The physiological impacts of changing snow cover on a montane leaf beetle

By

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Committee in charge:

Professor Caroline Williams, Chair
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Abstract

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Global climate change is altering not only average temperatures, but also the distribution and abundance of precipitation. During winter in montane habitats, changing precipitation patterns mean changing snow fall, which will have profound ecological impacts. Snow is an effective insulator and decouples below-snow temperatures from air temperatures, providing a stable environment and determining microclimate conditions in the soil. A wide range of organisms take advantage of stable below-snow winter environments by overwinter in soil. Winter soil microclimate modulates the degree of stress, performance, and ultimately fitness for organisms that overwinter in the soil. During the coldest portions of winter, the insulating effect of snow protects organisms from potentially damaging or even lethal cold stress. However, protection from cold extremes comes at a cost: in ectothermic animals, energy consumption is dependent on body temperature, and so relatively warm below-snow conditions drain energy stores more quickly. In winter resources are limited and energy stores cannot be replenished, and complete depletion of energy stores can cause mortality. Even if organisms survive, post-winter fitness may also be impacted by energy depletion by reducing reserves available for growth and reproduction. We don't yet understand the impacts of changing winter snow will have on overwintering organisms in montane environments. I hypothesize that winter snow cover, through regulation of winter microclimate, modulates the cause of stress and mortality (cold damage or energy depletion) for montane ectotherms overwintering in soil. By better understanding the impacts of changing winter snow cover on montane ectotherms, we may be better able to identify processes and populations that are vulnerable to climate change.

My dissertation aims to expand our understanding of the impact of snow cover on overwinter stress on insects that overwinter in the soil, by examining the impact of snow cover along elevation gradients, then extending the energy use model developed in Chapter 1 to test for impacts of plasticity. Finally I examine how overwinter conditions alter physiological processes upon emergence from overwintering. This work shows that winter snow cover has far reaching impacts on overwintering ectotherms, which are not equal across elevation, and have effects that can carry over into the growing season, expanding our understanding of changing winters and population impacts of climate change.

Chapter 1 establishes the energetic impacts and thermal challenges of changing snow cover along elevation gradients. Along elevational gradients snow cover increases but air temperature decreases, and it is unknown how these opposing gradients impact performance and fitness of organisms overwintering in the soil. I developed experimentally validated ecophysiological models of cold and energy stress over the past decade for the montane leaf beetle *Chrysomela aeneicollis*, along five replicated elevational transects in the Sierra Nevada mountains in California. Cold stress peaks at mid-elevations, while high elevations are buffered by persistent snow cover, even in dry years. While protective against cold, snow increases energy stress for overwintering beetles, particularly at low elevations, potentially leading to mortality or energetic trade-offs. Declining snowpack resulting in drier winters, will predominantly impact mid-elevation populations by increasing cold exposure, while high elevation habitats may provide refugia.

Chapter 2 focuses on the impacts of plasticity in metabolic-rate-temperature relationships in dormant organisms and their impacts on energy use estimates, expanding on the model developed in Chapter 1. Ecophysiological energy use models predict long-term energy use from metabolic rates, but we do not know the degree to which plasticity in metabolic intensity or thermal sensitivity impact energy use estimates. I quantified metabolic rate-temperature relationships of dormant willow beetles (*Chrysomela aeneicollis*) monthly from February to May under constant and variable acclimation treatments. Metabolic intensity increased through time, and acclimation altered both metabolic intensity and the thermal sensitivity. However, incorporating these two types of metabolic plasticity into energy use models did not improve energy use estimates, validated by empirical lipid measurements. Together, this indicates that while metabolic rate temperature relationships are plastic during winter, incorporating this plasticity does not improve prediction of energy use made by ecophysiological models, partly due to large individual variability in energy reserves.

Chapter 3 examines the impact that overwintering environment plays on prioritization of physiological processes upon emergence from dormancy, based on changes in gene expression. During winter, dormant organisms conserve resources through metabolic suppression and minimizing cellular processes. The transition from dormancy to active season requires a quick reversal of these processes and large-scale physiological transitions, enabling organisms to begin exploiting favorable environmental conditions. The impact of overwinter microclimate on the physiological processes involved in transitioning from dormancy to active season in insects are unknown. I conducted a field snow-cover manipulation and then profiled gene expression of willow leaf beetles *Chrysomela aeneicollis* during the transition out of dormancy using RNA-seq. Upon emergence from dormancy, beetles first prioritize up-regulation of transcripts associated with digestion and nutrient acquisition. The prioritization of nutrient acquisition is followed by investment into reproduction, but the timing is sex-specific with females investing sooner. Winter snow cover impacted the timing of these processes, with beetles that overwintered below snow being several days ahead of beetles that overwintered without snow. This highlights the importance of winter microclimate in regulating critical life history transitions.

Contents

List of Figures

List of Tables

Acknowledgements

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Chapter 1

Snow modulates winter energy use and cold exposure across an elevation gradient in a montane ectotherm

1.1 Introduction

Snow buffers the soil from cold air temperatures in winter, providing a thermally stable microhabitat for the vast array of terrestrial ectotherms (and hibernating endotherms) that overwinter in dormancy in the subnivium (below-snow habitat) or soil (Pauli et al., 2013; Wilsterman et al., 2021; Zhang, 2005). Anthropogenic climate change is causing decreased spring snowpack due to increased air temperatures, and earlier snowmelt dates across the Western United States (Mote et al., 2018; Stewart et al., 2004). Reductions in snowpack are exposing the soil to cold air temperatures, leading to the counterintuitive prediction of "colder soils in a warmer world" (Bale and Hayward, 2010; Groffman et al., 2001; Kearney, 2020; Marshall et al., 2020; Sinclair, 2001; Williams et al., 2015b; Zhu et al., 2019). Snow is thus one of the key factors determining exposure to climate change for organisms that overwinter beneath soil; and predicting vulnerability to climate change based on air temperature change alone will give very misleading conclusions. To develop our predictive understanding of how organisms will respond to climate change, we must develop general principles for how covariation in snow cover and air temperature through time and space will determine vulnerability for organisms that overwinter in the soil.

At the landscape level, coupled environmental gradients in snow cover and air temperature determine microclimate temperatures in the soil, and thus exposure to climate change. Towards higher latitudes and elevations, snow cover increases while air temperatures decrease (Ford et al., 2013; Rice et al., 2011). Across both latitudinal and elevational gradients in the USA, snow reduces or even eliminates the latitudinal gradients in soil compared to air temperature (Fitzpatrick et al., 2020; Fitzpatrick et al., 2019; Maurer and Bowling, 2014). Deep and persistent snowpack at high latitudes buffers the soil from cold air temperatures, and regions that are at risk of losing snow cover will experience greater exposure to climate change (Campbell et al., 2005; Fitzpatrick et al., 2019; Kearney, 2020; Zhu et al., 2019). To our knowledge, no studies have examined how spatial and interannual variation in snow cover along an elevation gradient impacts stress exposure, and correspondingly vulnerability to climate change, for organisms that overwinter in the soil. Vulnerability to climate change depends not only on exposure but also on sensitivity, determined by the intrinsic traits of the organism in question (Moritz and Agudo, 2013; Williams et al., 2008).

Two of the most important traits determining winter performance and survival are cold tolerance and energetics, which influence winter fitness and set range limits for a wide range of species (Humphries et al., 2003; Marshall et al., 2020; Osland et al., 2021; Overgaard et al., 2014; Shuter and Post, 1990). If microclimate temperatures drop below a population- or species-specific cold tolerance threshold, which depends on the combination of exposure time and temperature, then mortality or sub-lethal cold injuries can result (Bale, 1996; Sinclair et al., 2003). Cold damage can be mitigated by inducing synthesis of cryoprotective molecules, such as glycerol and other polyhydric alcohols, which increase cold hardiness by stabilizing membranes, binding ice crystals, and lowering supercooling point (Storey and Storey, 2012; Teets and Denlinger, 2013; Williams

et al., 2016a). Microclimate temperatures determine the rate of use of finite energy reserves, and increases in microclimate temperatures can cause energetic stress and lead to mortality or reduced ability to invest in subsequent reproduction (Bosch et al., 2010; Hahn and Denlinger, 2007; Irwin and Lee, 2003; Marshall and Sinclair, 2012; Sinclair, 2015). Snow increases energy use and reduces fecundity in some insects by increasing microclimate temperatures (Bosch et al., 2010; Hahn and Denlinger, 2007; Irwin and Lee, 2003; Marshall and Sinclair, 2012; Sinclair, 2015), and biophysical models suggest that snow will increase winter energy stress (risk of lethal energy depletion) but decrease cold stress (risk of lethal ice content) for overwintering ectotherms (Fitzpatrick et al., 2020; Fitzpatrick et al., 2019; Kearney, 2020). Snow also impacts energetics by determining winter length, with snowy years or locations associated with later springs and, correspondingly, longer and more energetically demanding overwintering periods (Inouye, 2008; Inouye et al., 2000; Sinclair, 2015). Overwintering ectotherms may thus face a widespread trade-off between risk of cold injury and use of energetic reserves, which is modulated by snow cover such that cold stress is increased but energy stress decreased by decreases in snow cover (Fitzpatrick et al., 2019). Here, we test the hypothesis that snow strongly determines vulnerability to climate change along an elevational gradient by modulating cold and energy stress.

We focus on a long-term study system consisting of replicated elevational transects in five drainages spanning 45 kilometers in the Eastern Sierra Nevada mountains in California, at the southern range edge of the Sierra willow leaf beetle, *Chrysomela aeneicollis* Schaeffer 1928 (Coleoptera: Chrysomelidae) (Dahlhoff and Rank, 2000; Rank and Dahlhoff, 2002; Rank et al., 2020). Interannual winter snow cover is highly variable in the Sierra Nevada, and two of the driest years on record have occurred within the last decade (Margulis et al., 2016), with 30 – 70% reductions in snowpack and increasing droughts predicted by 2100 (Hayhoe et al., 2004). *C. aeneicollis* overwinters in the soil as freeze-tolerant adults for 8-9 months of their one-year lifecycle, and organismal cold tolerance limits have been well-characterized (Boychuk et al., 2015). Population dynamics of *C. aeneicollis* are sensitive to variation in snow cover, with the range contracting northwards and up-slope during long droughts leading to local extirpations (Dahlhoff et al., 2019; Dellicour et al., 2014). In this study, we examine the impact of both interannual and spatial (elevational) variability in snow cover on beetle microclimate conditions; integrating environmental data with organismal traits using ecophysiological models to predict cold and energy stress along elevational gradients (Figure 1.1, Table S1.1). The combination of fine-scale resolution on microclimate variation through space and time, detailed information on organismal physiology, and estimates of spring phenology based on natural history provides an unparalleled opportunity to examine how changing snow cover impacts cold and energy stress for an ectotherm that overwinters in the soil.

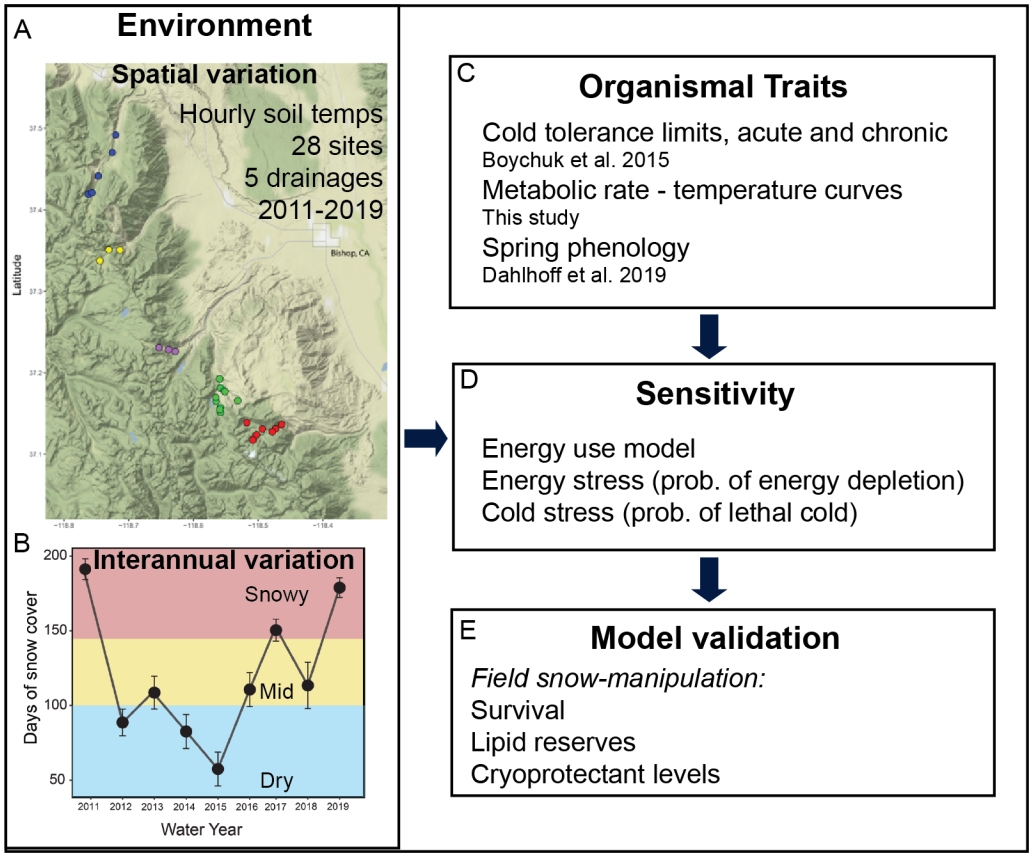

Figure 1.1) Integrating environmental conditions with organismal traits to describe how snow impacts sensitivity to cold and energy stress in a montane willow leaf beetle, *Chrysomela aeneicollis*. **A)** Microclimate monitoring network in the Eastern Sierra Nevada Mountains, providing hourly soil temperature data from 2011-2019 for 28 sites in 5 drainages representing replicate elevational transects (from north to south: Rock Creek, blue; Pine Creek, yellow; North Fork Bishop Creek, purple; South Fork Bishop Creek, green; and Big Pine Creek, red). B) The 9 years of soil temperature data were divided into snowy, moderate and dry years based on the duration of snow cover, to allow us to examine the impact of both spatial (elevation transects) and temporal (interannual) variation in snow cover. C) Organismal traits related to cold tolerance and energetics were characterized in the laboratory or obtained from the literature. D) Environmental data were integrated with organismal traits using ecophysiolgical models to estimate organismal energy and cold stress. E) Predictions of ecophysiological models were tested using a field snow-manipulation experiment at a single (low) elevation in the Rock Creek (northern) drainage.

1.2 Materials and Methods

Study system

Chrysomela aeneicollis inhabits high elevation (2700-3400 m) riparian and bog willow patches (Rank, 1992a). Both larvae and adult beetles feed on one of several *Salix* species, often spending most of their life on a single plant (Rank, 1992a) Beetles are univoltine and annual life cycle is closely tied to host plant phenology (Rank, 1992b). Mating and subsequent oviposition occurs on willow leaves in early summer (Rank et al., 2006), eggs hatch and larvae develop and pupate from June to August, eclosing as new adults in late August to mid-September (Smiley and Rank, 1986). After eclosion, adult beetles feed until they burrow into soil 5-10 cm below their host plant and enter dormancy, which can last from October to June (Smiley and Rank, 1986).

Microclimate monitoring network

Temperature data were collected using HOBO Pendant 64K temperature and light data loggers (Onset, MA, USA) that were buried at the base of a willow just below soil surface across a network of long-term monitored sites (n = 28; Figure 1.1, Table S1.1; other details in (Dahlhoff et al., 2019; Rank et al., 2020). Data were pre-processed to give complete hourly temperature records for every winter at each site, as well as an estimate of duration of snow cover in days for each site and winter (Supporting Methods, Microclimate data processing). Present study examined the winters of 2010 to 2019. Winters were categorized by water year (October 1st to September 30th), and named according to the calendar year in which it ends (i.e. "2015" encompasses 1 Oct 2014 – 30 Sept 2015). We categorized winters as snowy (> 145 days of snow cover; 2011, 2017, and 2019), moderate (101-144 days of snow cover; 2013, 2016, and 2018), and dry (< 100 days of snow cover; 2012, 2014, and 2015), based on analyses shown in Figure 1.1B.

Cold exposure across the landscape

For each site (n = 28), we used hourly soil temperature logger data described above to calculate monthly mean, extreme monthly minimum, and monthly mean daily minimum temperatures for December to April of each year, which preliminary analyses suggested were the coldest months. This created 15 variables (3 values for each of 5 months), which we summarized using principal components analysis. We used the first principal component (PC1) as a metric of cold exposure, because it captures extreme cold and mean temperature experienced during the coldest months. Cold exposure (PC1) was negatively correlated to all measures of temperature and effectively encapsulated winter environmental cold (Table S1.2, Table S1.3).

Energy use model

We measured thermal dependence of metabolic rate (metabolic rate – temperature curves) using stop-flow respirometry on wild-collected beetles (Supporting Methods, Respirometry). In August 2016, adult beetles (n = 15) were collected from a mid-elevation site in Rock Creek (Mosquito Flat; Table S1.1). and reared in the laboratory at 20/4 °C 12.5/11.5 h day night cycle until entering dormancy and held at 1°C in darkness for 6 months (Supporting Methods, Beetle husbandry). Stop-flow respirometry was used to estimate rates of O_2 consumption ($\dot{V}O_2$), which was used as a proxy for metabolic rate (Lighton, 2018). Repeated measurements were taken on

each beetle (n = 15) at minus 1, 1, 4 and 9 °C; beetles were given at least 72 hours to recover between measurements.

All statistical analyses were conducted in R 4.0.2 (R Core Team, 2019). We used a maximum likelihood approach using the package nlme (Pinheiro et al., 2020) to choose the most parsimonious model describing the relationship between temperature and oxygen consumption, using the approach described in Williams et al. (2012b). We evaluated power law and exponential models incorporating a general scaling factor, thermal sensitivity, mass scaling, and theoretical life-supporting minimal metabolic rate (Table S1.4). Models were compared and chosen based on Akaike Information Criterion scores and log-likelihood values.

The model transforms an input of soil temperature data (as a proxy for beetle body temperature) into instantaneous rates of oxygen consumption using empirically derived metabolic rate-temperature curves. *Chrysomela aeneicollis* adults are freeze tolerant during winter, with a freezing point of -5.3°C (Boychuk et al., 2015). Insect metabolic rates are suppressed during freezing (Irwin and Lee Jr, 2002; Marshall and Sinclair, 2012), and to account for this we reduced metabolic rate by a factor of three whenever temperatures were below -5.3°C. In this species, the thermal hysteresis gap (difference between freezing point and melting point of body fluids) is -0.22°C (Boychuk et al., 2015), which is smaller than the accuracy of the temperature loggers (±0.53°C), so we did not account for thermal hysteresis in our model (i.e., "freezing" and "melting" both occurred at -5.3°C). The model then takes an input of start and end dates and sums hourly oxygen consumption between those dates to output total oxygen consumed, which was then converted into lipid consumed based on a 2L O_2 consumed for every 1g of lipid consumed conversion (Schmidt-Nielsen, 1997), and then converted to energy use assuming 39 kJ g^{-1}lipid (Sinclair et al., 2013).

Field snow-manipulation experiment

The field-based snow manipulation experiment took place at Rock Creek Lodge (37°28'09.1"N 118°43'22.5"W, 2826 m elevation) in the winter of 2015-2016. We buried beetles on 7th November 2015 (n = 20 per treatment) at a depth of 5 cm either in the open (snowy plot, exposed to ambient snowfall) or beneath an open shed (dry plot, snow excluded but ambient air temperature). One the same day, a sub-sample of beetles were flash frozen (pre-winter sample, n = 8) and used for biochemical analysis. Beetles were excavated on 15th May 2016 (post-winter sample, n = 20/treatment) and assessed for survival by monitoring for movement 24 hours after emergence, then flash frozen for biochemical analysis. All samples were analyzed for energy reserves (triacylglyceride content using Thin-Layer Chromatography-Flame Ionization Detection)(Williams et al., 2011), glycerol, sorbitol, glucose and protein concentrations (using commercially available colorimetric assays, details in Supporting Methods, Biochemical assays).

We compared survival through winter between the snowy and dry plot using a chi-squared test. We assessed changes in energy reserves and cryoprotectant concentrations over winter by comparing pre- and post- winter samples using a paired-comparisons t-test with an expected value of 0. We tested for effects of snow on energy stores and cryoprotectants remaining in Spring using ANOVA implemented in the car package in R (Fox and Weisberg, 2018), with the fixed effects of treatment (snowy or dry plot), status (alive or dead), and sex (male or female), with total protein as a covariate representing body size. For general linear models, our modeling approach was to fit saturated models and sequentially drop non-significant interactions and terms, retaining the simplified model if it did not differ in explanatory power ($p > 0.05$), until the minimal adequate

model was determined (Crawley, 2012). All terms remaining in the minimal adequate model are reported. To assess effects of winter condition on cryoprotectant abundance, an ANOVA was run with treatment and status as independent variables and amount of glycerol as dependent variable.

We validated the energy use model by predicting energy use in experimental plots and comparing it to empirical lipid use estimates. We used temperatures recorded by iButton thermochrons (DS1922L, Maxim Integrated Products, Sunnyvale, CA) in each plot as input to the energy use model described in 4.4, with start and end dates (November 7th and May 15th) corresponding to timing of pre- and post-winter samples. We empirically estimated lipid used over winter by subtracting mean pre-winter lipid stores from mean lipid scores post-winter in snowy and dry plots.

Estimating phenology

We estimated the end date of winter based on the first observations of beetles at each site from annual early summer surveys from 2011-2019 (Table 1.1) (Dahlhoff et al., 2019). We excluded sites with only one abundance measurement throughout a given season. Spring emergence dates were analyzed using ANCOVA with elevation as covariate and snow category (snowy, moderate, or dry) as independent variables. Based on the results of these analyses, we allowed end dates of dormancy to vary as a function of elevation and of snow category for a given year (see Table 1.1 for day used in model). We held start of winter date constant at October 1st, which is the start of the water year and matches timing of defoliation of willows and beetle dormancy initiation timing in lab (Supporting Methods, Field snow manipulation experiment). To explore the impact of winter phenology (start and end dates of dormancy) on energy use, we additionally varied start and end dates in one-week intervals across the full range of ecologically relevant start and end dates (Start dates: September 17 to October 22; End dates: May 30 to July 3) based on field observations (NER, EPD and KTR, unpublished observations), and for each combination of start and end dates calculated the resulting mean energy use across all sites.

Spatial and temporal variation in cold exposure and energy use

We next tested how predicted energy use and cold exposure varied with elevation and interannual variation in snow cover. We fit linear mixed effect models using the lme4 package in R (Bates et al., 2014), using a Satterthwaite approximation in lmerTest package to approximate degrees of freedom and p-values (Kuznetsova et al., 2017). Dependent variables included predicted energy used or cold exposure (PC1); independent variables included the fixed effects of elevation, snow class (snowy, moderate, or dry) and their interaction; and random effects included water year and site nested within replicate elevation gradient (different colored transects in Figure 1.1). Predicted energy use was logarithmically ($\log_e$) transformed for normality in subsequent analysis. Preliminary analysis indicated a non-linear effect of elevation on cold exposure; thus, we included a second order polynomial of elevation in the subsequent model. To test for change in cold exposure and energy use by elevation class, models matching those described above were run with elevation as a categorical variable.

