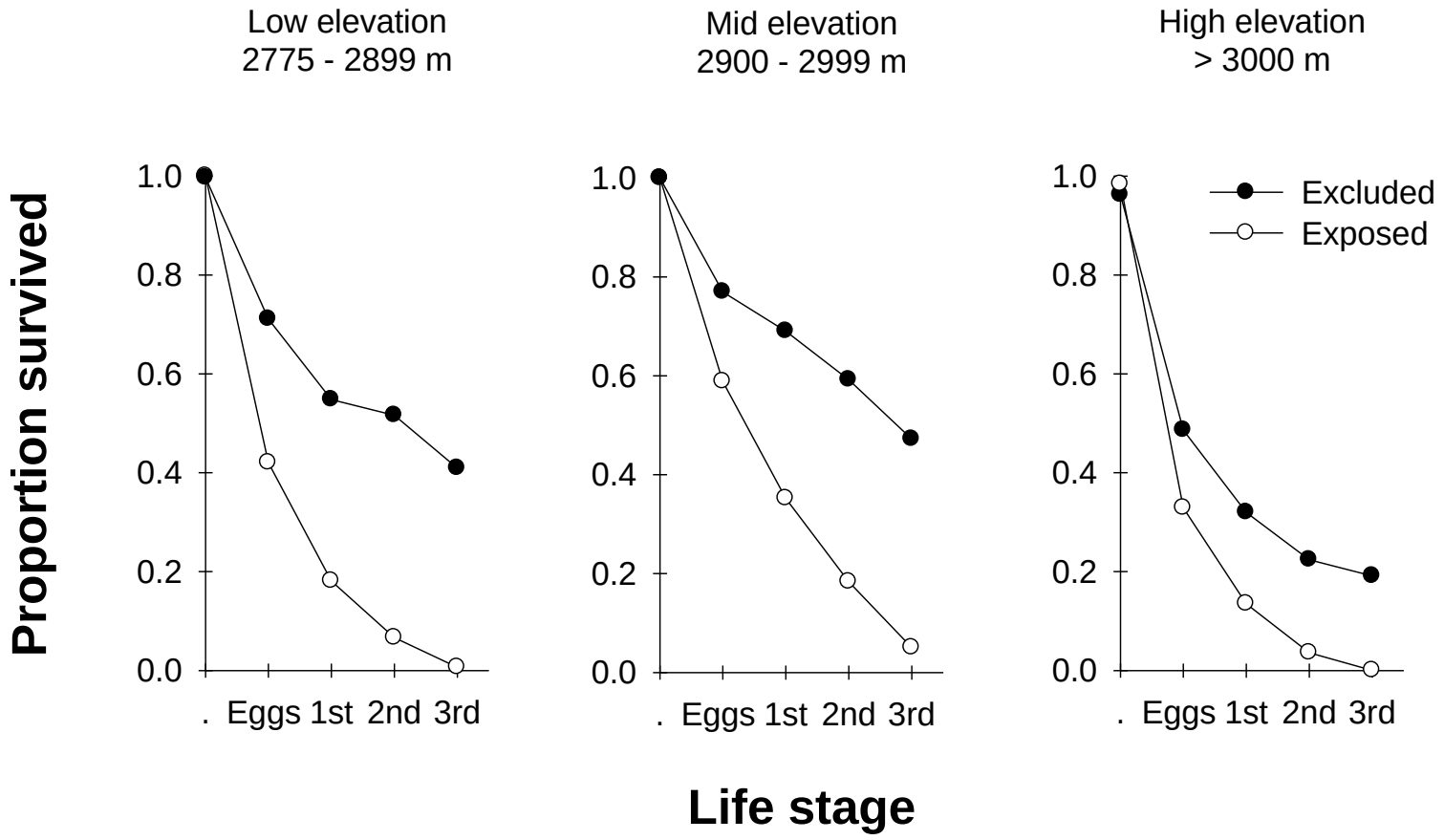

Figure 8 - Proportion survival of *C. aeneicollis* larvae excluded from or exposed to natural predators at three different elevations. Data show mean (±SE) proportion of larvae that survived to end of experiment (Low elevation: excluded n=25, exposed n=25; Mid elevation: excluded n=20, exposed n=20; High elevation: excluded n=25, exposed n=25).