Table 1.1) Average spring emergence dates of 28 populations of *Chrysomela aeneicollis* across an elevational gradient from 2011-2019. Spring emergence date is estimated from annual survey data (Dahlhoff et al., 2019). Years were categorized as Dry (2012, 2014, 2015), Moderate Snow (2013, 2016, 2018), and Snowy (2011, 2017, 2019) based on duration of snow cover (see text for details). Elevations were grouped Low (< 2900 m), Low-Mid (2901-3100 m), Mid-High (3101-3250 m), and High (> 3251 m). Observed Spring emergence dates were used as ending dates for energy use calculations.

Elevation group	Observed Spring emergence date	Standard error (days)
Dry Winters		
Low	June 3	1.9
Low-Mid	June 5	1.3
Mid-High	June 7	1.1
High	June 12	2.4
Moderate Snow Winters		
Low	June 8	2.2
Low-Mid	June 12	1.7
Mid-High	June 12	1.3
High	June 14	2.1
Snowy Winters		
Low	June 18	0.4
Low-Mid	June 23	1.6
Mid-High	June 23	1.2
High	June 26	2.4

Cold and energy stress

We identify cold mortality by identifying conditions that would likely cause cold induced mortality based off previously identified cold tolerances (Boychuk et al., 2015), which included temperatures below -5.3 °C (beetle freezing point) for 12 hours, and any temperatures below -15.0°C (beetle LT_{50}). We then identified energy depletion by identifying sites that our energy use model estimated total expenditure exceeded the 75th percentile of pre-winter energy stores measured from a subset of beetles sampled at the onset of our field snow manipulation experiment (n = 8). We fit binomial general linear mixed effect models using the lme4 package in R, with cold mortality or energy depletion as the dependent variable, and included the fixed effects of elevation, snow class (snowy, moderate, or dry) and their interaction; and random effects included water year and site nested within replicate elevation gradient (different colored transects in Figure 1.1). Best fit models were selected using methods describes above (*Field snow-manipulation experiment*).

1.3 Results

Cold exposure

Cold exposure showed a curvilinear relationship with elevation, increasing with elevation up to around 3200 m, then decreasing again at high elevations (Figure 1.2A). Dry winters are colder than snowy winters up to mid-elevations, after which cold exposure does not differ between years ($F_{4,150} = 5.6$, $p = 0.0003$; moderately snowy winters are intermediate, see Figure S1.1A). The degree of cold exposure increase is dependent on elevation, and with low-mid and mid-high elevations experiencing the greatest degree ($F_{6,146}=8.6$, $p = 0.001$; Figure 1.2B).

Energy use model – development and validation

In our snow manipulation experiment soil temperatures were higher and more stable in the snowy plot compared to the dry plot, and temperatures in the snowy plot never dropped below 0°C (Figure 1.3A, Table S1.5). The energy use model accurately predicted lipid use based on microclimate temperatures in both plots (model accuracy 99.7% and 95.2% respectively in snowy and dry plots; Table 1.2). Survival of beetles did not differ between snowy and dry plots ($\chi^2_{1,40} = 0.0$, $p = 1.0$). Beetle storage lipid stores decreased through winter in both snowy and dry plots (dry: $t_{19} = -19.1$, $p < 0.0001$; snowy: $t_{19} = -8.14$, $p < 0.0001$), and storage lipid stores were lower at the end of winter in the snowy plot compared to the dry plot ($F_{1,38} = 23.1$, $p < 0.0001$; Figure 1.3B). Among beetles that died during the winter, those in the snowy plot had almost completely depleted their lipid stores (0.029 ± 0.01 mg; Figure 1.3B) while dead individuals from the dry plot had substantial lipid reserves remaining (0.37 ± 0.09 mg; Figure 1.3B), and survival status interacted with snow treatment to predict lipid stores ($F_{1,38} = 9.7$, $p = 0.0036$). Glycerol, a functional cryoprotectant in insects, increased from the beginning to the end of winter in beetles that overwintered in the dry but not the snowy plot (dry: $t_{19} = 7.87$, $p < 0.0001$; snowy: $t_{19} = -1.70$, $p = 0.11$). At the end of winter, glycerol was higher in beetles that overwintered in the dry compared to the snowy plot (Table S1.6; $F_{1,36} = 44.2$, $p < 0.0001$), and in both treatments, beetles that survived winter had higher free glycerol than those that died ($F_{1,36} = 12.4$, $p = 0.0012$; Figure 1.3C; Table S1.6). Neither glucose ($F_{1,38} = 0.4$, $p = 0.55$) nor sorbitol ($t_{19} = 1.98$, $p = 0.63$) differed between treatments (Table S1.6).

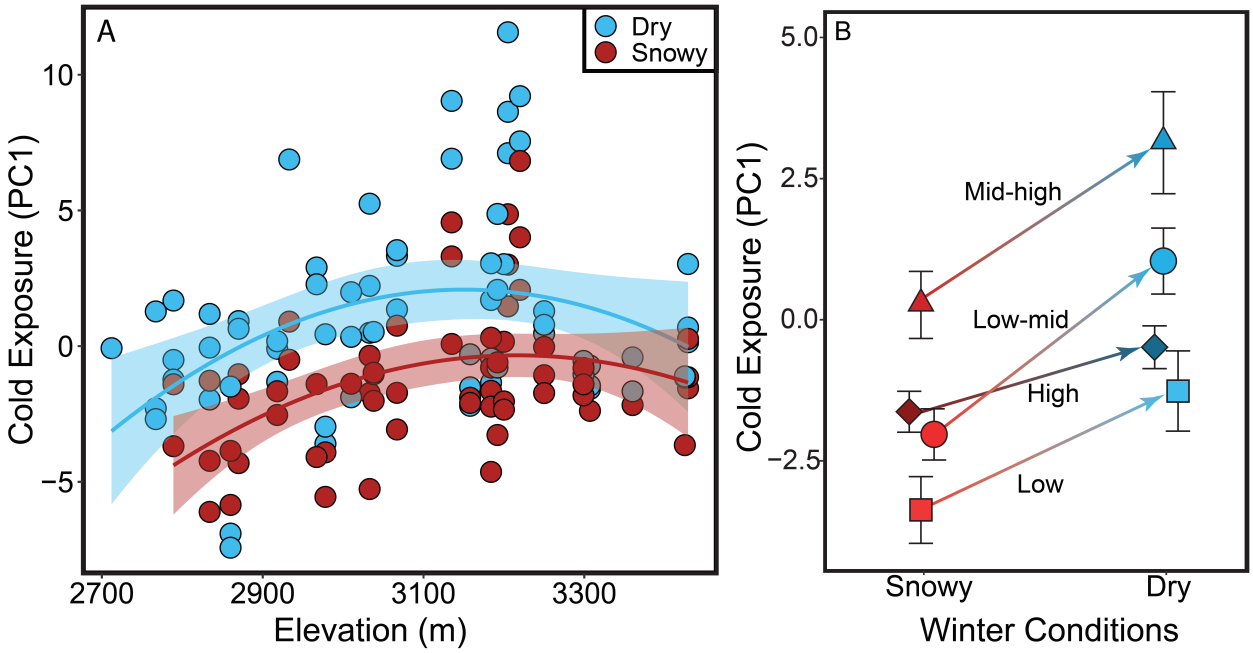

Figure 1.2) Snow alters cold exposure in the soil along an elevational gradient and between years in the Eastern Sierra Nevada mountains. A) Cold exposure (scores on the first principal component [PC1] summarizing winter soil temperatures) as a function of elevation for the three snowiest (red) and driest (blue) years from 2009-2019 (all years are shown in Figure S1.1). Each point represents one site/year, and shaded regions show 95% confidence intervals on the fitted non-linear regression. B) Average change in cold exposure between snowy and dry years (mean ± SE), grouped by elevation band (Low: < 2900 m; Low-Mid: 2901-3100 m; Mid-High: 3101-3250 m; High: > 3251 m). Arrows indicate the shifts that populations will experience under projected climate change in the Sierras.

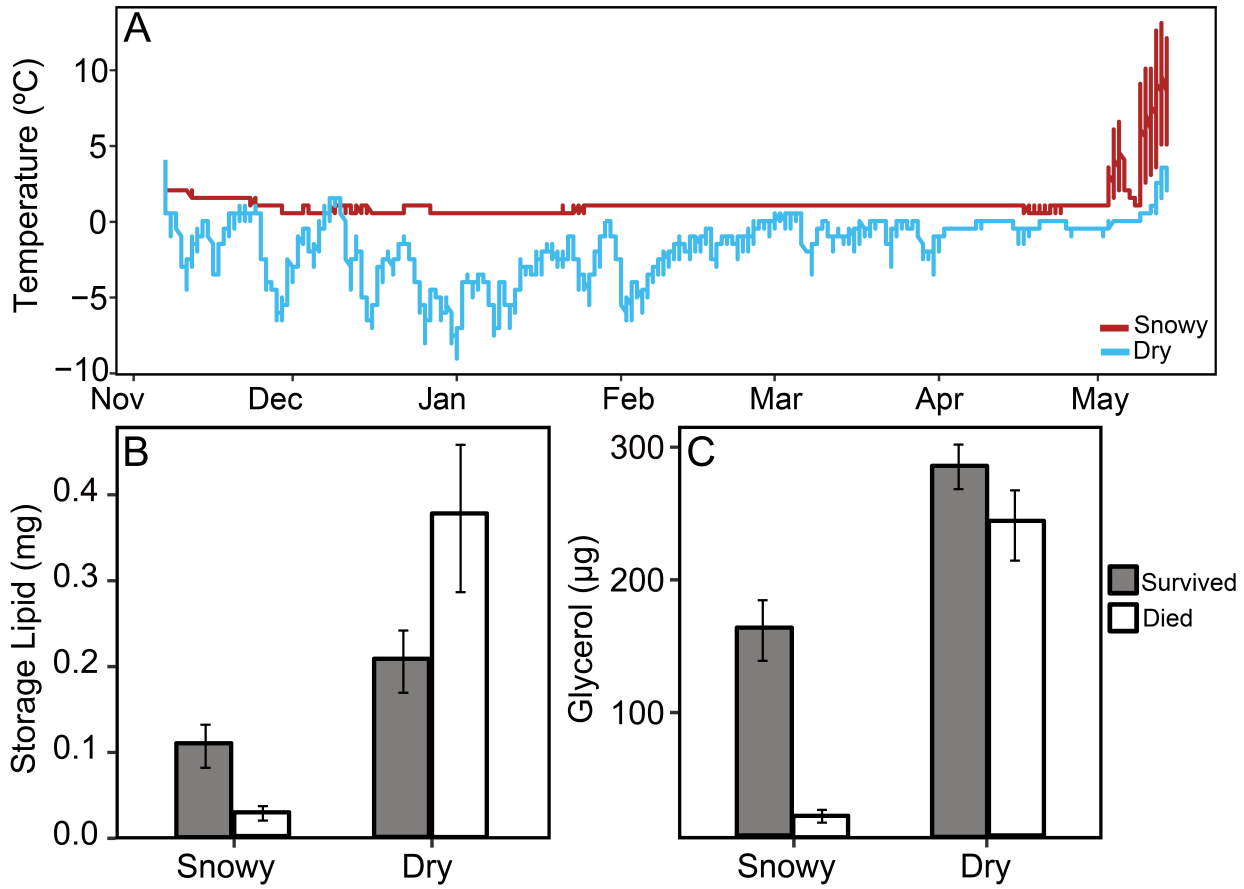

Figure 1.3) A field snow-manipulation experiment at a low-elevation site in the Sierra Nevada mountains supports the hypothesis that snow modulates energy use and cryoprotectant production in the willow leaf beetle, *Chrysomela aeneicollis*. (A) Microcliamte temperatures for beetles (N = 20/treatment) buried at 5 cm depth in the Snowy (red, open to natural snow accumulation) and Dry (blue, snow excluded but open to ambient temperatures) plots, measured by iButton thermochrons. (B) Storage lipid (mg triacylglceride) or (C) glycerol content (a functional cryoprotectant) of beetles at the end of winter (May) that survived (grey) or died (white) in each plot. Bars show mean ± SEM.

Table 1.2) Comparison of storage lipids used over winter predicted from the energy use model, to empirical measurements of storage lipid depletion of *Chrysomela aeneicollis* from a field snow-manipulation experiment. In field experiment, storage lipid used was calculated as the difference between average lipid stores at the beginning and end of winter, quantified using TLC-FID.

	Snow Present	**Snow Excluded**	**% Energy Cost of Snow**
Field Experiment	0.589 mg	0.398 mg	47.99%
Model Estimate	0.591 mg	0.417 mg	41.73%
Model Accuracy	99.7%	95.2%	

Energy use through time and space

Spring emergence dates were later at higher elevations ($F_{1,191}$ = 10.4, p = 0.0015) and in snowy years ($F_{2,191}$ = 97.7, p < 0.0001), leading to increased snow cover being associated with longer winters through space and time (Table 1.1). To estimate energy use at our 28 sites, we therefore allowed winter length to vary with elevation and winter type (snowy or dry). Based on these site- and year-specific winter durations, total winter energy expenditure varied more than 2-fold across the elevational range and between years (Figure 1.4A with moderate winters included see Figure S1.2). Total winter energy expenditure decreased monotonically with increasing elevation in both snowy and dry years ($F_{1,22}$ = 16.4, p = 0.0005). Snowy winters were more energetically costly than dry winters ($F_{6,154}$ = 6.3, p = 0.0024), except at high elevations, where total winter energy expenditure converged between snowy and dry winters. This led to a steeper elevational decline in total winter energy expenditure in snowy compared to dry years (elevation × winter type; $F_{2,151}$ = 5.9, p = 0.0034). The degree of energy use increase in snowy winters was dependent on elevation, with low elevations having the greatest degree ($F_{6,247}$ = 4.1, p = 0.001, Figure 1.4B)

Total winter energy expenditure is driven by the combination of soil microclimate temperatures and winter duration (here, set by spring phenology), so we also examined energy expenditure at comparable winter lengths to explore the relative impacts of these two factors with respect to variation through time (interannual variation), and space (along the elevational gradient). With respect to interannual variation, we found that snowy winters were less energetically demanding than dry winters for any given winter duration, contrary to our prediction that snow would decrease soil temperatures and thus suppress energy use, (Figure S1.3A). In our Sierra Nevada field sites, this occurs because in snowy years, snow buffers the soil from warm energetically demanding temperatures in spring (Figure S1.4). Thus, early snow melt in dry winters leads to an earlier uptick in energy use and consequently a higher overall energy use in dry winters, for any given end date (Figure S1.3A). The importance of spring conditions in determining total winter energy use can also be seen from the steeper slope that results from altering end date compared to start date (Figure S1.3B). However, when we account for the fact that beetles emerge from dormancy on average 17 days later in snowy compared to dry winters [June 6 in dry winters, June 22 in snowy winters, ($F_{2,191}$ = 97.67, p < 0.0001)], total winter energy use is higher in snowy compared to dry winters. Thus interannual variation in snow cover will impact energetics primarily through winter duration, and the microclimate temperature differences among years (lower energy use in snowy years with cool springs) will counteract the impact of phenology (higher energy use in snowy years with long winter) (Figure S1.5). Along the elevational gradient, energy use driven by microclimate temperatures decreases with increasing elevation, and increases due to longer winters at high elevations only partially counters that effect (Figure S1.6). Thus spatial variation in energy use is primarily driven by differences in microclimate temperatures across elevational gradients.

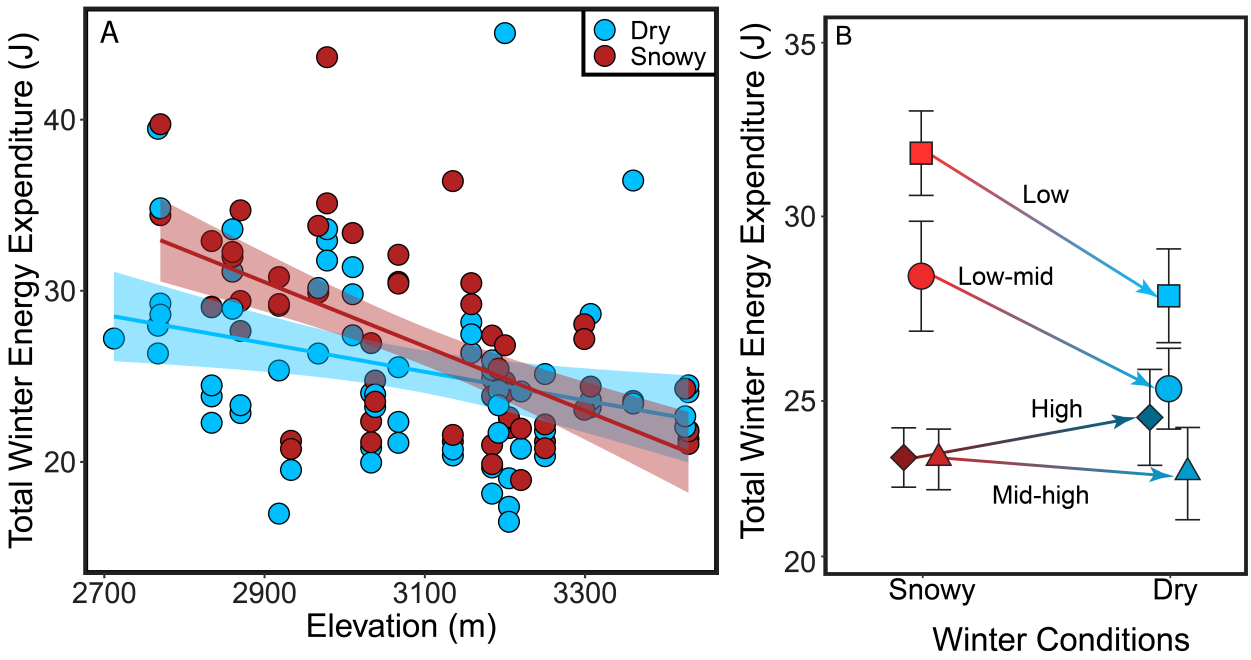

Figure 1.4) Projected total winter energy use of overwintering *Chrysomela aeneicollis,* along an elevation gradient and between years in the Eastern Sierra Nevada mountains. (A) Total winter energy expenditure (J) across elevation for the three snowiest (red) and driest (blue) years from 2009-2019 (all years are shown in Figure S1.2, predicted by the energy use model. Grey dashed line indicates the 75th percentile of prewinter lipid stores of a subset of beetles. Each point represents one site/year, and shaded regions show 95% confidence intervals on the fitted linear regression. B) Average change in total winter energy use between snowy and dry years (mean ± SE), grouped by elevation band (Low: < 2900 m; Low-Mid: 2901-3100 m; Mid-High: 3101-3250 m; High: > 3251 m). Arrows indicate the shifts that populations will experience under projected climate change in the Sierras.

Cold and energy stress

Across all sites and years, the temperatures in the soil never dropped below the LT_{50}, however, the soil was frequently frozen for more than 12h, suggesting that cold mortality of this beetle is predominantly caused by prolonged relatively mild cold exposure rather than acute cold exposure. would be unlikely to cause mortality in this beetle species (Figure S1.7). We therefore used the frequency of > 12h exposure to temperatures below the freezing point as our metric of cold stress. Similar to cold exposure, cold stress increased with elevation and peaked at mid-elevations (Z_{182} = -2.601, p = 0.01; Figure 1.5A). At the coldest sites (~3100-3300m), almost 50% of sites were predicted to cause cold stress for overwintering beetles. Cold stress did not differ between snowy and dry winters (Z_{183} = -0.915, p = 0.33). We inferred energy stress by comparing predicted energy use for a site and year to empirically measured energy stores of beetles from these populations (n = 8), measured at the start of winter (> 75% percentile). Energy stress was highest at low elevations and depended on snow cover (Z_{174}=4.22, p=0.042 ;
Figure 1.5B), with 75% of sites at low elevation predicted to exhaust available energy reserves in snowy years.

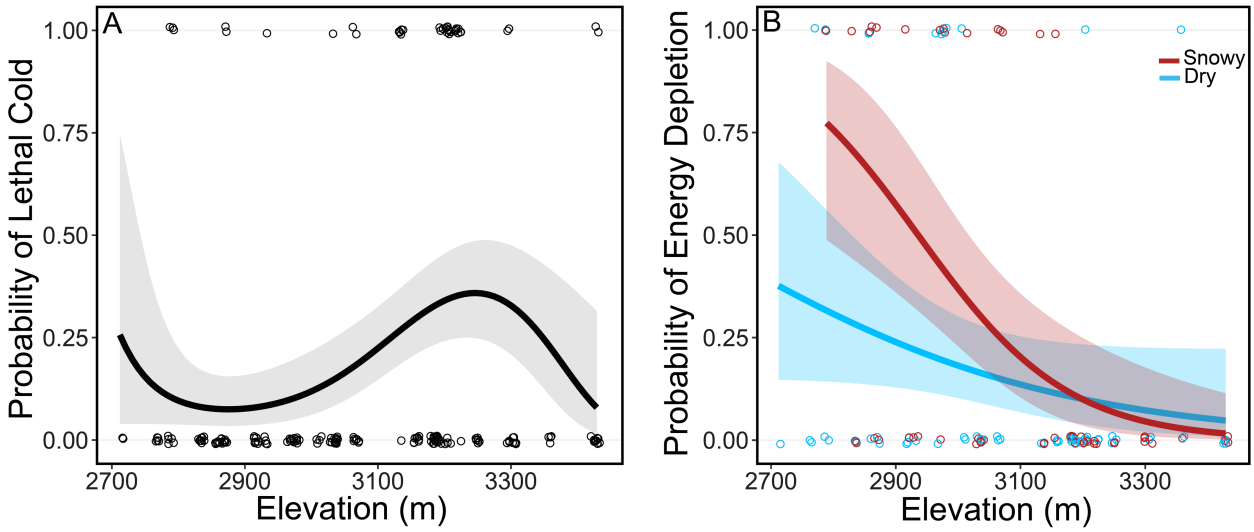

Figure 1.5) Cold and energy stress as a function of elevation for overwintering *C. aeneicollis*. The probability of environmental temperatures (A) crossing cold tolerance limits (>12 hours below supercooling point of -5.3°C), or (B) causing complete energy depletion (predicted energy use exceeds 75th percentile of estimated pre-winter energy stores) across all sites and years at a given elevation in snowy (red) and dry (blue) winters. Each point indicates whether a threshold crossing event occurred (1) or not (0) in each site/year, and shaded regions show 95% confidence intervals on the fitted logistic binomial regression. Snowy and dry winters are seperated when there was a significant difference between their impact on mortality across elevation.

1.4 Discussion

Snow is an effective insulator that decouples soil temperatures from winter air temperatures, critically determining winter stress exposure for the many ectothermic organisms that overwinter in the soil. Here, we show that snow strongly alters cold exposure and energy stress for the willow leaf beetle *Chrysomela aeneicollis*, and by extension other ectotherms overwintering in the soil in snowy mountains. Climate change-induced reductions in snow cover will therefore have very different fitness implications for organisms living at different elevations, and these fitness impacts cannot be predicted based on air temperatures alone. This highlights the importance of refining our understanding of how snow interacts with air and soil temperatures to determine organismal performance and fitness, in order to predict the biological impacts of climate change(Fitzpatrick et al., 2020; Fitzpatrick et al., 2019; Kearney, 2020; Kelsey et al., 2020).