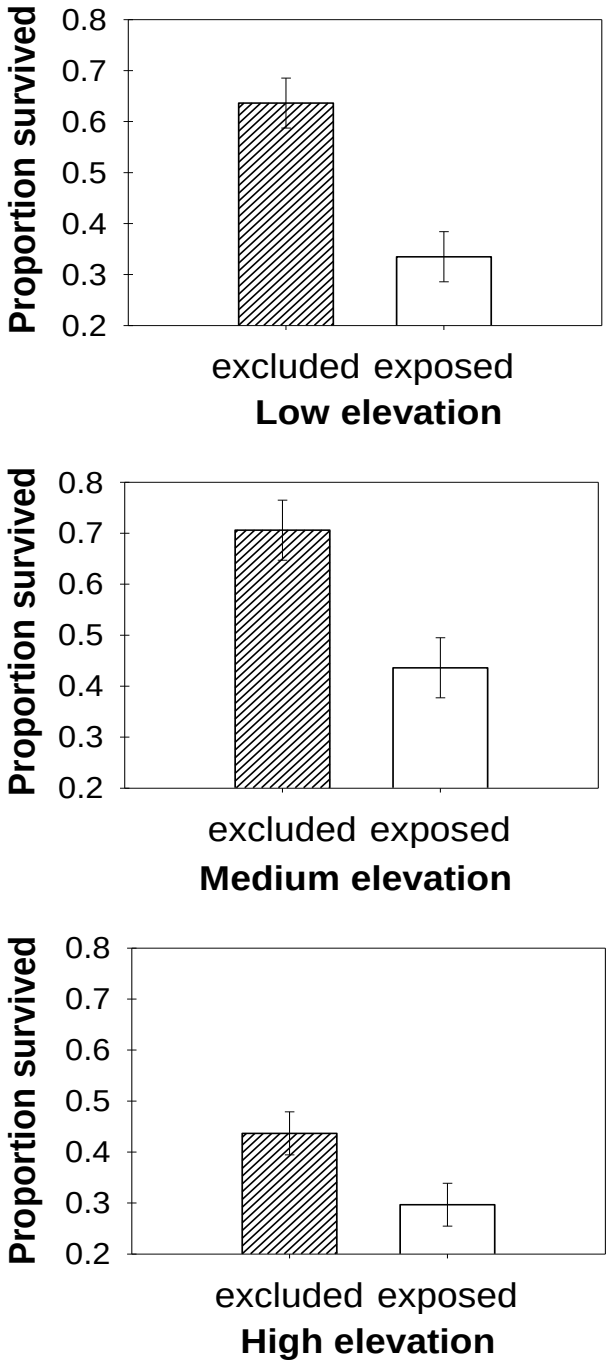

Figure 9 - Relationship between the sum of growing degree days and larval development rate of beetle families during summer 2011. See text for growing degree day calculation methods and statistical analysis.

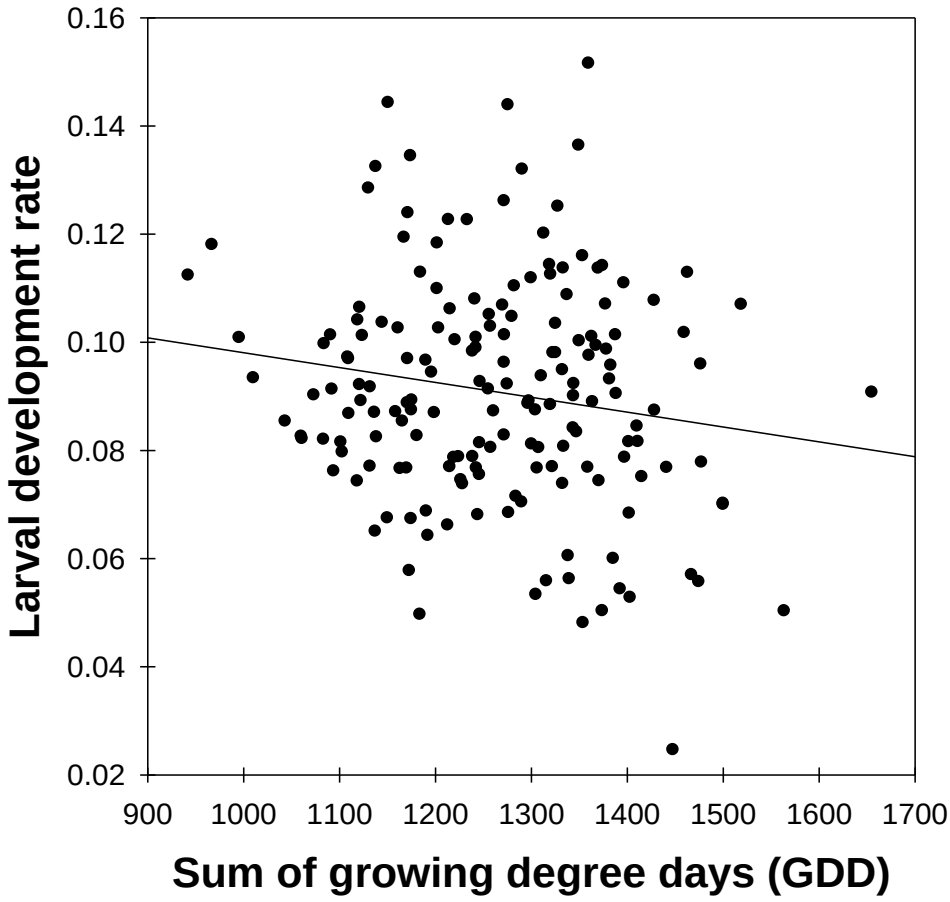

Figure 10 - Effect of maternal *PGI* genotype and *COII* haplotype on larval development rate. Open bars, mt-*COII*-3 haplotypes; crosshatched bars, mt-*COII*-1 haplotypes. Data shown are least square means (±SE) of each *PGI* and *COII* combination (1-1, *COII*-1, n=31; 1-1, *COII-3*, n=26; 1-4, *COII*-1, n=53; 1-4, *COII*-3, n=42; 4-4, *COII*-1, n=10; 4-4, *COII*-3, n=10).