Impacts of snow on cold exposure and cold stress

We found strong support for our hypothesis that snow modulates the elevational gradient in cold stress, for organisms that overwinter in the soil. Air and bare soil temperatures decline linearly with elevation, with a moist adiabatic lapse rate of around 3-7 °C/km (Maurer and Bowling, 2014), matching our findings of a 3.7°C/km lapse rate in soil in both conditions, with snow being on average 1.4°C warmer. Cold exposure in the soil was strongly non-linear, peaking at mid-elevation (3100-3200 m). Across the SNOTEL monitoring network in the western US, snow cover completely eliminates the elevational gradient in mean January soil temperature (Maurer and Bowling 2014), but our more nuanced characterization of cold exposure represents an organismal view, by focusing on the monthly extreme low temperatures that are likely to cross thresholds for lethal or sub-lethal damage. The conclusion that cold exposure will peak at mid-elevations was consistent in 4/5 replicated mountain drainages, and occurred in both snowy and dry years (Figure S1.8). This parallels predictions that mid-latitude soils are predicted to become colder (longer durations of frozen snow-free ground), driven by increasing mid-winter air temperatures and loss of snow cover, while high-latitude soils are buffered by persistent snowpack and experience smaller magnitudes of cooling (Zhu et al., 2019).

Cold stress for our model ectotherm, *Chrysomela aeneicollis,* was also predicted to peak at mid-elevations, occurring due to chronic (>12h) exposures to temperatures below the freezing point of body fluids. This is the first demonstration of the impact of snow cover variation along an elevational gradient on organismal function, and supports conclusions from latitudinal studies that snow will strongly modulate cold exposure in the soil along environmental gradients in snow cover and air temperature (Fitzpatrick et al., 2019; Zhu et al., 2019). Although the degree of cold stress will depend on proximity of soil temperatures to organismal cold tolerance limits (analogous to thermal safety margin (Sunday et al., 2014)), we believe the general conclusion that winter cold stress will peak at mid-elevations in snowy mountains may generalize broadly, given that cold tolerances frequently closely match environmental cold extremes (Marshall et al., 2020).

Interannual variation in snow cover also modulated cold exposure, but the conclusions with respect to organismal impacts were less clear. While cold exposure was increased in dry compared to snowy years (Figure 1.2), our models did not predict increased stress for *C. aeneicollis*. Supporting this, survival did not differ between the snow- and snow-exclusion plots in our field snow manipulation experiment, although the increased cryoprotectant concentrations of beetles in the dry plot suggests a plastic up-regulation of cold-protective mechanisms (Figure 1.3, Boychuk

et al. 2015). However, at the population level *C. aeneicollis* populations are negatively impacted by dry years (Dahlhoff et al., 2019), consistent with a role for interannual variation in cold stress impacting population dynamics (although it remains possible that population declines are driven by the impacts of snow on soil water content, microbial respiration, and primary productivity (Kelsey et al., 2020)). We conclude that there is good evidence that interannual variation in snow cover can impact fitness of ectotherms that overwinter in the soil, but more research is required to fully demonstrate the mechanisms.

Impacts of snow on energy use

Winter energetics is a key fitness component for overwintering animals, and the ability to survive winter on stored energy can determine survival and limit distributions (Humphries et al., 2002; Shuter and Post, 1990). Our study is the first to model energy use across an elevational gradient, and our empirical validation provided strong evidence that our model captures organismal energetics well (>95% accuracy under both snowy and dry conditions). As would be predicted from declining air temperatures, we found that both energy use and energy stress decline with increasing elevation, despite increasing winter length at high elevations. This is in contrast to conclusions from a mechanistic niche model using the wood frog *Lithobates sylvatica*, which suggested that the opposing gradients in winter length and soil temperatures would completely cancel each other out across a latitudinal gradient in North America (Fitzpatrick et al., 2020; Fitzpatrick et al., 2019). One key difference between these approaches is that mechanistic niche models initiate and terminate "winter" according to the microclimate conditions rather than calendar date (Fitzpatrick et al., 2020; Fitzpatrick et al., 2019), whereas we used fixed thresholds based on natural history observations, effectively assuming that phenology is independent of site-to-site microclimate variation. Spring emergence can be either environmentally cued or endogenously determined in animals that undergo programmed dormancy (Wilsterman et al., 2021), suggesting that both approaches may capture ecologically relevant responses. If beetles are emerging from dormancy as soon as energy use increases in the spring, the energetic costs of winter will be overestimated in our analysis, which could lead to a more shallow (or even absent) elevational cline in energy use. We therefore conclude that snow will strongly alter energy use clines compared to predictions based on air temperature, and that although the shape of the cline (and fitness impacts) will depend on species traits, it will be considerably more shallow than would be predicted by air temperature.

We found strong support for the hypothesis that interannual variation in snow cover will impact energy use and energy stress for overwintering *C. aeneicollis*. Over the past decade, snowy years increased energy use and the probability of lethal energy depletion (Figures. 1.4 & 1.5), particularly at low elevations, and the snowy plot in the field snow-manipulation experiment (which was a low elevation site) caused almost complete (and possibly lethal) energy depletion. This adds to a growing body of evidence that snow can cause energy depletion in insects at low elevations (Irwin and Lee, 2003; Marshall and Sinclair, 2012), and additionally suggests that this constraint is lifted at high elevations due to low air temperatures on the shoulder seasons and persistent snow cover during winter. Winter starvation and energy depletion causes mortality and impacts demography and distributions in fish (Finstad et al., 2004; Shuter and Post, 1990), and is likely to cause life history bottlenecks in insect and other ectotherm populations as well. Energy depletion may impact fitness not only by causing starvation, but also through reduced reproductive output, compromised locomotor performance, or reduced ability to invest in cryoprotection or

other energetically demanding somatic maintenance (Irwin and Lee, 2003; Kaufmann et al., 2013). Energy drain due to snowy winters could be compounded by the reduced subsequent growing season after a late snowmelt, making it difficult for offspring to reach the appropriate developmental stage before the onset of cold temperatures (Rank et al., 2020; Sheriff et al., 2017). Although the general trend of climate change will be to reduce energetic limitations on low – mid-elevation populations, the periodic snowy years with very late spring phenology are likely to impact population dynamics. Similar to cold stress, high elevations were buffered from swings in energy stress between years by persistent snow cover, even in dry years, supporting conclusions that organisms at high elevations or latitudes with persistent snow cover will be buffered from climate change for some period of time.

Somewhat surprisingly and in contrast to our predictions, average energy use at a fixed winter duration was lower for snowy compared to dry winters (Figure S1.3A). This occurred because spring snowpack buffered the soil from warm air temperatures and lowered cumulative energetic costs, cancelling out the energetic gains during the cold mid-winter months. This illustrates an important nuance to the maxim of "colder soils in a warmer world" (Groffman et al., 2001) – although snow does indeed increase extreme minimum winter temperatures, during the shoulder seasons snow has an important cooling effect. Thus, in systems where air temperatures warm rapidly in spring while snowpack is still present, such as the relatively low latitude Sierra Nevada mountain ranges, cumulative energy use can be lower when snow is persistent, for a given duration of winter. However, when we take into account that snowy years also have delayed spring phenology and are thus longer than dry winters, we found support for our prediction that snowy winters are more energetically demanding. This suggests that impacts of interannual variation in snow cover on winter energy use are likely to come largely from the effects on spring phenology, rather than by increasing energy use due to soil warming (Fitzpatrick et al., 2019; Kelsey et al., 2020).

Limitations and assumptions of models

Our physiological measurements came from a single population, and it is possible that either cold tolerance or energetic traits differed among populations due to local adaptation or neutral processes (Brandt et al., 2020; Lester and Irwin, 2012; Sinclair et al., 2012) . More research is needed to understand how clinal variation in cold tolerance or energetics interacts with exposure to drive sensitivity of a range of species to climate change. Fine-scale microclimate data, such as we have drawn upon in this study, are not available for most systems, but daily weather data from spatial grids or climate models can be downscaled to organismal body temperatures and physiology using mechanistic modelling approaches such as Niche Mapper (Kearney, 2020; Kearney and Porter, 2017; Kearney and Porter, 2020). The recent addition of a snow module will facilitate rapid advances in our knowledge of how snow impacts overwintering organisms (Kearney, 2020; Kearney and Porter, 2017; Kearney and Porter, 2020). However, significant fine-scale climate heterogeneity in mountainous regions makes projections from course-scale climate grids a significant challenge (Ford et al., 2013). The other key assumptions of the model (loggers approximate beetle body temperatures, fixed phenology within an elevation band) are discussed further in supporting information (See Supporting Discussion, Assumption of models).

Snow in a changing world

Anthropogenic climate change is leading to increased frequency of extreme climatic events (and thus snow cover extremes), against a general backdrop of declining snow cover due to increase air temperatures, in the Western USA and many regions globally (Mallakpour et al., 2018; Mote et al., 2018; Williams et al., 2015b). Interannual variation in snow cover could lead to swings in selective pressures on traits underlying cold hardiness and energetics, maintaining genetic variation that will respond to a changing climate. Our work supports the conclusion that ectotherms overwintering in soil face a trade-off between cold risk and energy use, with snow cover driving that trade-off towards increased energy stress and decreased cold stress (Fitzpatrick et al., 2019). If a negative genetic correlation exists between these key winter fitness traits (i.e. genes that enhance cold tolerance also increase metabolic costs; (MacMillan et al., 2012; Williams et al., 2016b), then interannual variation in snow cover may contribute to maintaining polymorphisms in populations of ectotherms. Areas where the swings in selective gradients are most pronounced, such as mid-latitude and mid-elevation regions, may thus be critical reservoirs of genetic diversity that will determine adaptive potential in response to climate change (Dahlhoff et al., 2008; Rank and Dahlhoff, 2002).

Environmental clines in snow cover decouple the above- and below-ground thermal niche, and challenge the paradigm that climate change will drive uniform upslope range shifts for montane organisms. Individuals moving up from lower elevations may not find refugia in mid-elevations, where they may encounter dangerously cold extremes that intensify during drought years, preventing upward elevational shifts and instead accelerating local extirpations as we have seen during the recent droughts (Dahlhoff et al., 2019). Although high elevations may be able to act as a refugia as conditions continue to become drier, populations shifting to higher elevations are faced with additional physiological challenges, like hypoxia, which causes insects to develop slower at higher elevations (Dahlhoff et al., 2019; Harrison et al., 2015), and colder growing season temperatures (McMillan et al., 2005). Because of these challenges, populations may no longer be viable if appropriate climatic conditions only exist at high elevations.

Conclusions

The *C. aeneicollis* study system is a microcosm for understanding processes through which climate change drives range contractions at the trailing range edge of a species range; which we can use as a model for other montane ectotherms that overwinter in soil in snowy mountains with long, harsh winters. We have shown that snow cover strongly modifies elevational gradients in cold exposure and energy use, with concomitant alterations to cold and energy stress for organisms that overwinter in the soil. Mid-elevation populations will be most vulnerable to winter climate change due to increasing cold stress, while high elevation populations will be buffered from interannual fluctuations in snow cover for time by persistent snow cover. Some of the most important impacts of snow arise through its impacts on spring phenology, which strongly determines winter energy use. These findings highlight the importance of incorporating snow and impact that it has on soil microclimate in predicting organismal responses to climate change. Snow decouples shifts in the above- and below-ground thermal niche, and is thus a vital component of predicting responses to climate change for terrestrial organisms.

Chapter 2

The impact of metabolic plasticity on winter energy use models

2.1 Introduction

Understanding the energetic impact of climate change is an important aspect of global change biology, which can reveal critical patterns of vulnerability that temperature alone cannot detect (Dillon et al., 2010; Fitzpatrick et al., 2019). Ecophysiological energy use models predict energy use by mapping body temperatures (often estimated from microclimate temperature) into energetic expenditures using metabolic rate-temperature relationships (Kearney and Porter, 2020; Sinclair et al., 2016; Sinclair et al., 2013). However, these models assume that metabolic rate-temperature relationships are static, an assumption that is clearly violated given the large amount of phenotypic plasticity that occurs in response to microclimate variation or developmental change (Sinclair, 2015; Sinclair et al., 2016). The impact of plastic variation in metabolic-rate temperature curves on energy use modelling has not been well explored, which may lead to misleading energy estimates, or overlook important periods of energetic drain. Here, we examine the importance of incorporating metabolic plasticity into winter energy use models in dormant overwintering insects.

Energy conservation is particularly relevant to insect fitness in winter, when finite energy stores must sustain life and also fuel subsequent reproduction (Hahn and Denlinger, 2011; Sinclair, 2015). To conserve limited energy stores, many insects overwinter in diapause; a programmed dormancy characterized by arrested (or slowed) development and metabolic suppression (Koštál, 2006; Wilsterman et al., 2021). Insect metabolic rates during diapause are a fraction (varying from as low as 10% up to 85%) of active metabolic rates of comparable developmental stages (Hahn and Denlinger, 2011; Ragland et al., 2009). Metabolic rates can be altered in two main ways: 1) by altering the intercept, which represents overall metabolic intensity, or 2) by altering the slope, representing thermal sensitivity (Terblanche et al., 2009). Both metabolic intensity and thermal sensitivity change as a result of phenotypic plasticity, including developmental plasticity and acclimation or acclimatization (Bozinovic et al., 2013; Lachenicht et al., 2010). Metabolic intensity varies throughout diapause time as a result of developmental plasticity, reaching lowest intensity several weeks after onset (Toxopeus et al., 2021), followed by a gradual increase in metabolic intensity throughout the spring (Gray et al., 1995; Lester and Irwin, 2012). Winter microclimates can also alter metabolic rate-temperature relationships through acclimation (in the laboratory) or acclimatization (in the field); with the general pattern emerging that variable winter environments lead to decreased thermal sensitivity, while warmer winter microclimates lead to decreased metabolic intensity, both leading to energetic savings in energetically demanding environments (Sgolastra et al., 2010; Williams et al., 2015a; Williams et al., 2012b). Developing a general method to incorporate these widespread patterns of phenotypic plasticity into energy use models may improve our ability to accurately predict winter energy use.

Chrysomela aeneicollis (Schaeffer 1928) populations in the Sierra Nevada mountains are an important model system for understanding physiological and genetic basis of responses to climate change (Dahlhoff et al., 2019; Dahlhoff et al., 2008; Rank and Dahlhoff, 2002). Freeze-

tolerant adults overwinter in diapause in the soil, often beneath snow, for up to eight months of their one-year life cycle (Boychuk et al., 2015). Snow cover modifies the thermal environment of soil by buffering from cold air temperatures (Pauli et al., 2013), providing a relatively warm and stable thermal environment for overwintering insects that can impact energetics and fitness (Irwin and Lee, 2003). Ecophysiological energy use models suggest that energetic costs of winter decrease across elevation, and that snowy years are more energetically demanding than dry years due to longer winter periods (Chapter 1), but previous energy use models used a single metabolic rate-temperature curve, with no plasticity taken into account. In this study, we address two main objectives: first, we test how metabolic rate-temperature relationships of *C. aeneicollis* change throughout dormancy and in response to thermal acclimation to ecologically relevant microclimates associated with winter snow; and second, we use ecophysiological models to assess how these modifications will impact energy use estimates; validating these models using empirical measurements of energy reserves.

2.2 Materials and Methods

Beetle collection and acclimation treatments

Beetles were collected in August 2018 from the Rock Creek Drainage (37°26'25.8"N 118°44'46.0"W) in the Eastern Sierra Nevada Mountains in California. Beetles were then housed in incubators (MIR-154-PA incubators; Panasonic Scientific, Wood Dale, IL, USA) in the laboratory at a 20/4°C 12L/12D day night cycle until they entered dormancy, following protocols in Chapter 1. Once beetles entered dormancy (determined by cessation of feeding in the presence of food; between September 21st and October 11th), they were housed in 50mL conical tubes filled to around 40% with coconut husk substrate, and moved to an incubator held at 1°C in 24 h darkness. On November 1st the beetles (N=120) were haphazardly divided between a constant and variable acclimation treatment in separate incubators under constant darkness. The constant treatment simulated conditions beneath the snow (constant 1°C), while the variable treatment simulated uncovered ground (-2.5/-1/0/-1C in a 6/6/6/6 h cycle). Temperature regimes were selected to mimic late-winter conditions, based on temperature measurements from plots subject to an experimental snow manipulation (Chapter 1). Beetles were kept in these acclimation treatments, with moisture being added monthly, until their respiration rates were measured.

Measuring respiration rates

To capture changes in the metabolic rate-temperature relationship through the end of dormancy, oxygen consumption ($\dot{V}O_2$) was measured using a Sable Systems FoxBox respirometer (Sable Systems International, North Las Vegas, NV) stop-flow respirometry system once a month from February to May, following protocols from Chapter 1. We chose these timepoints to best reflect metabolic rates through the transition out of the coldest portion of dormancy. Briefly, at each timepoint a subset of 15 beetles from each acclimation treatment (constant and variable) were individually placed into a 10mL syringe and flushed with CO_2 and H_2O-free air, generated using a Drierite-Ascarite-Drierite column, and then incubated at -1°C, 4°C, 9°C for 48 h or 20°C for 24 h before having $\dot{V}O_2$ measured. The order of temperature exposures for the three lowest temperatures was randomized, but always finished with the 20°C measurement to prevent any downstream impacts of prolonged exposure to warm temperatures on respiration rates. Beetles

were given a minimum of 48 hours to recover between each measurement, during which time they were returned to their acclimation treatment. After each beetle was measured at all temperatures, beetles were frozen for biochemical analysis.

Fitting metabolic rate-temperature curves

All analyses were performed in R 4.0.2 (R Core Team, 2019) unless otherwise specified. $\dot{V}O_2$ was log-transformed ($\log_e$) prior to analyses to approximate a linear relationship with temperature. Metabolic rate-temperature curves were fit to the data using a linear mixed effect model with the lmer function from the lme4 package. We used a Satterthwaite approximation in the lmerTest package to approximate degrees of freedom and p-values (Bates et al., 2015; Kuznetsova et al., 2017). Model selection was done by starting with a full factorial model and eliminating non-significant interactions that improved model fit evaluated by Akaike Information Criterion (AIC) (Crawley, 2012). The initial model included $\dot{V}O_2$ as the dependent variable and month, measurement temperature, acclimation treatment, and mass as independent variables along with their interaction terms. Since $\dot{V}O_2$ measurements were repeated measures, beetle identity was included in the model as a random effect. Once the best fit model was chosen and significant terms were identified, we then made models that identified the slope and intercepts of monthly values and treatment independently. Slopes and intercepts were extracted and used to approximate thermal sensitivity and metabolic intensity in energy use models.

Ecophysiological energy use models

We compared the performance of three ecophysiological energy use models. The first model was from Chapter 1, based on a single metabolic rate-temperature curve. We refer to this as the Constant model, and all models were modified from this starting model (Eq. 1).

(Eq. 1)

$$\log_e \dot{V}O_2 = S \times t - b$$

S indicates thermal sensitivity, *t* indicates temperature, and *b* represents metabolic intensity. The second model incorporates a stepwise monthly increase in the intercept, simulating the gradual increase in metabolic intensity that we documented through time in the respiration rate measurements, with an intercept of -1.952 and a slope of 0.173. We determined the rate of change in the intercept by fitting a linear regression to the intercept of the metabolic rate-temperature relationship as a function of month (Feb – May), and used the slope of this regression as a scaling factor in the static model. We refer to this as the Dynamic model (Eq. 2).

(Eq.2)

$$\log_e \dot{V}O_2 = (S \times t) + (b_m \times m - b)$$

Where m is month of measurement, and b_m is the monthly rate of change in metabolic intensity. The third model incorporated the impact of plasticity in response to acclimation treatment (Results). The empirical metabolic rate-temperature relationships from Chapter 1 was measured in beetles maintained under constant acclimation conditions. Using the constant model as a base, we modified both *S* and *b* by the amount that they were modified in the variable compared to

constant acclimation treatment in the present study. We refer to this as the Variable model, and it is used only in predictions of energy use in the variable acclimation treatment (Eq. 3).

Eq. 3

$$\log_e \dot{V}O_2 = (S + S_a) * t + (b + b_a)$$

Where S_a refers to the difference in thermal sensitivity and b_a the difference in metabolic intensity between the two acclimation treatments.

Hourly oxygen consumption throughout the winter was calculated using Eqs 1-3, using incubator temperatures *(t)* from the constant and variable acclimation treatments. Monthly energy use was calculated by summing hourly oxygen consumption, and converted to lipid consumed assuming 2L oxygen per 1g lipid metabolized (Schmidt-Nielsen, 1997), giving rise to monthly lipid use estimates under each model, for each acclimation treatment.

Empirically validating model estimates

We quantified lipid (triacylglyceride) stores of beetles (15/month/treatment) using thin-layer chromatography coupled to a flame ionization detector (TLC-FID; Iatroscan MK-6s TLC-FID Analyzer; Shell-USA, Spotsylvania VA, USA), with cholesterol as an internal standard (Williams et al., 2011). We tested if lipid stores changed through time by ANOVA.

To validate model predictions of lipid use against these empirical measurements of lipid stores, we subtracted the monthly lipid use estimates for each model (Eqs. 1-3) from the average February lipid stores for constant and variable incubators separately, giving us predicted monthly lipid stores. We expressed cumulative model errors (over the entire period of February to May) as the absolute difference between lipid stores of individual beetles in May and predicted lipid stores in May under each model, separately for constant and variable incubators. Model accuracy was compared between models using ANOVA with absolute errors as the response variable and model as the predictor, separately for each acclimation treatment.

2.3 Results and Discussion

Plasticity in metabolic rate-temperature relationship

Beetle metabolic rate – temperature relationships were plastic both throughout development and in response to thermal acclimation. Beetle metabolic rates increased between February and May, resulting in a gradual increase in metabolic intensity with no corresponding change in thermal sensitivity ($F_{3,108}$=32.37, p<0.0001; Figure 2.1). A similar increase of metabolic intensity during winter has been observed in pine beetles (Lester and Irwin, 2012), gypsy moths (Gray et al., 1995), and solitary bees (Sgolastra et al., 2010) but not goldenrod gall flies (Irwin et al., 2001), and occurs during the transition from diapause to post-diapause quiescence (Hahn and Denlinger, 2011; Lester and Irwin, 2012; Ragland et al., 2009). Paralleling our results of no change in thermal sensitivity, thermal sensitivity remains consistent throughout winter in gypsy moth eggs (Gray et al., 1995), but increases towards the end of winter in Fall webworm pupae (Williams et al., 2015a), suggesting that changes in thermal sensitivity throughout winter may be less important and consistent than changes in overall metabolic intensity. .

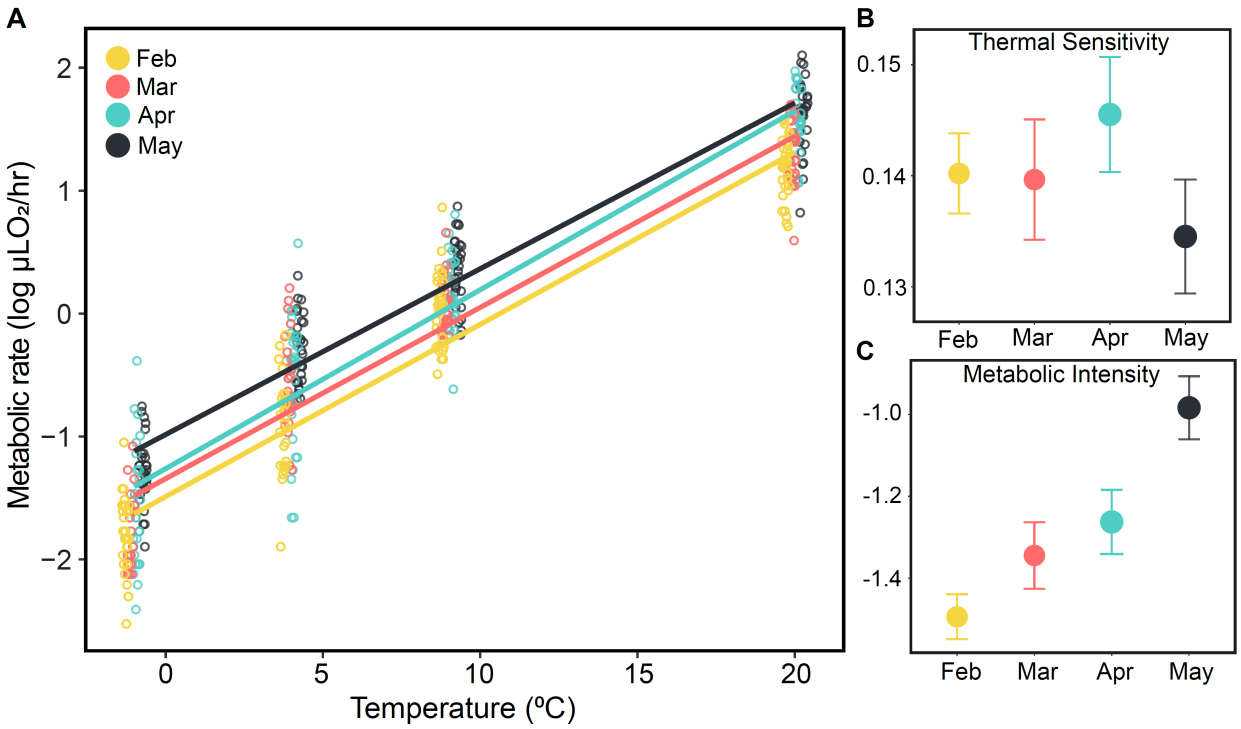

Figure 2.1) Developmental plasticity in metabolic rate – temperature relationships in *Chrysomela aeneicollis*. (A) Metabolic rates (estimated using rates of oxygen consumption ($\dot{V}O_2$) as a function of temperature for beetles measured in February (yellow), March (pink), April (teal), and May (black). (B) Thermal sensitivity (slope) and (C) Metabolic intensity (intercept) of metabolic rate-temperature relationships in panel A. Error bars in panel B and C denote standard errors on the parameter estimates.

Beetles acclimated to variable temperatures had higher metabolic intensity than beetles acclimated to constant conditions ($F_{1,237}$=9.74, p=0.0020; Figure 2.2C), and lower thermal sensitivity ($F_{1,332}$=5.48, p=0.0198; Figure 2.2B). Variable winter temperatures also resulted in lower thermal sensitivity in overwintering larval Lepidoptera (Williams et al., 2012b). Thermal regimes approximated soil temperatures below snow (constant) or with snow removed (variable) at a high elevation site in the Eastern Sierra Nevada mountains (Chapter 1), so it is likely that natural variation in snow cover would elicit a plastic response in these beetles

Impact of metabolic plasticity on long-term energy use

Beetle lipid stores decreased linearly through time ($F_{3,106}$ =3.54, p = 0.017), and did not differ between constant and variable acclimation treatments ($F_{1,106}$=0.27, p=0.60, Figure 2.3). All models predicted energy use accurately, with estimates falling within the 25th-75th percentile of empirically measured beetle lipid stores, with the exception of the constant acclimation treatment in May (Figure 3). Neither the Dynamic model, approximating the magnitude of observed developmental plasticity, nor the Variable model, incorporating acclimation plasticity, improved accuracy of model estimates relative to the base Constant model used in Chapter 1 ($F_{2,84}$=0.33, p=0.72; Figure 2.3C). The Dynamic model predicted accelerating rates of energy use throughout time, corresponding to the observed increase in metabolic intensity, but we did not see any increase in rates of empirical lipid depletion through time (if anything, rates of lipid depletion slowed through time, particularly in the constant conditions). This suggests that the increase in metabolic intensity that we observed in beetles towards the end of winter does not influence long-term trends in energy use.

In this case, the magnitude of intraspecific variation in lipid stores overshadowed any effect of plasticity on model accuracy. The largest magnitude of plasticity in our experiment was developmental, and, in the constant acclimation treatment, the difference in predicted energy use resulting from developmental plasticity was 0.04 mg (Constant versus Dynamic model, Figure 2.3A). However, there was large individual variation in observed lipid stores (SD=0.15 mg), swamping the magnitude of plasticity. Based on observed levels of variability, the magnitude of difference between model estimates would have to exceed ~0.18 mg of lipid in order to detect a statistically significant effect (power analysis; power=0.8, n=15, α=0.05). To detect the observed predicted difference in energy use between the Constant and Dynamic model in the constant environment (0.04 mg lipid), we would need a sample size of 112 per treatment (or 7.5 times what was used in this study). The large degree of variation among individuals in lipid stores not only makes it difficult to detect the impact of plasticity in models, but is likely to have more profound impacts on energetic stress in winter than the magnitude of plasticity that we observed. Individual lipid stores are vital in determining an organism's fasting endurance in winter (Trondrud et al., 2021), and may provide an interesting avenue for future research.

The degree of metabolic plasticity that we observed may be more impactful in natural thermal environments, which exhibit greater variation in winter energetic demands than did our two acclimation treatments (i.e. due to a wider range of temperatures, or longer overwintering periods). Energy use estimates from models incorporating developmental plasticity (increase in metabolic intensity) would be particularly sensitive to warm temperatures due to the non-linear relationship of metabolic rate with temperature (Jensen's inequality; (Ruel and Ayres, 1999)) combined with the observed increase in metabolic intensity. This could have large impacts on overwinter energy use, where warm end of winter temperatures can heavily influence energy expenditure (Chapter 1). Adding the developmental plasticity to model estimates of winter energy

use from Chapter 1 led to an average increase of 24.3% or 0.149 mg, and when including thermal acclimation there was an 11.0% or 0.064 mg increase. When we compare these energy use differences to the threshold ~0.18 mg lipid difference needed to detect an effect given intraspecific variability in lipid stores, it is clear that although the impact of acclimation plasticity will not be detectable even in ecologically relevant microclimates; developmental plasticity may at least come close to causing detectable differences in energy use in more ecologically relevant thermal regimes.

The energetic costs of winter are a critical and understudied aspect of understanding biotic responses to climate change (Fitzpatrick et al., 2020; Sinclair, 2015; Williams et al., 2012a). Metabolic rate temperature relationships in dormancy are plastic; changing as a result of developmental plasticity and thermal acclimation. We developed a novel method to incorporate these types of plasticity into energy use models in a generalizable way, but this did not improve our model estimates relative to empirical lipid quantifications. We instead found that individual variation in lipid stores far overshadowed the effects of plasticity on energy use. This suggests that estimating winter energy use based on a single metabolic rate-temperature will give us reasonable estimates that will help understand the energetic impacts of environmental change, although developmental plasticity may need to be accounted for when energy demands diverge very strongly between environments.

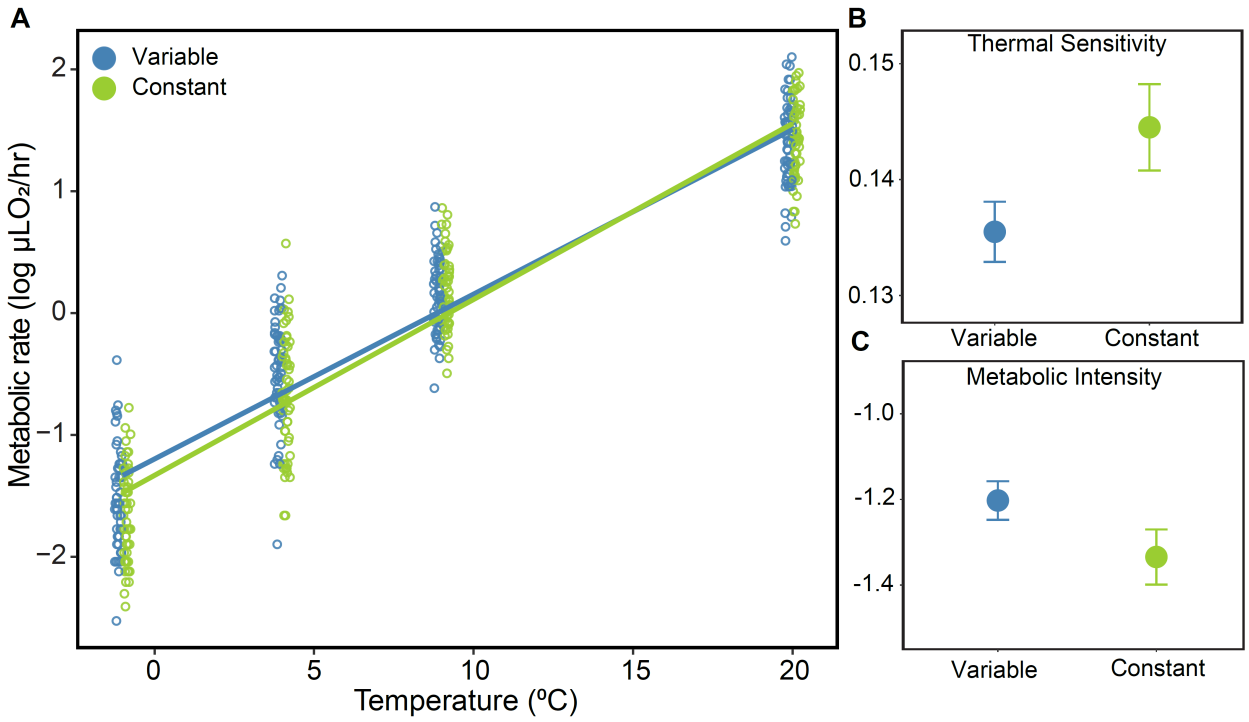

Figure 2.2) Plasticity in metabolic rate – temperature relationships due to thermal acclimation in *Chrysomela aeneicollis*. (A) Metabolic rate data from beetles that overwintered in variable (temperatures between -2.5 and 0 °C daily) and constant (1°C) acclimation treatments, with monthly measurements pooled. (B) Thermal sensitivity (slope) and (C) Metabolic intensity (intercept) of metabolic rate-temperature relationship in panel A. Error bars in panel B and C denote standard errors on the parameter estimates.

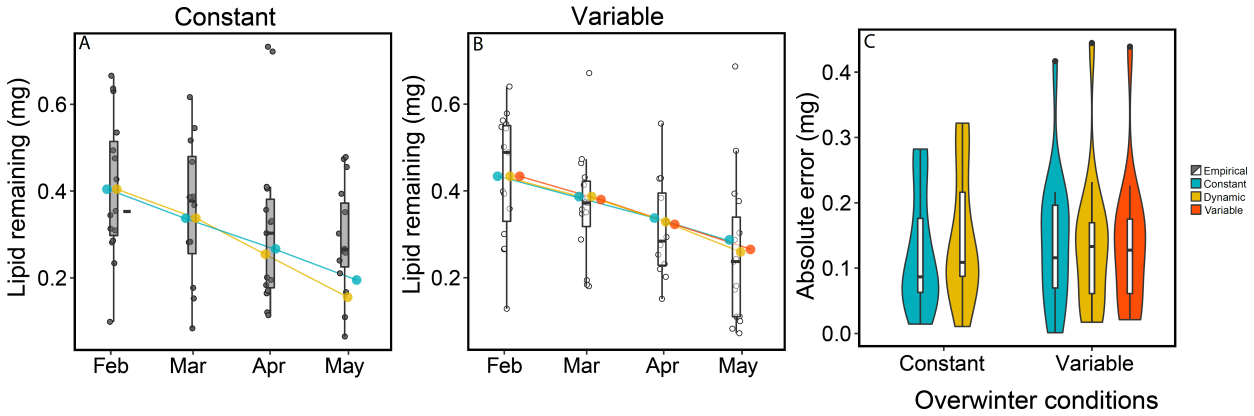

Figure 2.3) Energy use model estimates are not improved when incorporating metabolic rate plasticity when compared to empirical lipid measurements. Data shown are energy use estimates from energy use models incorporating no plasticity (Constant, teal), developmental plasticity (Dynamic, yellow), and acclimation plasticity (Variable, orange) overlain on empirical lipid quantifications (grey bars) for *C. aeneicollis* beetles that overwintered in constant (A) or variable (B) acclimation treatments. Estimates from the constant base model are provided for both constant and variable acclimation treatments to allow comparison of model estimates in the same environment. (C) Errors between model predictions of lipid stores compared to empirically measured lipid stores of beetles in May.

Chapter 3

A time course of gene expression suggests that montane leaf beetle prioritize digestion and reproduction during emergence from dormancy

3.1 Introduction

For animals inhabiting seasonal environments, winter poses challenges that require extensive physiological modifications to survive. To cope with these challenges, many animals wait out the challenging season by entering a state of dormancy (Wilsterman et al., 2021). For insects this comes in the form of diapause, which is an endogenously programmed dormancy that is associated with suppressed metabolism, increased stress tolerance, and stalled development (Koštál, 2006). Diapause requires extensive physiological modification that can begin months in advance, including accumulation of energy stores, increasing desiccation resistance, and cessation of reproduction (Hahn and Denlinger, 2011; Yoder et al., 1992). The transition out of a dormant state into an active growing and reproductive season requires reversal of many of the dormancy related physiological modifications to successfully meet the energetic demands of the active season (Sinclair, 2015). This large shift of physiological processes requires energetically costly cellular modifications that cannot be all achieved at once and require prioritization (Raubenheimer et al., 2007; Roncalli et al., 2018). While we have a partial understanding of the pathways and physiological mechanisms underlying the transition out of dormancy (Poelchau et al., 2013; Ragland et al., 2010; Yocum et al., 2015), we do not have a good understanding of how environmental conditions influence the timing, magnitude, and prioritization of these responses in natural environments. Understanding the role of the environment, and more specifically the microclimate, in determining the intensity of seasonal physiological shifts will help us expand our understanding of the biological impacts of climate change. Here we examined how microclimate variation, driven by presence or absence of winter snow cover, impacted gene expression upon emergence from dormancy in a montane leaf beetle.

At the onset of diapause there is down-regulation of genes associated with metabolism, DNA replication and transcription, endocrine signaling, stress response, and morphogenesis (Bonizzoni et al., 2013; Koštál, 2006). During the deepest maintenance phase of diapause, morphogenesis has stopped, metabolic rate is maximally suppressed, and resistance to cold and oxidative stress is maximized; with corresponding expression changes in genes regulating metabolism, development, and stress responses (Koštál, 2006; Ragland et al., 2010). During the latter part of winter, metabolic suppression is gradually released and diapause is terminated along with the endogenous lock of development, after which insects often remain dormant in a state of post-diapause quiescence until environmental conditions become permissive for development, allowing them to coordinate timing with seasonal change (Yocum et al., 2015). The termination of diapause is accompanied by up-regulation of genes associated with transmembrane transport, cellular signaling pathways, anaerobic glycolysis, and neuroendocrine control, and down-regulation of the oxidation-reduction process (Ragland et al., 2011; Yocum et al., 2015). After diapause termination, upon transition from post-diapause quiescence to active season there are

large scale life history transitions, but the trajectories of gene regulation during this process remain unknown.

Microclimatic conditions of overwinter hibernacula determine survival through diapause and physiological condition upon emergence (Irwin and Lee, 2003; Marshall and Sinclair, 2018; Turnock and Fields, 2005; Williams et al., 2002). During diapause, metabolic rates remain temperature sensitive, although suppressed (Gray et al., 1995; Lester and Irwin, 2012; Williams et al., 2012b). Because dormant insects overwinter with fixed energy reserves, any increase in energy use can either lead to mortality from energy depletion or compromise future reproduction (Hahn and Denlinger, 2007; Sinclair, 2015). Cold microclimate temperatures increase the risk of cold stress, which can directly cause mortality, or reduce performance and fitness due to cellular damage and associated energetic costs (Bale, 1987). Winter cold extremes determine range limits in many insects (Crozier, 2004; Lehmann et al., 2015; Lynch et al., 2014)

Winter snow is a pivotal regulator of winter microclimate for organism that overwinter in soil. Snow is an effective insulator that decouples air and soil temperatures, leading to relatively warm and stable soil temperatures when it is present (Pauli et al., 2013). Higher average temperatures below-snow increase energy use through winter (Irwin and Lee, 2003; Marshall and Sinclair, 2012). In contrast, when snow is not present, organisms overwintering in the soil are more frequently exposed to stressful cold temperatures (Fitzpatrick et al., 2019; Zhang, 2005). In addition to thermal regulation, snow also directly impacts phenology of dormancy by determining when it is even possible to physically emerge from soil, and by impacting the availability of resources (i.e. timing of plant budburst). By modulating energy use and cold exposure, and determining seasonal timing, snow can directly impact organismal fitness in both winter and in the subsequent growing season. Despite the large organismal effects of snow cover variation demonstrated during the winter stage, we lack an understanding of how variation in snow cover over winter modifies the regulation of physiological processes upon emergence from dormancy.

Populations of the willow beetle *Chrysomela aeneicollis* in the Eastern Sierra Nevada are living at their southern range edge, where they are restricted to high elevation riparian areas. Beetles living in these habitats overwinter in soil below their willow host plant as pre-reproductive adults, where they spend up to nine months of their one-year life cycle in dormancy. These habitats have highly variable interannual snow cover, driven by cycling of El Niño and La Niña oscillations and increasingly frequent drought (Allen and Anderson, 2018; Berg and Hall, 2017; Lute and Abatzoglou, 2014), exposing beetles to energy stress in snowy winters and colder microclimates in dry winters (Chapter 1). Snow impacts population dynamics of *C. aeneicollis*, with range contractions occurring after several consecutive winters with low snow (Dahlhoff et al., 2019). Here, we manipulated snow cover and profiled gene expression using RNAseq during the first five days after emergence from dormancy, to examine how winter conditions influence physiological processes during a critical life history transition.

3.2 Materials and Methods

Field snow manipulation

Beetles were collected in August 2018 from Above Bull Lake (37°08' 59.2"N; 118°W33' 05.6"W) in the South Fork Bishop Creek drainage of the Sierra Nevada Mountains in California. We brought beetles back to the laboratory in Berkeley CA, where they were they were housed in 475 mL plastic containers containing moist coconut husk, and kept in an incubator (Panasonic MIR-154-PA, Wood Dale, IL) at constant temperature and light cycles (20ºC : 4ºC day : night; 12.5L:11.5D photoperiod). We fed the beetles *ad libitum* with several *Salix lasiolepis* leaves at a time, which were replaced every 3 to 4 days. We considered beetles dormant when they stopped feeding in the presence of food, and at that point they were transferred to 50 mL conical tubes and moved to an incubator held at 1°C with 24 h darkness.

In October 2018, beetles were haphazardly selected to be overwintered either in a plot exposed to ambient snowfall (Snowy plot) or one below an overhang (Dry plot) to keep snow removed, following methods outlined in Chapter 1. We first placed beetles into 10 cm long sections of PVC pipe half filled with sterile coconut husk (n = 10 beetles/tube), and sealed with fiberglass window screening over each opening. Seven PVC tubes were then placed inside each rubber container (n=12 containers) and sealed with aluminum window screening. We recorded temperature using by an iButton thermochron (DS1922L, Maxim Integrated Products, Sunnyvale, CA) in each rubber container. Two rubber containers were then buried per hole (3 holes total per treatment). We returned to collect the beetles in June 2019, where we dug up each container, and flash-froze beetles in liquid nitrogen as soon as we could assess they were alive based on movement (0 h timepoint). We then took the remaining beetles to the Sierra Nevada Aquatic Research Laboratory (SNARL; 37º36'51" N, 118º49'47"W, 2159 m elevation), where they were kept in an incubator (Panasonic MIR-154-PA, Wood Dale, IL) (20ºC : 4ºC day : night cycle and 14L : 10D photoperiod) with fresh *Salix orestra* leaves, which were never fed on. We flash-froze 12 beetles in liquid nitrogen from each overwinter condition 24, 48, 72, and 120 hours after they were excavated, ensuring that they were sampled the same time of day to minimize any influence of circadian effects on gene expression.

Temperature data processing

The iButton temperature loggers used in the overwinter plots inadvertently stopped recording in March, leaving a gap of three months of missing temperature data. To fill in this gap for the snowy plot we used data from a nearby site, Pine Grove (RCPG, 37°28'13.4”N; 118°W43'33.7"W) that was collected using HOBO Pendant 64K temperature and light data loggers (Onset, MA, USA) buried ~5cm below the soil at the base of a willow (Chapter 1). We fit a linear regression of temperature data from the Snowy plot to the RCPG logger temperature data. The model was then used to transform the RCPG temperature data to into estimated Snowy plot temperatures for winter. The RCPG soil temperature closely matched the Snowy plot temperature data ($t=62.78$, $p<0.0001$), with an R^2 of 0.428, an intercept of -0.53 and a slope of 0.65 (Figure 3.1). We then repeated this procedure for the Dry plot; by regressing Dry plot temperature data from early winter to air temperature data from the Rock Creek Lakes weather station (RCL; CA Dept of Water Resources; 37°27'26.2074"N, 118°44'6.072"W; 2957 m elevation). The model fit was then used to transform the RCL air temperature data into estimated Dry plot data for winter.

RCL weather station data matched Dry plot microclimate data ($t=54.69$, $p<0.0001$), with an R^2 of 0.498, an intercept of -0.46 and a slope of 0.32 (Figure 3.2).

RNA extraction, Library prep and sequencing

Beetles were stored at -80°C until we extracted whole body RNA. First, we homogenized whole beetles in TRIzol (Thermo Fisher, Waltham, MA), then performed extractions using Directo-zol RNA miniprep kits (Zymo Research, Irvine, CA) following the standard protocol. RNA was quantified using a Qubit Fluorometer (Thermo Fisher, Waltham, MA) with Quant-iT RNA Assay Kits (Thermo Fisher, Waltham, MA), and quality was assessed using a Bioanalyzer 2100 (Agilent, Santa Clara, CA). Samples with minimal degradation and appropriate concentrations were used in subsequent steps.

Libraries were constructed for each beetle (n = 12) from each overwintering condition (Snowy and Dry) at each of the five timepoints, giving rise to 120 libraries. Libraries were prepared by Novogene using NEBNext UltraTM II Directional RNA Library Prep Kit (New England Biolabs, Ipswich, MA). During library construction, mRNA was enriched using oligo(dT) beads (Thermo Fisher Scientific, Waltham, MA). Libraries were then multiplexed and sequenced on an Illumina NovaSeq with 150PE reads.

RNAseq data processing

Upon receiving the data, we used Cutadapt (Version 1.15; (Martin, 2011)) to remove adapters and low-quality portions of reads (Phred score > 20). Resulting reads that were less than 30bp were then removed.

Transcriptome assembly

To make a transcriptome, we mapped whole body RNAseq reads from three beetle samples to the assembled *C. aeneicollis* genome (Bracewell et al. in prep) using hisat2 (Kim et al., 2019). The mapped reads were then used to create a transcriptome with stringtie 2 for the MAKER annotation (Cantarel et al., 2008; Kovaka et al., 2019). We then functionally annotated the transcriptome against the Swiss-Prot database using Trinotate (v3.2.1; Bryant et al. (2017)) with BLASTx for nucleotide sequences, and BLASTp and Pfam for TransDecoder (v5.50) translated peptide sequences. We also used Signalp (v4.1) to predict signal peptides and tmhmmm (v2.0) to predict transmembrane helices in proteins.

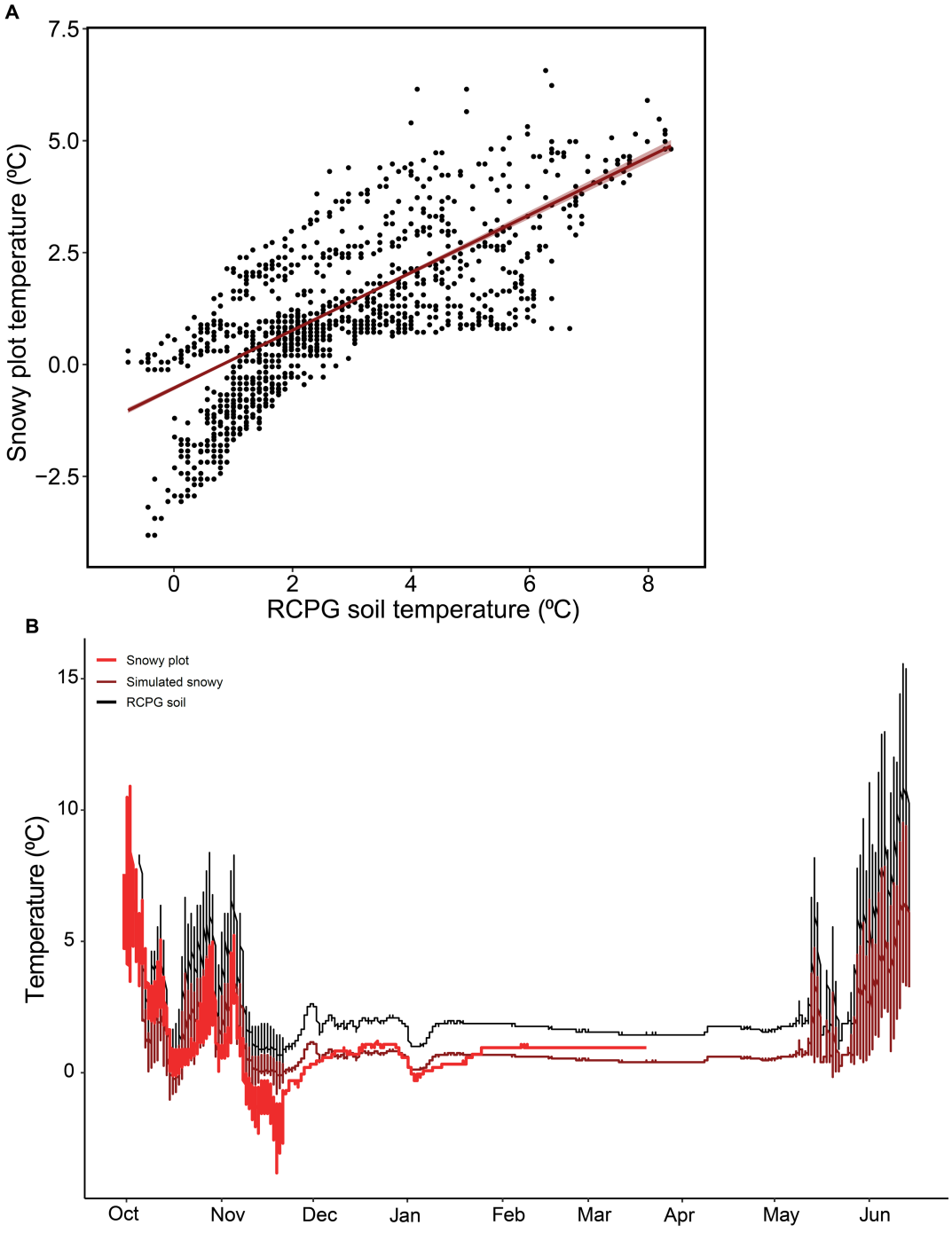

Figure 3.1 Comparison of Snowy plot temperatures to RCPG soil and simulated snowy temperatures. (A) Relationship of RCPG site temperatures and Snowy plot temperatures, with regression used to generate simulated snowy temperatures. (B) Winter temperatures from data loggers in the Snowy plot (bright red) and the nearby soil temperatures from the RCPG site (black). These two temperatures were used to make the simulated snowy temperatures (dark red). For more details see methods of temperature simulation.

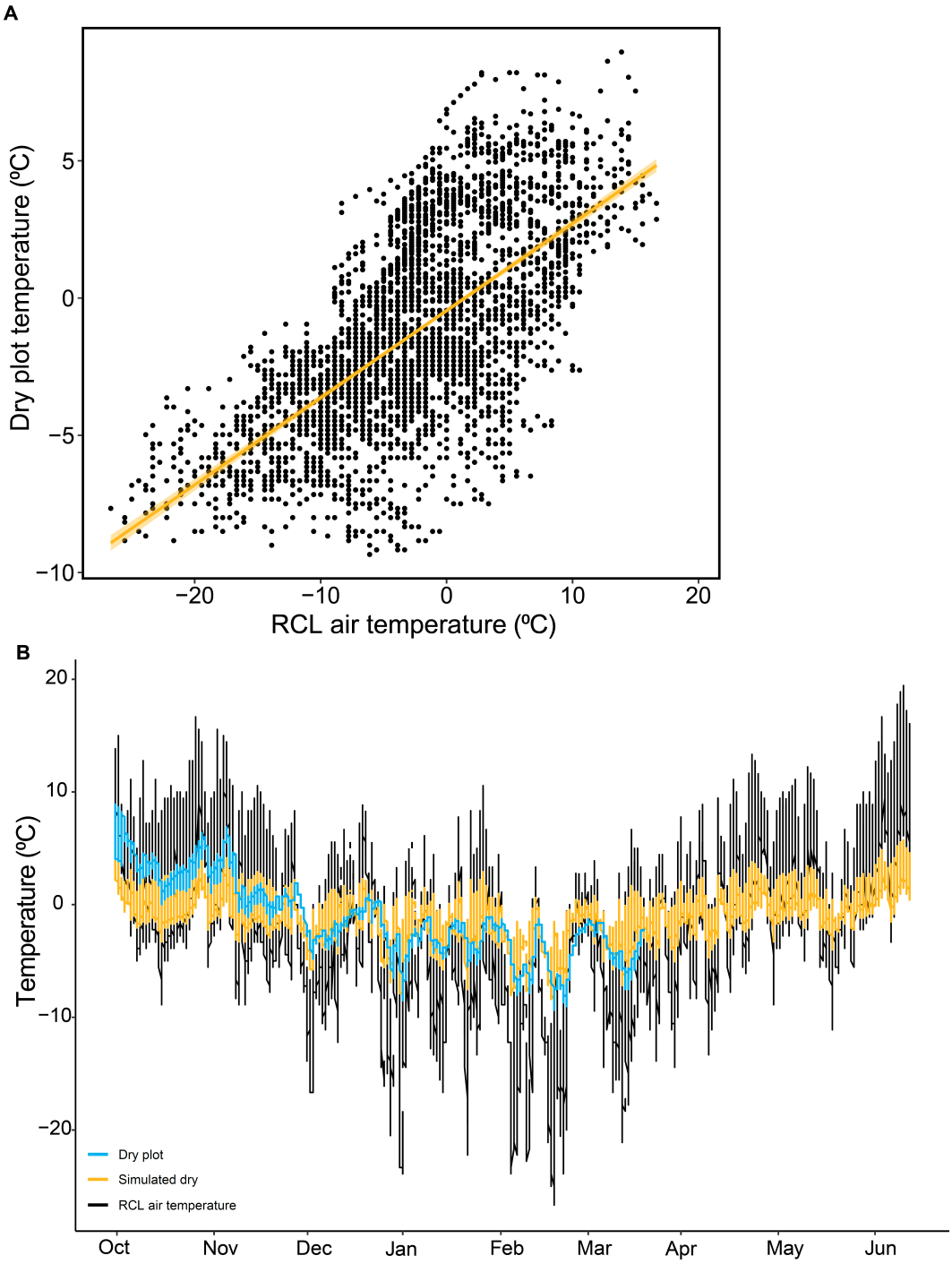

Figure 3.2 Comparison of Dry plot temperatures to RCK air temperatures and simulated dry temperatures. (A) Relationship of RCL air temperatures and Dry plot temperatures, with regression used to generate simulated dry temperatures. (B) Winter temperatures from data loggers in the Dry plot (blue) and the nearby air temperatures from the RCK weather station (black). These two temperatures were used to make the simulated dry temperatures (yellow). For more details see methods of temperature simulation.

Differential gene expression analysis

We first used Kallisto (v0.46.2) (Bray et al., 2016) to quasi-map reads of each library to each contig in the transcriptome to generate read counts for each transcript. The number of mapped reads were compared across all samples visually using boxplots, following Stillman et al. (2020), and no samples were removed.

Differential expression (DE) analysis was done in R (v4.2.0; R Core Team (2019)) using the package EdgeR (Robinson et al., 2010). We first generated multidimension scaling of filtered read counts using the *plotMDS* function. We then identified the sex of the beetles based on expression of three transcripts of the doublesex gene, which is important in sex determination in beetles and have sex-specific isoforms (Price et al., 2015; Shukla and Palli, 2012). Individuals clearly expressed high levels of either male- or female-specific doublesex transcripts, leaving little ambiguity in sex assignment. Sex assignments were validated with reference to other sex-specific transcripts (three vitellogenin transcripts for females, and sperm flagellar protein and two testis-specific protein kinases for males); confirming in all cases that individuals with a male-specific pattern of expression at doublesex also expressed testis-specific proteins, and individuals with a female-specific pattern of expression at doublesex expressed ovarian proteins. Males and females were then analyzed separately, to identify sex-specific patterns of gene expression. Read count data were normalized using a transcripts per million normalization, then a fully factorial generalized linear model (negative-binomial error distribution) was fitted, with main and interaction effects for winter snow conditions and time of sample. Likelihood ratio tests, with a fold change of > 2 and a false discovery rate of < 0.01, were used to identify differentially expressed transcripts. Each likelihood ratio test was done as a pairwise comparison, first comparing snowy and dry plots at each timepoint to get transcripts that changed with winter snow cover, then comparing each timepoint within a plot to the Time 0 samples, to identify transcripts that changed in expression levels through time. We combined all unique transcripts from each comparison to create a set of differentially expressed genes for each sex separately, for clustering and GO enrichment analysis, which were then median centered and $\log_2$ transformed. We clustered transcripts using k-means clustering in Cluster (v3.0) (De Hoon et al., 2004). Optimal number of k-means clusters was determined by starting with n=10 clusters, and reducing the numbers of clusters until all remaining clusters visually showed unique expression profiles (Stillman et al., 2020). The optimal number of clusters was independently confirmed as the number of clusters that minimized the sum of squared errors (SSE) using the package cluster (v2.1.2) (Maechler et al., 2021) in R.

GO enrichment analysis

To identify the molecular functions and biological processes that were over-represented in each cluster, we performed a GO (gene ontology) enrichment analysis using the R package GOseq (v1.42) (Young et al., 2010) on differentially expressed genes in each cluster. We considered a GO term to be over-represented if it had a Benjamini and Hochberg adjusted p-value < 0.05.

Pathway analysis

Based on over-represented GO terms, we conducted pathway-wide analyses on the central metabolic pathways glycolysis, the citric acid cycle and β-oxidation. We identified all genes involved in these pathways in the *Tribolium castaneum* genome using KEGG (Kyoto Encyclopedia of Genes and Genomes), and extracted matching transcripts from our transcriptome. We then compared the matching transcripts to our DE transcript set, and manually compared the direction of mean expression change through time separately for males and females that overwintered in Dry and Snowy conditions (four separate comparisons), giving rise to transcripts that increased, decreased, or did not change through time for each treatment group.

3.3 Results

Field snow manipulation

Snow first accumulated in late November, and was present consistently until mid-May (Figure 3.3A). The peak snow depth was 200.6cm in March. During the winter the Snowy plot was on average 1.9^{o}C warmer than the Dry plot, with average temperatures of 0.9 ± 1.1^{o}C (Snowy) and -0.8 ± 2.3^{o}C (Dry) respectively. Extreme minimum temperatures were substantially colder in the Dry compared to the Snowy plot (Dry: -8.9^{o}C [Feb 19]; Snowy: -1.0^{o}C [Oct 15]), and the extreme maximum temperature was higher in the Snowy plot (Dry: 5.7^{o}C; Snowy: 9.5^{o}C, both occurring June 16; Figure 3.3B). In the last weeks before emergence, temperatures were warmer in the Snowy plot with an average temperature of 2.5^{o}C in the Dry plot and 4.6^{o}C in the Snowy plot and maximum temperatures of 4.8^{o}C in the Dry plot and 7.8^{o}C in the Snowy plot. There was 76.2% survival of beetles that overwintered in the Dry plot, which is higher than 58.6% of beetles in the Snowy plot. Low survival of beetles in the Snowy plot is largely driven by one of the three replicate plots having 11.9% survival, while the two others combined had 81.6% survival.

Differential gene expression patterns

The most prominent driver of differences in gene expression was sex, with females falling exclusively below and males exclusively above 0 on MDS Dimension 1 (Figure 3.4A). Correspondingly, female-specific transcripts of doublesex were expressed higher (Doublesex X1: $F_{1,118}$=680, p<0.0001; Doublesex X2: $F_{1,118}$=717, p<0.0001) in individuals that had an MDS1 value less than 0, while male-specific transcripts (doublesex X3) were higher in individuals with an MDS1 value greater than 0 ($F_{1,118}$=1380, p<0.0001; Figure 3.4B). The second main pattern in overall gene expression in the MDS plot on dimension 2 is the effect of time, which is sex dependent. Overall, the lower the value of MDS2, the later after emergence the sample is taken. Beetles that overwintered in Dry plots have higher values of MDS2 than beetles that overwintered in Snowy plots.

The number of differentially expressed transcripts (DETs) was higher in males (4,532 unique DETs), than females (1,451 unique DETs, Figure 3.5A). Annotation was generally robust, with each pairwise comparison having between 75-88% of the DETs annotated from either blastx, blastp, or Pfam; except for the between treatments comparison at 72 h in females, which had 62% annotated (Table 3.1). Males had higher numbers of differentially expressed transcripts than females at every equivalent pairwise comparison, with the exception of the 0 h to 24 h comparison in the Snowy plot, which both had 17 differentially expressed transcripts (Figure 3.5A).

Differential gene expression increased with time after emergence in both sexes, with the greatest differentiation from the initial timepoint occurring at 120 h post-emergence (Figure 3.5B). Differential expression between treatments was highest upon emergence (0 hours) and after 72 hours in females and 120 hours in males (Figure 3.5C).

Functional analysis

The optimal number of k-means clusters was independently determined to be five for both males and females. Clusters had between 65-72% GO term annotation, with the exception of cluster iv, which only had 46.5% annotation, overall giving a comprehensive representation of the biological processes involved in each cluster (Tables 3.2 & 3.3). In male cluster i, there is minimal change in expression through time for beetles that overwintered in the Snowy plot, but beetles that overwintered in the Dry plot emerged with much lower expression levels, which converged with beetles from the Snowy plot after 120 hours. The GO terms most enriched in cluster i are associated with nutrient acquisition, including “digestion”, “carbohydrate metabolic processes”, “cellulose catabolic processes”, and “cellulase” (Table 3.2). Cluster ii was enriched for GO terms involved in nucleic acid biosynthesis including “'de novo' IMP biosynthetic process" and “purine nucleotide biosynthetic process” (Table 3.2). Cluster iii was only enriched for a single GO term in each of biological processes and molecular functions; “regulation of development, heterochronic” and “DNA binding” respectively, and changed little through time or between plots (Table 3.2). Cluster iv was enriched for GO terms associated with ciliated movement including “cilium assembly” and “microtubule-based movement” (Table 3.2). There was little change through time in cluster iv for beetles that overwintered in the Dry plot, and beetles that overwintered in the Snowy plot started with similar expression as beetles from the Dry plot, but rapidly increased through time. Finally, cluster v was enriched for GO terms associated with metabolism including “fatty acid beta-oxidation”, “ATP synthesis coupled electron transport”, and “reactive oxygen species metabolic process” (Table 3.2). Cluster v decreased through time in both Snowy and Dry beetles, with beetles that overwintered in the Snowy plot starting lower and decreasing more dramatically(Figure 3.6).

In females, clusters 1, 4 and 5 all show overwinter plot-specific changes through time, while cluster 2 increased in both plots, but was overall greater in beetles that overwintered in the Snowy plot, and cluster 3 decreased through time with overall lower expression in beetles that overwintered in the Snowy plot (Figure 3.7). Cluster 1 showed a similar pattern to cluster i in males, with little change through time in beetles from the Snowy plot, while beetles from the Dry plot have very low expression upon emergence, which converges with beetles from the Snowy plot after 120 hours (Figure 3.7). Interestingly, functional annotation of Cluster 1 revealed very similar results as cluster i, being enriched for GO terms related to nutrient acquisition, including digestion, carbohydrate metabolism, and lipid metabolism (Table 3.3). Cluster 2 was enriched for the GO terms: negative regulation of epidermal growth factor receptor signaling pathway and nutrient reservoir activity (Table 3.3). Cluster 3 was enriched for GO terms associated with protein modification including co-translational protein modification, macromolecule modification, and protein deglutathionylation (Table 3.3). Cluster 4 was only enriched for two GO terms that were associated with saccharopine dehydrogenase (Table 3.3). Cluster 5 was enriched for GO terms that were associated with biosynthesis, including valine, leucine, histidine, pyridoxine, methionine, and inosine monophosphate biosynthetic processes (Table 3.3). Both clusters 4 and 5 show similar patterns of increase in expression among all beetles, with beetles from the Snowy plot having a

more rapid increase in expression through time, a pattern that is more exaggerated in cluster 5 (Figure 3.7).

Metabolic impacts

To look at metabolic regulation upon emergence from dormancy, we examined expression changes in genes involved in glycolysis, β-oxidation, and TCA cycle. In males, transcripts of genes involved in glycolysis do not have a coordinated pattern within beetles that overwintered in a given plot, but there are significant changes through time in hexokinase (HK), phosphoglucose isomerase (PGI), glyceraldehyde 3-phosphate dehydrogenase (GADPH), phosphoglycerate mutase (PGM), and lactate dehydrogenase (LDH) (Figure 3.8A). Both HK and PGI show interesting counter patterns among two separate transcripts that map to each gene, whereby one transcript is up-regulated through time, while the alternate transcript is down-regulated (Figure 3.8B). In PGI, the pattern of regulation of alternate transcripts is flipped between microclimates; whereby the PGI transcript that is up-regulated in beetles that overwintered in the Snowy plot is down-regulated in beetles that overwintered in the Dry plot, and vice versa. Counter expression patterns of transcripts that map to HK are only observed in Snowy beetles, and both show matching down-regulation in beetles that overwintered in the Dry plot. Change in expression of PGM is only observed in beetles that overwintered in the Dry plot, where it is up-regulated. LDH is up-regulated through time in both Snowy and Dry plots. Expression of GAPDH is up-regulated through time in beetles that overwintered in the Snowy plot; and down-regulated in beetles that overwintered in the Dry plot. There were no changes in any transcripts of glycolytic genes in females.

Overall, transcripts of TCA genes in males slightly increased through time in a highly coordinated manner in beetles that overwintered in the Dry plot, while the majority of transcripts were down-regulated in beetles that overwintered in the Snowy plot, with the exception of α-KDH (Figure 3.8D). There is an increase in transcripts coding for both the A and B subunits of α-KDH in all males, with a particularly sharp increase 72 hours after emergence in beetles that overwintered in the Snowy plot. There is less change in TCA encoding transcripts in females, with only IDH and MDH having significant changes in expression through time, both of which match expression patterns in males.

Transcripts for genes involved in β-oxidation closely match TCA transcripts, slightly increasing from emergence to 120 hours in beetles that overwintered in the Dry plot, and decreasing 48 hours after emergence in beetles that overwintered in the Snowy plot. Acyl-CoA oxidase (ACOA1/2) is an exception in both conditions; it has a much greater increase in expression than other transcripts in the pathway through time in beetles that overwintered in the Dry plot, and is the only transcript in the pathway that increases in beetles that overwintered in the Snowy plot. In females, expression of transcripts in β-oxidation show a much larger and more significant change through time than glycolysis or TCA. Most β-oxidation transcripts change through time in females, and closely match the expression profiles of males.

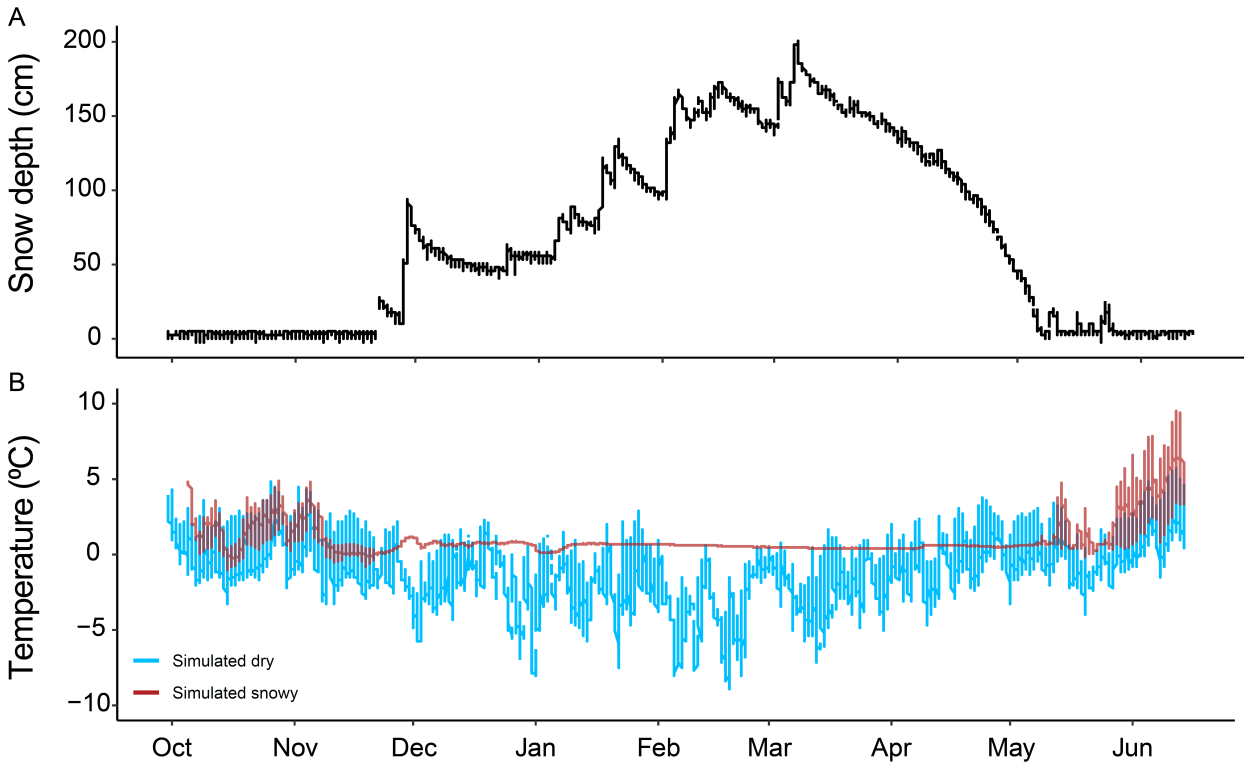

Figure 3.3) Microclimate conditions in experimental overwintering plots. (A) Snow depth throughout experiment. Data are from the Rock Creek Lakes weather station (CA Dept of Water Resources), 1.7km from where experiment took place. B) Simulated microclimate temperatures in Snowy plot (red) and Dry plot (blue) for the duration of the overwintering period (see methods for details of temperature simulation).

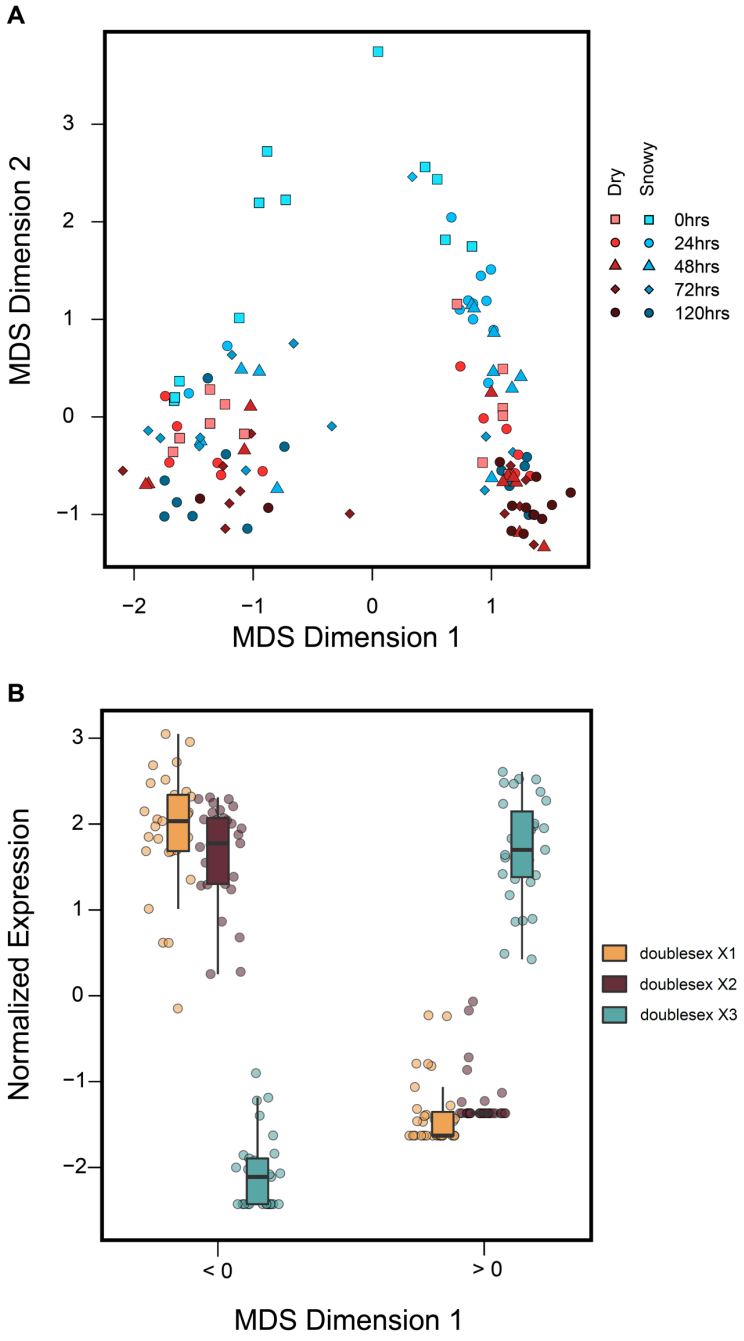

Figure 3.4) Global gene expression patterns of *C. aeneicollis* during emergence from winter dormancy. (A) Multidimensional scaling plot (MDS) of global gene expression of beetles overwintered in Snowy (red) or Dry (blue) conditions, and sampled at five timepoints after emergence from overwintering (square = 0 h; light circle = 24 h, triangle = 48 h, diamond = 72hrs, and dark circles = 120 h). (B) Expression of three isoforms of the sex determining gene *doublesex*, separated by MDS dimension 1.

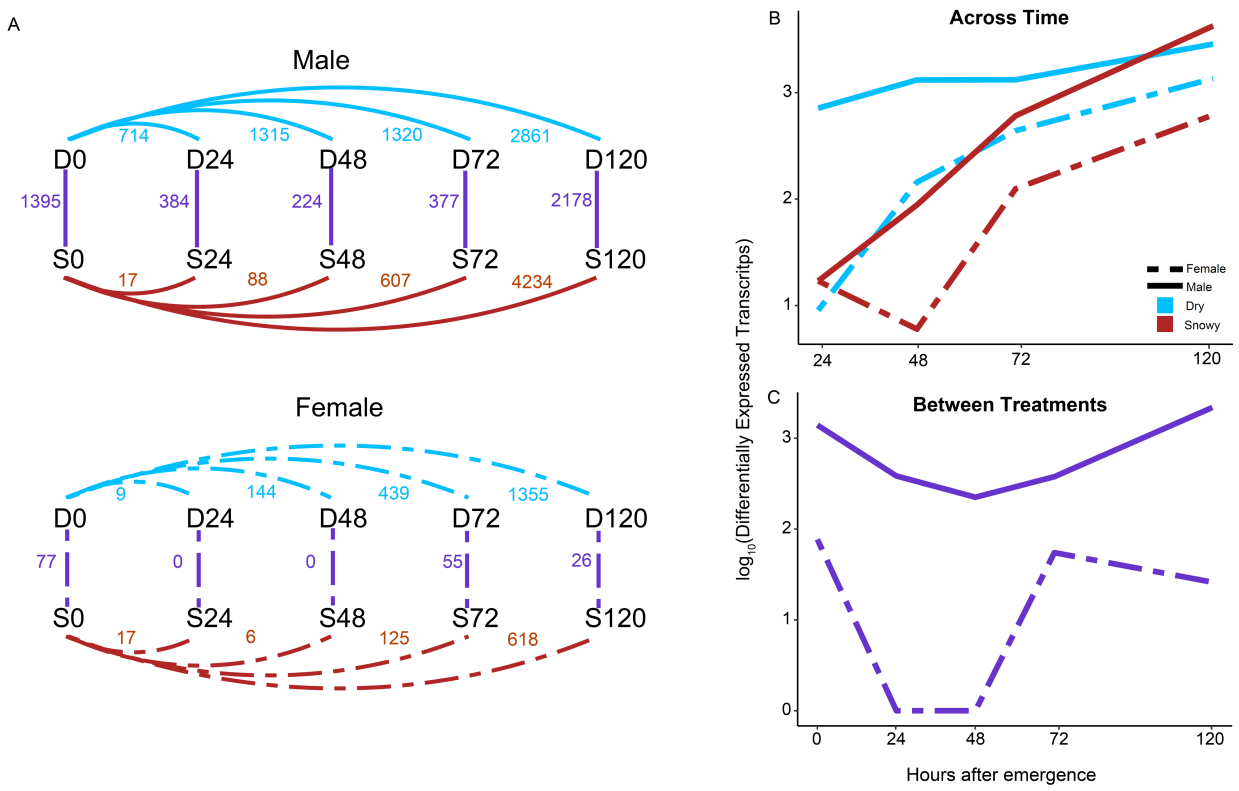

Figure 3.5) Magnitude of differential gene expression of *C. aeneicollis* during emergence from dormancy. (A) Pairwise comparisons for likelihood ratio tests (fold change of > 2 and FDR < 0.01), with number of identified differentially expressed genes for both males and females. Color indicates the level of comparison and is conserved across all panels: blue indicates Dry plot through time, red indicates Snowy plot through time, purple indicates comparisons between overwinter microclimate at a given timepoint. Solid lines represent males, and dashed lines, females. (B) Log_{10} transformed number of differentially expressed genes across time (corresponding to blue and red pairwise comparisons from panel A). (C) Log_{10} transformed number of differentially expressed genes between treatments.

Table 3.1) Differentially expressed transcripts between pairwise comparisons. Number of differentially expressed transcripts (DET), transcripts upregulated (showing a 2 fold log2 change in expression), and downregulated (lower than 0.5 fold log2 change in expression) for each pairwise comparison.

Group 1	Group 2	DET	Up (FC >2)	Down (FC<0.5)	Annotated
Male					
0 h Dry	24 h Dry	714	147	341	0.84
0 h Dry	48 h Dry	1315	229	720	0.83
0 h Dry	72 h Dry	1320	222	886	0.84
0 h Dry	120 h Dry	2861	523	1519	0.85
0 h Snowy	24 h Snowy	17	7	13	0.75
0 h Snowy	48 h Snowy	88	19	56	0.88
0 h Snowy	72 h Snowy	607	157	236	0.87
0 h Snowy	120 h Snowy	4234	1301	1224	0.83
0 h Dry	0 h Snowy	1395	173	853	0.83
24 h Dry	24 h Snowy	384	49	253	0.83
48 h Dry	48 h Snowy	224	27	139	0.85
72 h Dry	72 h Snowy	377	120	146	0.85
120 h Dry	120 h Snowy	2178	485	832	0.85
Female					
0 h Dry	24 h Dry	17	2	14	0.85
0 h Dry	48 h Dry	6	0	6	0.83
0 h Dry	72 h Dry	125	17	85	0.82
0 h Dry	120 h Dry	618	331	274	0.81
0 h Snowy	24 h Snowy	9	1	8	0.77
0 h Snowy	48 h Snowy	144	44	83	0.77
0 h Snowy	72 h Snowy	439	125	168	0.83
0 h Snowy	120 h Snowy	1355	353	574	0.82
0 h Dry	0 h Snowy	77	35	33	0.81
24 h Dry	24 h Snowy	0	-	-	-
48 h Dry	48 h Snowy	0	-	-	-
72 h Dry	72 h Snowy	55	40	24	0.62
120 h Dry	120 h Snowy	26	17	8	0.86

Table 3.2) Top enriched GO terms for each cluster in males. Top 5 enriched GO terms for Biological Processes and Molecular Function associated with each k-means cluster in male beetles. The number of differentially expressed transcripts (#DET) and percent of DETs that are annotated in each cluster (%Ann) are also shown.

Cluster	#DET	% Ann	**Biological processes**	p-value	**Molecular Function**	p-value
i	457	68.7	digestion	7.E-47	methyl indole-3-acetate esterase activity	2.E-20
			carbohydrate metabolic process	5.E-39	polygalacturonase activity	5.E-19
			cell wall organization	5.E-19	cellulase activity	9.E-18
			cellulose catabolic process	6.E-17	thioglucosidase activity	3.E-16
			collagen catabolic process	4.E-15	glucosinolate glucohydrolase activity	3.E-16
ii	1821	67.8	'de novo' IMP biosynthetic process	1.E-07	alditol:NADP+ 1-oxidoreductase activity	7.E-09
			cellular response to forskolin	2.E-06	retinal dehydrogenase activity	3.E-08
			purine nucleotide biosynthetic process	2.E-06	allyl-alcohol dehydrogenase activity	1.E-07
			galactose catabolic process via UDP-galactose	7.E-06	alcohol dehydrogenase (NADP+) activity	4.E-06
			galactose metabolic process	7.E-06	heme binding	7.E-06
iii	451	70.5	regulation of development, heterochronic	5.E-07	DNA binding	1.E-08
iv	230	46.5	cilium assembly	2.E-09	O-phospho-L-serine:2-oxoglutarate aminotransferase activity	3.E-10
			L-serine biosynthetic process	2.E-08	pyridoxal phosphate binding	4.E-07
			microtubule-based movement	3.E-06		
			pronephros development	3.E-06		
			microtubule depolymerization	2.E-05		
v	1517	69.7	fatty acid beta-oxidation	2.E-15	dolichyl-diphosphooligosaccharide-protein glycotransferase	5.E-10
			ATP synthesis coupled electron transport	3.E-08	acyl-CoA dehydrogenase activity	8.E-08
			reactive oxygen species metabolic process	1.E-06	pyruvate carboxylase activity	1.E-07
			co-translational protein modification	2.E-06	NADH dehydrogenase (ubiquinone) activity	2.E-06
			mitochondrion morphogenesis	4.E-06	protein disulfide isomerase activity	7.E-06

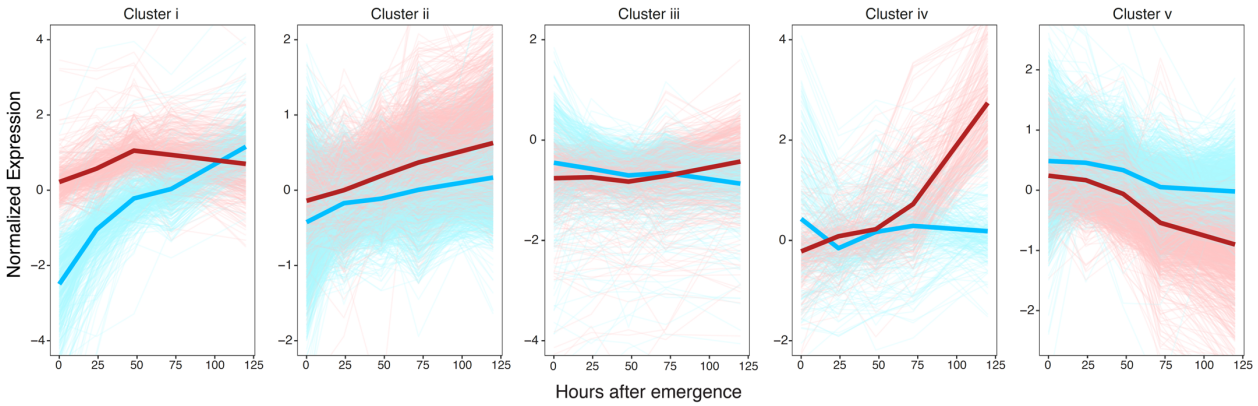

Figure 3.6) Patterns of gene expression during emergence from diapause in male *C. aeneicollis* beetles. Normalized expression of differentially expressed transcripts from five clusters generated via k-means clustering. Dark lines depict the mean expression of all transcripts in cluster for male beetles that overwintered in Snowy (red) and Dry plots (blue), and light lines depict each individual transcript in the cluster. For identities of GO terms enriched in each cluster see Table 2.

Table 3.3) Top enriched GO terms for each cluster in females. Top 5 enriched GO terms for Biological Processes and Molecular Function associated with each k-means cluster in female beetles. The number of differentially expressed transcripts (#DET) and percent of DETs that are annotated in each cluster (%Ann) are also shown.

Cluster	#DET	% Ann	**Biological processes**	p-value	**Molecular Function**	p-value
1	224	68.3	digestion	2.E-34	cellulase activity	8.E-14
			carbohydrate metabolic process	3.E-19	cysteine-type peptidase activity	8.E-11
			cellulose catabolic process	2.E-13	methyl indole-3-acetate esterase activity	5.E-10
			cellular carbohydrate catabolic process	2.E-08	chitin binding	1.E-09
			chitin catabolic process	4.E-08	L-fuconate dehydratase activity	2.E-08
2	109	65.1	negative regulation of epidermal growth factor receptor signaling pathway	5.E-06	nutrient reservoir activity	3.E-07
3	605	71.4	co-translational protein modification	2.E-08	dolichyl-diphosphooligosaccharide-protein glycotransferase activity	5.E-11
			macromolecule modification	6.E-07	protein disulfide isomerase activity	6.E-08
			protein deglutathionylation	6.E-07		
			response to unfolded protein	1.E-06		
			protein N-linked glycosylation	2.E-06		
4	443	65.7			saccharopine dehydrogenase (NADP+, L-lysine-forming) activity	3.E-06
					saccharopine dehydrogenase (NAD+, L-glutamate-forming) activity	3.E-06
5	70	72.9	purine nucleotide biosynthetic process	1.E-11	O-phospho-L-serine:2-oxoglutarate aminotransferase activity	5.E-14
			L-serine biosynthetic process	4.E-11	pyridoxal phosphate binding	2.E-11
			purine nucleobase biosynthetic process	5.E-11	xanthine oxidase activity	1.E-08
			nucleoside metabolic process	5.E-10	methenyltetrahydrofolate cyclohydrolase activity	2.E-08
			tetrahydrofolate interconversion	2.E-09	methylenetetrahydrofolate dehydrogenase (NADP+) activity	2.E-08

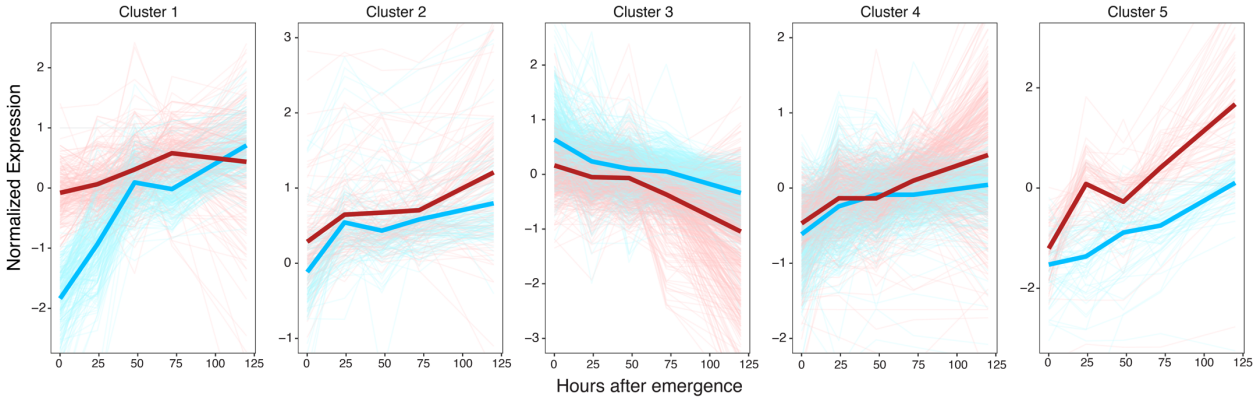

Figure 3.7) Patterns of gene expression during emergence from diapause in female *C. aeneicollis* beetles. Normalized expression of differentially expressed transcripts from five clusters generated via k-means clustering. Dark lines depict the mean expression of all transcripts in cluster for female beetles that overwintered in Snowy plot (red) and female beetles that overwintered in the Dry plot (blue), and light lines depict each individual transcript in the cluster. For identities of GO terms enriched in each cluster see Table 3.

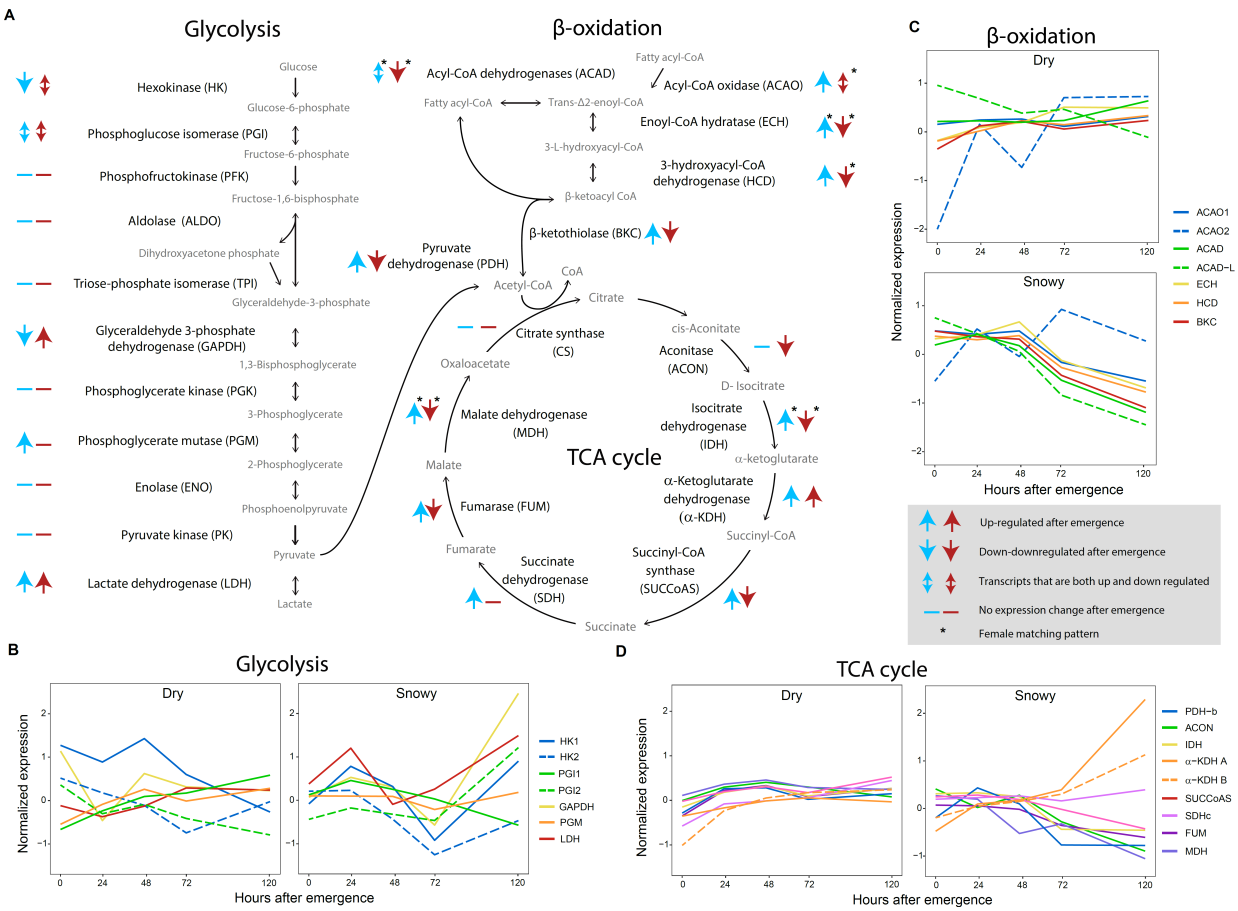

Figure 3.8) Transcript expression patterns of Glycolysis, β-oxidation, and TCA cycle pathways upon emergence for beetles that overwintered with or without snow cover. (A) Change in expression patterns of transcripts annotated as each enzyme in each pathway from emergence (0 hours) to 120 hours after emergence in male beetles. Up arrows represent a general increase in expression through time, down arrows indicated a general decrease through time, and double-sided arrows indicate a mixture of increasing and decreasing expression levels through time among multiple transcripts annotated as that enzyme. Colors identify overwinter conditions, with beetles overwintering in Dry plot as blue, and Snowy plot in red. Asterisk denotes when a pattern is matched in females. (B) Mean normalized expression patterns of transcripts annotated as enzymes in glycolysis, (C) β-oxidation, or (D) TCA cycle

3.4 Discussion

The transition from dormancy to active season requires extensive physiological modification and reprioritization of energy allocation, but is an understudied phase of insect lifecycles. This is the first study to profile the impact of winter microclimate on gene expression in an insect during the period between emerging from dormancy and resuming activity. As such, this work provides a window into the timing and prioritization of cellular processes during the transition out of dormancy, highlighting the magnitude of sex-specific gene expression and the impact of variation in snow cover on gene regulation.

Digestion, assimilation, and metabolism

Both males and females up-regulated transcripts related to digestion and macronutrient catabolism immediately upon emergence from dormancy, suggesting that nutrient assimilation and processing is a key priority. Indeed, the most dramatic changes through time for both males and females occur in clusters that are enriched for transcripts associated with digestion, cellulose, and carbohydrate metabolism (cluster i in males and 1 in females; Tables 3.1 & 3.2). Diapausing insects do not feed and thus reduce their gut volume and activity (Wilsterman et al., 2021), and therefore upon emergence, *C. aeneicollis* must rebuild the digestive tract and remodel metabolic pathways in order to begin feeding. Beetle host plants, willow in the genus *Salix*, are primarily made up of cellulose and carbohydrates (McCabe and Barry, 1988), explaining the up-regulation of cellulose and carbohydrate metabolic processes. In the present experiment beetles did not feed during the five-day sampling period, although food was available to them. Thus the changes to digestion and nutrient assimilation were preparatory, analogous to the large-scale preparatory digestive remodeling in birds preparing for migration (McCabe and Guglielmo, 2019). Up-regulation of transcripts of several digestion related genes have been found to be up-regulated post-diapause in ladybird beetles (Qi et al., 2015), but the time course of up-regulation was previously unknown. Here, it appears that beetles in the snowy plot emerged with digestive systems largely activated, while beetles from the dry plot rapidly up-regulated gene expression to converge by 5 days after emergence. Rapid up-regulation of genes associated with digestion immediately upon emergence likely allows beetles quickly compensate for the energetic drain occurring from winter.

In addition to up-regulation of digestion and nutrient assimilation, we demonstrated extensive remodeling of the TCA cycle and β -oxidation during the transition from dormancy to active season. Altered metabolic regulation is the main hallmark of diapause, but previous work has focused on the transition into or metabolism during diapause (Hahn and Denlinger, 2011; Sinclair, 2015). β-oxidation stood out as being particularly strongly impacted, with significant expression changes in every gene in the pathway in both males and females, and a pronounced impact of snow cover. Gene expression in β-oxidation and TCA cycle decreased strongly over the five days post-emergence in beetles that overwintered in the Snowy plot, consistent with a shift towards catabolizing carbohydrates and cellulose and away from stored lipid reserves. Copepods also down-regulate β-oxidation during diapause termination as they shift from metabolizing primarily lipid to protein (Skottene et al., 2019), suggesting this could be a general pattern associated with transition out of dormancy. In copepods, expression of TCA genes increases several hours after diapause termination, but are then down-regulated 24 h after diapause termination (Roncalli et al., 2021). In the current experiment there was no observed early increase in TCA expression in the snowy plot, there is down-regulation that matches patterns in copepods, but in our case the down-regulation is several days after emergence. The down-regulation of TCA

genes may be a result of an early increase in expression of TCA genes to meet increased energetic demands, and then a downregulation once enough enzyme is produced after the first 24 h, but we would need further data to test this. Beetles from the Dry plot showed contrasting patterns of metabolic regulation beetles from the Snowy plot, with gene expression in β-oxidation and TCA cycle maintained or even slightly increasing over the five-days post-emergence. With their delayed digestion and nutrient assimilation capacities, it appears beetles from the dry plot maintained their capacity to catabolize overwinter lipid stores while digestion capacity ramped up. These coordinated shifts in β-oxidation and TCA highlight the early reliance on lipid metabolism to fuel the early energetic costs of emergence including reestablishment of digestion, and illustrate that microclimate conditions can modulate the timing of these metabolic shifts in substrate use.

Several key central metabolic enzymes are at the heart of metabolic flexibility, and can be involved directly through pathway flux control, or indirectly through moonlighting functions or nutrient signaling (Eanes, 2017; Marden, 2013). Phosphoglucose isomerase (PGI) in *C. aeneicollis* is polymorphic, with allele frequencies fluctuating with climate variation and impacting running speed, thermal tolerance, fecundity, and larval development rate (Dahlhoff and Rank, 2000; McMillan et al., 2005; Neargarder et al., 2003; Rank and Dahlhoff, 2002). We found two isoforms that map to PGI and show opposite patterns of change through time in snowy versus dry conditions, with PGI isoform 1 increasing through time in beetles that overwintered in the Dry plot, while decreasing through time in Snowy beetles, and PGI isoform 2 increasing in the Snowy plot beetles and decreasing in beetles that overwintered in the Dry plot. These isoforms may represent isozymes or alternative splicing, and alternate transcripts may be expressed in specific tissues giving rise to the patterns we see through time, but we are unable to determine the cause from this data alone and need to be cautious in interpretation. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) has the largest change through time in expression of any glycolytic transcript in beetles that overwintered in the Snowy plot, and changes minimally in beetles that overwintered in the Dry plot. The large change in beetles that overwintered in the Snowy plot may be due to GAPDH's many moonlighting functions including regulation of apoptosis, involvement in metabolic/redux sensing, and involvement in nutrient sensing via the mTOR pathway (Marden, 2013). Interestingly, the TOR pathway has been identified as a critical pathway in diapause termination (Ragland et al., 2011), implicating GAPDH as a candidate metabolic gene involved in a broader diapause phenotype. Of all of the TCA cycle transcripts, α-ketoglutarate dehydrogenase (α-KDH) is the most interesting, because it is an enzyme that has potential to control flux through the TCA cycle (Tretter and Adam-Vizi, 2005). Of all TCA transcripts, α-KDH was the only that was up-regulated in beetles that overwintered in the Snowy plot, indicating that its differential regulation may be due to an moonlighting function. Like GAPDH, α-KDH functions as a nutrient and redox sensor (Eanes, 2017; McLain et al., 2011), potentially highlighting the role of redox sensing in regulating oxidative stress associated with the transition from dormancy to active season (Jovanović-Galović et al., 2007; Jovanović-Galović et al., 2004). Future work will interrogate the functional consequences of variation in these key metabolic regulators.

Reproduction

Genes involved in reproduction were up-regulated after emergence in both males and females, but the transcripts involved and the timing were sex-specific. Clusters iv in males and and 5 in females are enriched for GO terms reflecting investment in reproduction, whereby the male cluster (iv) is enriched with cilium assembly and microtubule-based movement associated with sperm production, and the female cluster (5) includes many GO terms for amino acid biosynthesis and cofactors associated with them (tetrahydrofolate interconversion). Up-regulation of the reproduction cluster (5) in females begins before the up-regulation seen in males (cluster iv), potentially reflecting the higher cost of reproduction for females (Hayward and Gillooly, 2011), and the greater time needed to be prepared. Gene expression profiles found that female copepods invest in reproduction immediately upon termination of diapause, matching our observations. In both males and females, reproduction-related transcripts increased much earlier and in the case of males to a much larger degree in beetles from the snowy compared to the dry plot, suggesting that snow accelerated reproductive development.

Impacts of snow

The general pattern that we observed was that the snowy plot accelerated gene expression profiles related to digestion, reproduction, and metabolic shifts away from lipid catabolism and towards carbohydrate and cellulose metabolism. In natural populations, spring phenology is delayed by a few weeks in snowy compared to dry conditions, leading to a shorter growing season with associated requirements for rapid development (Chapter 1). In the year of the experiment, snow melt occurred several days before excavation, meaning that the snowy plot was exposed to solar radiation while the dry plot was shaded by the overhang keeping the snow off, leading to higher temperatures in the snowy plot which may have accelerated development (Figure 3.3B). Warm end of winter temperatures lead to advanced reproductive development in the stink bug (Takeda et al., 2010), and may have played a role in the accelerated development we observed in the beetles from the snowy plot. Alternatively, longer-term conditions including moisture levels or low average, or extreme minimum temperatures may have resulted in developmental delays in beetles from the Dry plot. We haven't replicated across years so can't determine the precise factors driving gene expression differences, but that they are likely multifarious factors that differ between plots that could impact beetle fitness.

Despite considerably colder minimum temperatures in the snowy plot, we did not see any sign of carry-over effects in the gene expression patterns. If damage did occur in winter we would expect to see an overrepresentation of GO terms associated with cellular repair (i.e autophagy and apoptosis) in beetles that overwintered in the Dry plot. Survival was also slightly higher in beetles that overwintered in the Dry plot, indicating that there was no substantial cold-induced mortality. If cold damage did occur, it would have happened in the coldest months (Jan-Mar; Figure 3.3B), followed by several months of warmer conditions. Moderate cold injury can be repaired during periods of warmer temperatures (Turnock and Bodnaryk, 1993), so conditions between March and June may have been favorable enough to repair any accumulated cold damage. Overwinter conditions are associated with stress, but the impacts of these stressors may be cleared upon emergence or drowned out by the extensive physiological shifts associated with emergence.

Impacts of Climate Change

Highly seasonal environments are some of the most rapidly changing environments on the planet, and, in many of these habitats, winters are warming more rapidly than summer (Cohen et al., 2014; MacDonald, 2010). Temperate and polar environments are seeing shifts in both mean temperatures and precipitation patterns (Williams et al., 2015b). Changing precipitation patterns in winter will impact these environments by altering the amount and duration of snow cover, in turn changing the amount of cold and energy stress that organisms overwintering in these environments will experience (Fitzpatrick et al., 2020; Kearney, 2020). In addition to shifts in amount of precipitation, increasing mean temperatures are leading to earlier snow melt dates and shorter winters (Marshall et al., 2020). Given our lack of understanding of winter climate on the transition from dormancy to growing season we may be missing a mechanism in which changing winters can impact summer fitness.

Drought is becoming more frequent in the Sierra Nevada leading to low snow winters to become more commonplace, and winters are projected to become drier (Hayhoe et al., 2004; Margulis et al., 2016; Mote et al., 2018). Our findings suggest that preparation for digestion and nutrient acquisition is delayed after dry winters. Emergence timing in *C. aeneicollis* is closely timed with willow budburst, providing food shortly after or upon emergence (Rank, 1992b; Smiley and Rank, 1991). Delayed digestion preparation after dry winters would lead to the inability to take advantage of food upon emergence. Mismatched timing of herbivorous insect and host plant can have large fitness impacts (van Asch et al., 2007).

Snowmelt occurs earlier in dry winters and beetle emergence date is up to two weeks earlier than after snowy winters (Chapter 1), meaning that there are longer growing seasons after dry winters. Our findings suggest that there is a delay in reproductive timing after a dry winter, leading to an inability to take advantage of increased growing season. Increasingly dry winters may not have negative impacts on reproductive timing in *C. aeneicollis*, but they may not benefit from increased growing seasons that are observed in other insects (Buckley et al., 2015; Macgregor et al., 2019).

Conclusion

The transition from dormancy to active season is a critical time that links winter impacts to summer fitness. We found that digestion and nutrient catabolism are rapidly up-regulated during emergence from dormancy, concordant with a shift away from catabolism of stored energy reserves. Reproduction was up-regulated soon after emergence in a sex-specific manner. The timing and prioritization of these processes was strongly impacted by snow, with beetles emerging from a snowy plot able to more rapidly transition to active season physiology compared to those from a dry plot. The delayed timing observed in beetles after a dry winter may negate any positive impacts of longer growing seasons that come with earlier snow melt in dry winters. Together, this work suggests that increasingly frequent dry winters in the Sierra Nevada will have physiological impacts that extend beyond winter and carry over into the growing season.

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Appendix 1: Supplementary information for Chapter 1

Supporting Results

Energy use model

The metabolic rate-temperature curve was a simple exponential function of temperature (Table S1.2):

(Eq. 1)

$$\dot{V}O2 = 0.16e^{0.167t}$$

Where $\dot{V}O2$ is the rate of oxygen consumption (µl/hr) and t is temperature (°C). Mass was excluded from the model because it was not significantly correlated with oxygen production ($R2 = 0.005$, $F_{1,45} = 0.21$, $p = 0.65$).

Supporting Discussion

Assumptions of models

Our models have a number of assumptions which impact the conclusions to some degree. First, we assume that beetle body temperatures over winter are approximated by soil logger temperatures. We believe this is a reasonable assumption for a small-bodied soil-dwelling organism such as a beetle, where thermal environment is relatively simple and conductive heat transfer dominates, thus body temperatures closely approximate substrate temperatures. We made this assumption because the prevalence of conductive heat exchange in soil makes it a less heterogenous environment than surface temperatures (Beltrami, 1996). In situations where convective or radiative heat gain are important fluxes, biophysical models to estimate body temperatures would be preferred (Fitzpatrick et al., 2019; Kingsolver and Buckley, 2015). Our soil loggers were at fixed depth (3-5 cm), and thus estimates of cold and energy use do not account for variation in beetle overwintering depth, which could have far-reaching impacts on winter energy use (Huey et al., 2021). As a general rule, it's likely that organisms that overwinter deeper in the soil would experience less fluctuations in soil temperature compared to those near the soil surface, and would thus be less impacted by spatial and temporal fluctuations in snow cover (Huey et al., 2021). Secondly, we assume that phenology is fixed at a given elevation band on a given year, according to the average snow duration for that year (which leads to it being classified as a snowy, mid, or dry year). Although we based our estimates on empirical data on first observation of beetles along these regularly sampled transects, there is considerable variation in phenology from site to site due to local topological effects.

Supporting Methods

Beetle husbandry

Beetles housed in Sanyo MIR-154-PA incubators (Sanyo Scientific, Bensenville, IL) where they were kept at constant temperature and light cycles (20ºC : 4ºC day : night; 12.5L :

11.5D photoperiod) and were fed *ad libitum* with a bunch of three *Salix lasiolepis* leaves, which were replaced every three days. Beetles were considered dormant once they ceased feeding (which happened between September 21st to October 11th, even in the presence of food), at which point they were moved into an incubator held at a constant 1ºC with 24 h darkness.

Respirometry

Beetles were placed in a 10 mL syringe then flushed with CO_2 and H_2O free air (generated by scrubbing using Drierite-Ascarite-Drierite column) for 20 seconds at a flow rate of 100 mL min^{-1}, then sealed at 5 mL volume. Samples were then placed in dark incubators at -1°C, 1°C, 4°C, for 48 hours or 9°C for 24 hours. These extended times were necessary to generate sufficient oxygen depletion to give an accurate measurement. All beetles were measured at each temperature with at least 72 hours to recover between each treatment, and the order of treatments were assigned randomly. Of the 15 beetles used for respiration measurements, three died between measurements. Beetles that died were removed from the final data set. After incubation, rate of O_2 consumption ($\dot{V}O_2$) and CO_2 production rate ($\dot{V}CO_2$) produced were quantified as a proxy for metabolic rate, by injecting 3 mL of the 5 mL sample into a FoxBox respirometer (Sable Systems, Las Vegas, USA) with a flow rate of 96-98 mL min^{-1}. $\dot{V}O_2$ consumption and $\dot{V}CO_2$ production were calculated using conversions from Lighton 2018 (Lighton, 2018).

Field snow manipulation experiment

Chrysomela aeneicollis adults and late instar larvae were collected in September 2015 from South Fork Bishop Creek (37°08'58.0"N 118°33'01.1"W). Beetles were reared following protocols described above. In November 2015, after the first snow fall, five beetles were placed into one of eight 9mm bamboo tubes filled with shredded coconut husk and the tops were sealed with fiberglass screening. The tubes were then clustered into one of four 10x10cm PVC flexible coupling (n=20beetles/treatment). Two iButton temperature loggers (DS1922L, Maxim Integrated Products, Sunnyvale, CA) were placed in each rubber container. The containers were then buried at a mid-elevation site in the Rock Creek Drainage, CA (37°28'09.1"N 118°43'22.5"W, 2826m elevation), beneath 5cm of soil, in either a Snowy (in the open, exposed to ambient snow fall), or Dry plot (beneath an overhang, snow excluded). In May 2016, beetles were collected and survival was assessed by observing if beetles could stand and walk during a three-minute observation, which was repeated three times in the first 24 hours of emergence. If beetles were unable to stand after removed, they were considered dead. All beetles were then flash frozen for biochemical analysis.

Biochemical assays

Triacylglycerides were quantified using TLC-FID as a measure of energy stores remaining at the end of winter and compared to pre-winter beetle energy stores to calculate energy used through winter. Total beetle lipids were extracted using a Folch extraction (Folch et al., 1957). Lipid quantification was done using thin layer chromatography with an flame ionization detector (TLC-FID) on an Iatroscan MK-6s TLC-FID Analyzer (Shell-USA), using a diacylglycerol internal standard, following methods from Williams et al. 2011 (Williams et al., 2011).

Glycerol was quantified by enzymatic quantification using a Free Glycerol Reagent (Sigma Aldrich, USA) using a glycerol standard (Sigma Aldrich, USA). Sorbitol was quantified using a D-Sorbitol Colormetric Assay Kit (Sigma Aldrich, USA) using a D-sorbitol standard S(igma Aldrich, USA). Glucose was quantified using Glucose Assay Reagent (Sigma Aldrich, USA) using a glucose standard (Sigma Aldrich, USA). Total Protein was quantified using a bicinchoninic acid assay Reagent (Sigma Aldrich, USA) using a bovine serum albumin standard (Sigma Aldrich, USA). Absorbance for all assays was measured using a Synergy H1 plate reader (BioTek, Winooski, Vermont, USA).

Microclimate data processing

Data loggers recorded every 30 minutes from June 2010 – June 2019, and we averaged half-hourly measurements to give into hourly soil temperature records. Temperature data from each site were manually scanned for gaps in temperature data, and years that were missing more than a week of data were removed. We also scanned for and removed any year with recorded light exposure, which indicated that the loggers were exposed to surface temperatures and would not be representative of soil microclimate. All data used are summarized in Table S1.

Duration of snow cover was determined by summing total days in winter where temperature data were stable (fluctuating less than a degree over two hours) and in the range of 1 to -2°C, which is indicative of snow buffering (Schmid et al., 2012). Days were only counted if snow buffering was observed for the entire day.

To compare soil temperature to air temperatures (Fig. S3), soil temperatures were used from our South Lake site (Table S1), and compared to the nearby CA Dept of Water Resources South Lake weather station (37.175903°, -118.562660°; data available at cdec.water.ca.gov). Temperature date were matched up by time and air temperature was subtracted from soil temperature.

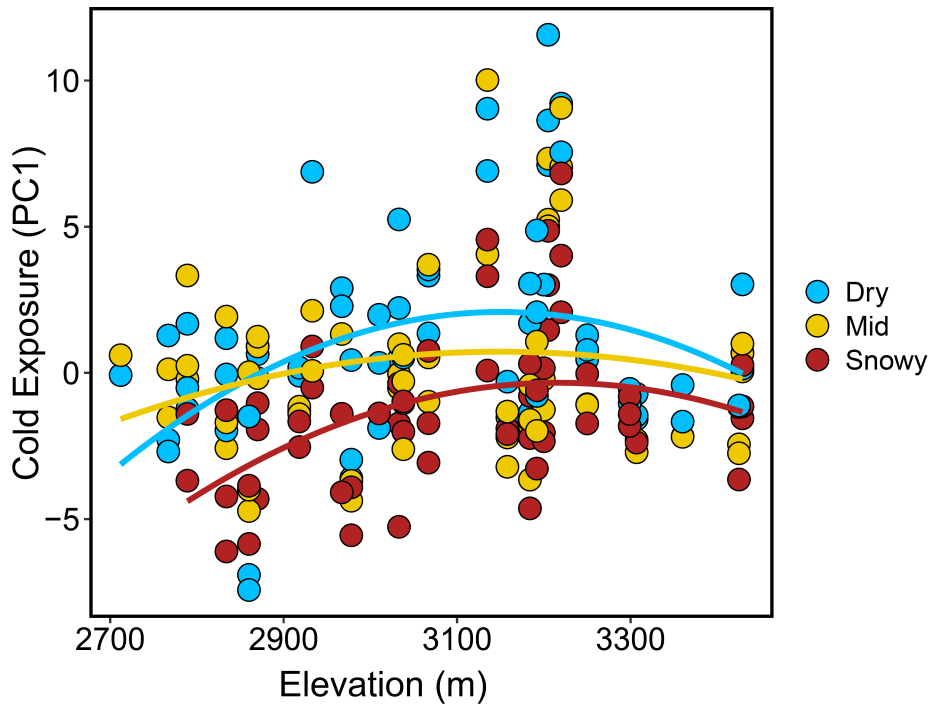

Figure S1.1) Predicted cold exposure of overwintering *C. aenecollis* across elevation in snowy, moderate, and dry winters. Value from principal component analysis encompassing winter temperature in winter across elevation for dry (snowy), moderate (yellow), and dry (blue) winters. Each point represents one site in one of the three most snowy three driest or three intermediate winters from 2009-2019.

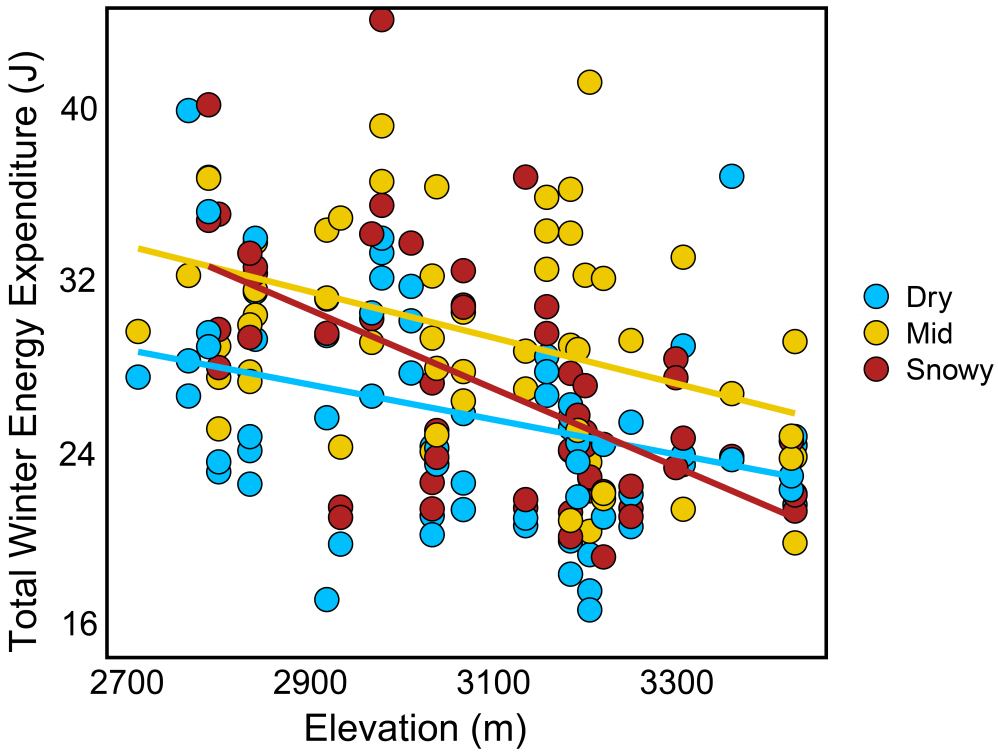

Figure S1.2) Predicted energy used of overwintering *C. aeneicollis* across elevation in snowy, moderate, and dry winters. Total winter energy expenditure from energy use model across elevation for dry (snowy), moderate (yellow), and dry (blue) winters. Each point represents one site in one of the three most snowy three driest or three intermediate winters from 2009-2019.

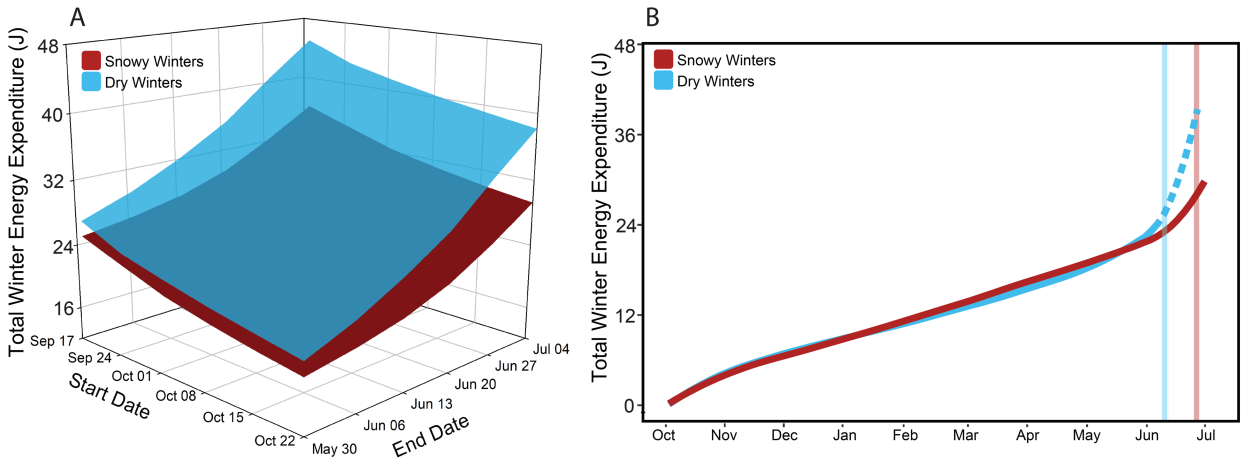

Figure S1.3) Predicted winter energy expenditure of populations of *C. aeneicollis* in snowy vs dry winters. (A) Total winter energy expenditure in snowy and dry years across the range of ecologically relevent start and end dates. Planes represent the average value of all 28 sites in each winter condition. (B) Cumulative winter energy expenditure averaged across all sites in snowy (red) and dry (blue) winters. Light vertical bars represent average day of first obeservation of beetles in respective years (June 6 in Dry, June 22 in Snowy). Dashed lines indicate energy drain occuring after average emergence date.

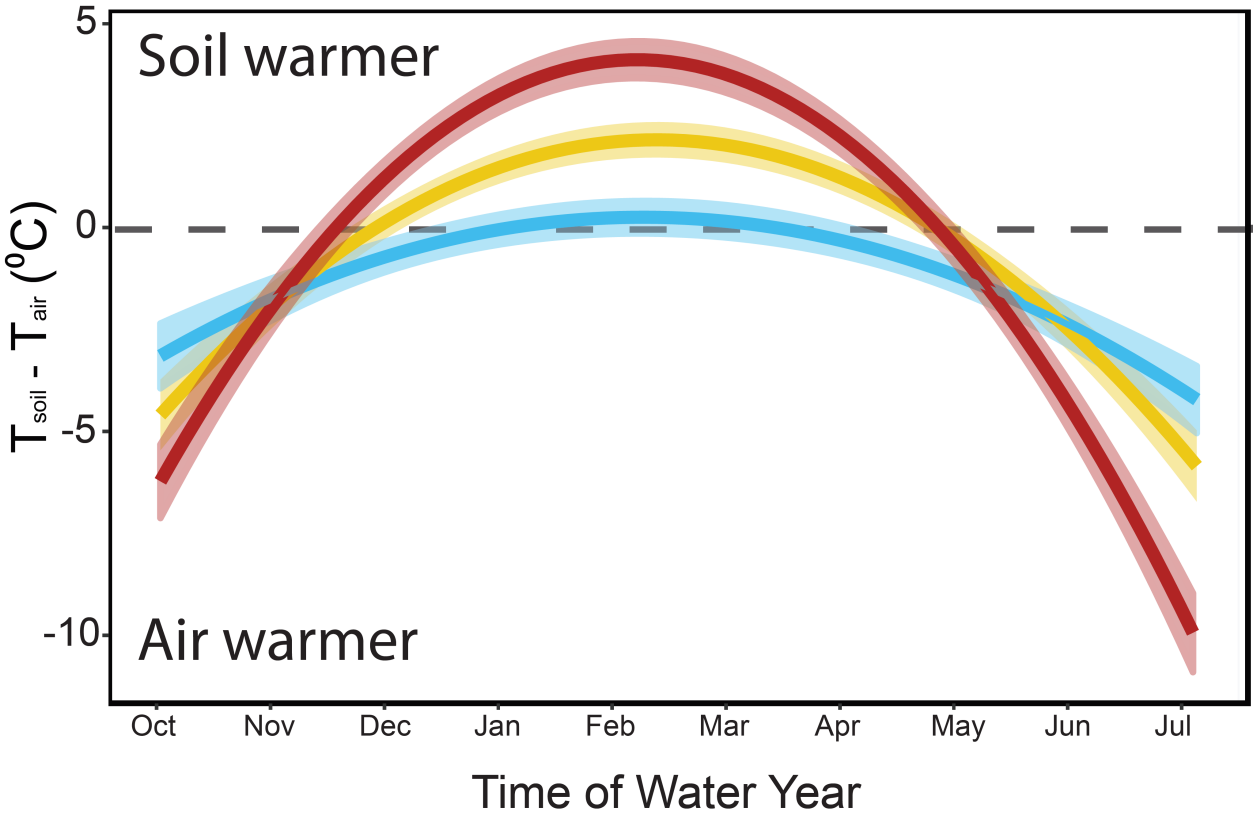

Figure S1.4) Intensity of snow buffering from air temperatures in Eastern Sierra Nevada Mountains. The difference between soil temperature and air temperature at (3001m) between snowy, intermediate, and dry winters. Lines are best fit second order polynomial model to data with 95% confidence interval.

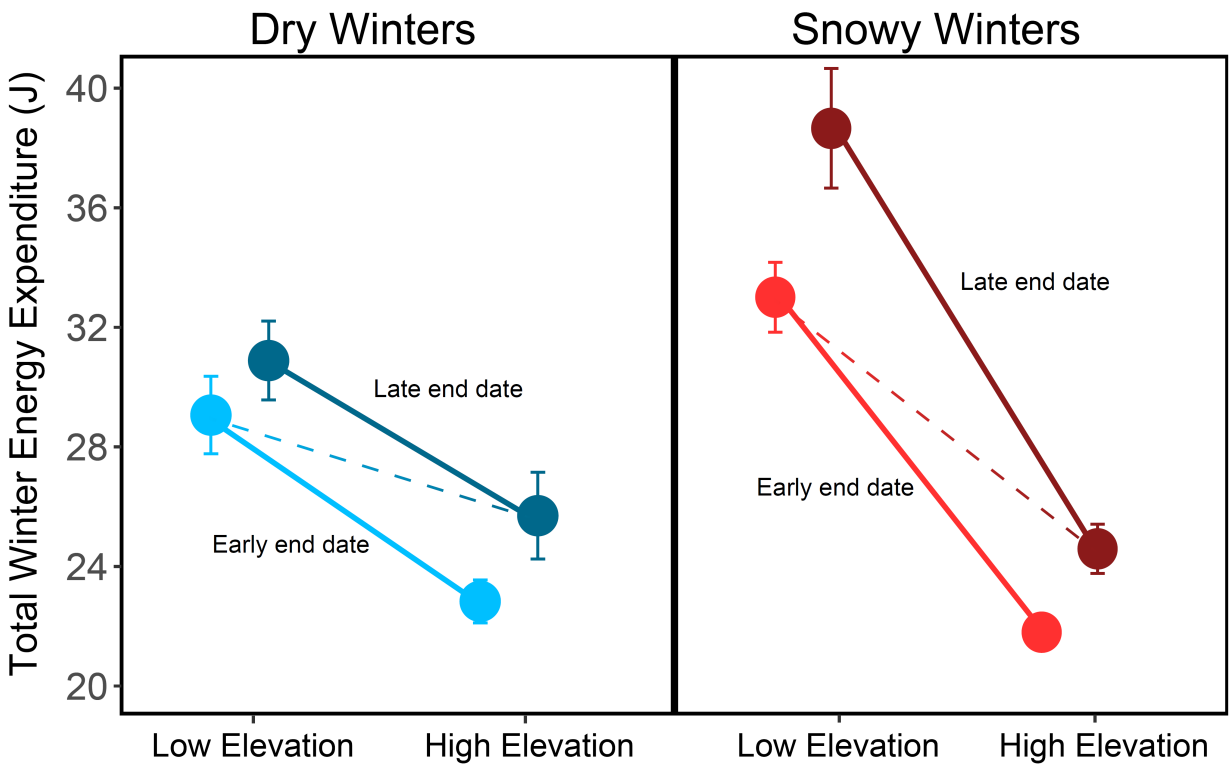

Figure S1.5) Energy use of *Chrysomela aeneicollis* at low (<2900m) and high elevation (<3250m) sites using phenological end dates observed at high and low elevation sites. Energy use output from model estimates based of temperature data starting on October 1st and ending at the date of first observation of beetles at low elevation sites (Early end date; June 3rd in dry winters and June 18 in snowy winters) and high elevation sites (Late end date, June 12th in dry winters and June 26 in snowy winters).

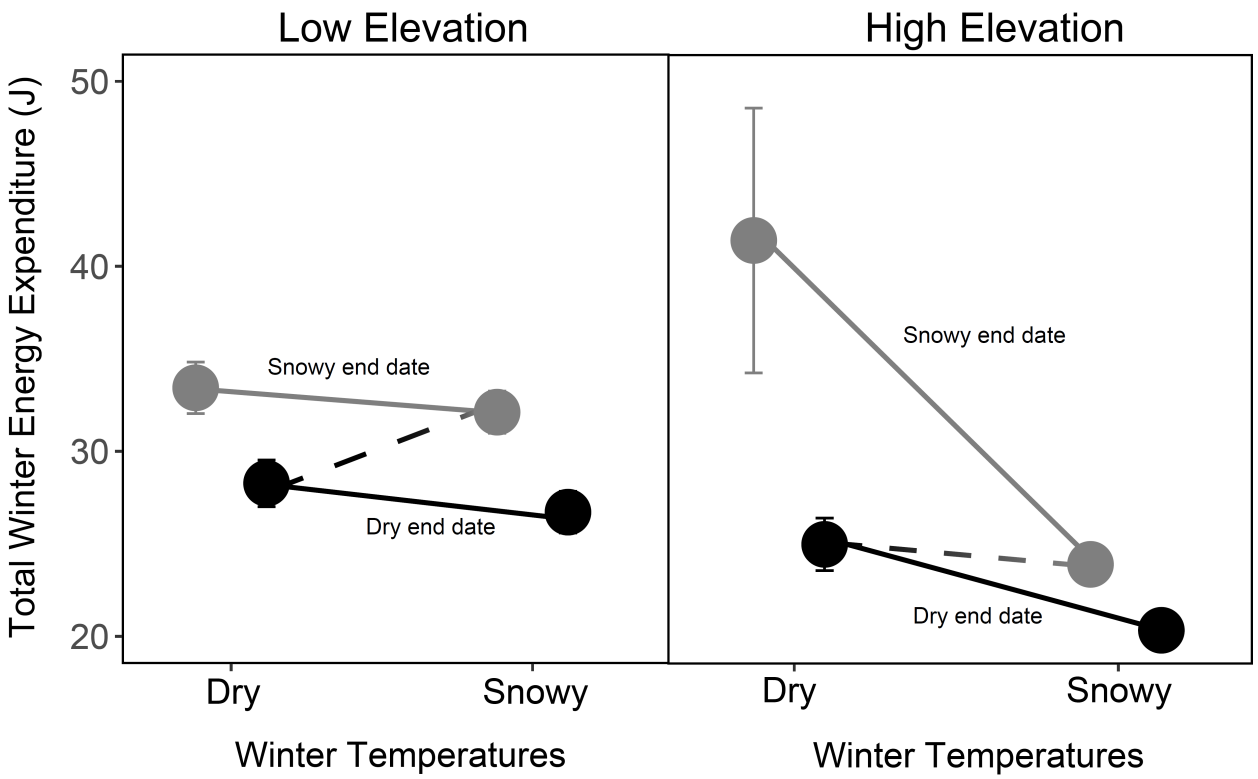

Figure S1.6) Energy use of *Chrysomela aeneicollis* in snowy and dry winters at low (<2900m) and high elevation (<3250m) sites using phenological end dates observed in snowy and dry winters. Energy use output from model estimates based of temperature data starting on October 1st and ending at the date of first observation of beetles at low elevation sites (end dates: June 3rd in dry winters and June 18 in snowy winters) and high elevation sites (end dates: June 9th in dry winters and June 26 in snowy winters).

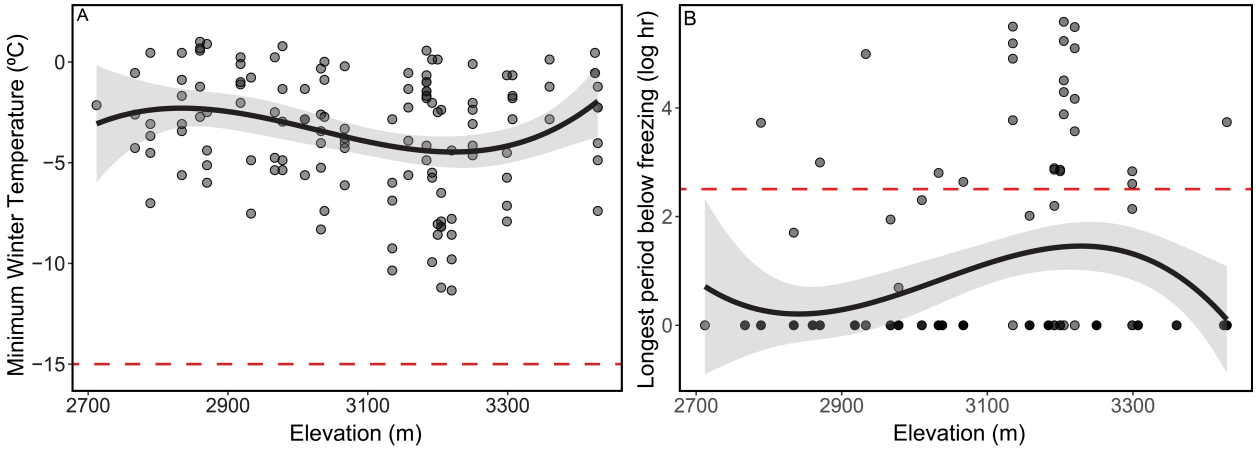

Figure S1.7) Prevalence of lethal cold threshold crossing events for *C. aeneicollis* across elevation between 2009-2019. A) Minimum winter temperature that each site experienced each winter. Red dashed line indicated the temperature at which 50% of beetles die upon 1 hour of exposure (-15°C). B) Longest duration below beetle freezing temperature (-5.3°C) experienced at each site in each winter. Red dashed line indicates the duration that 100% of beetles die after (12hrs) (Boychuk et al., 2015).

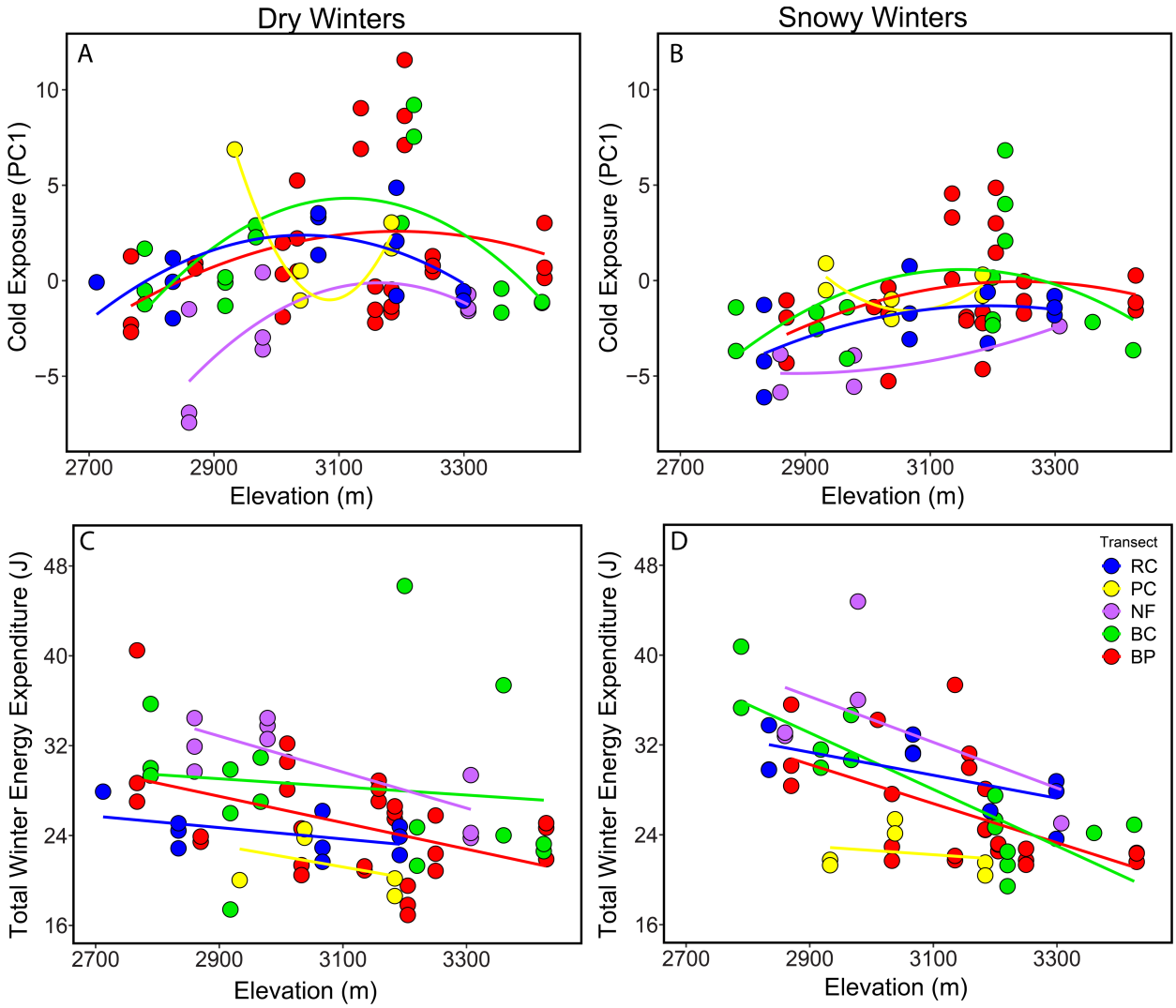

Figure S1.8) Predicted energy used and cold exposure of overwintering *C. aenecollis* across five replicate elevation gradients in snowy and dry winters. Total winter energy expenditure from our energy use model across elevation for dry (A) and dry (B) winters. Each point represents one site in one of the three most snowy or three driest winters from 2009-2019. Value from principal component analysis encompassing winter temperature in winter for dry (C) and snowy (D) winters across elevation. Plots are seperatred by elevation replicate starting from northernmost to southernmost. For list of sites see Table S1

Table S1.1) Sites in the Easter Sierra Nevada that included in study by replicate elevation gradient and the winters that were included in analyses. Site location laid out in Dahlhoff et al 2019. Each x indicates a water year that had temperature data for the entire winter. Colors of the columns indicate whether it was a snowy (red), moderate (black) or dry (blue) winter.

	Elevation (m)	*2011*	*2012*	*2013*	*2014*	*2015*	*2016*	*2017*	*2018*	*2019*
Rock Creek										
1. East Fork Campground	2712		x	x						
2. Pine Grove	2834	x	x	x	x	x	x	x	x	x
3. Mosquito Flat	3067	x	x	x	x	x	x	x	x	x
4. Heart Lake	3192	x	x	x	x	x			x	x
5. Below Ruby Lake	3299	x	x			x		x		x
Pine Creek										
6. Falls Crossing	2933	x	x	x			x	x		
7. Pine Lake	3038	x	x	x	x		x	x	x	
8. Honeymoon Lake	3184	x	x	x	x		x	x	x	
Piute Pass										
9. North Lake Campground	2860		x	x	x	x	x	x	x	x
10. Upper Bog	2978	x	x	x	x	x	x			x
11. Loch Leven	3298		x	x	x	x	x	x		
South Bishop Creek										
12. Willow Campground	2767		x	x	x	x	x			
13. La Hupp	2800	x		x	x	x	x	x	x	x
14. South Lake	3010		x		x	x		x		
15. 1st Stream Crossing	3033	x	x	x	x	x	x	x	x	x
16. Pipeline	3135	x			x	x	x	x	x	x
17. Mary Louise Creek	3158		x	x	x	x	x	x	x	x
18. Lower Bull Creek	3184	x	x	x	x	x	x	x		x
19. High Stream Crossing	3205	x	x	x	x	x	x	x	x	x
20. Below Bull Lake	3250	x	x	x	x	x		x		x
21. Above Green Lake	3429	x	x	x	x	x	x	x	x	x
Big Pine Creek										
22. 26 Bog	2789	x	x	x	x	x	x	x	x	
23. 40 Bog	2918	x	x	x	x	x	x		x	x
24. Falls Site	2967	x	x	x		x		x		
25. Below Black Lake	3200	x	x	x			x	x		x
26. Upper Site	3220	x		x	x	x	x	x	x	x
27. Sam Mack Meadow	3360	x	x	x	x					
28. Above 7th Lake	3425	x			x	x	x		x	

Table S1.2) The top four loadings in a principle component analysis of measures of winter cold across 28 sites in the Sierra Nevada Mountain range between 2010-2019. Loading one was chosen to represent overall winter cold exposure due to its negative correlation with every measure included in the analysis.

	PC1	PC2	PC3	PC4
JanMin	-0.2626	-0.2729	0.2703	0.0415
JanMed	-0.2768	-0.1301	0.1721	0.0018
FebMin	-0.2604	-0.1431	-0.3646	0.1381
FebMed	-0.2803	0.0267	-0.3054	-0.0459
MarMin	-0.2633	-0.0650	-0.3754	0.1306
MarMed	-0.2594	0.2247	-0.1798	-0.3716
AprMin	-0.2154	0.0606	0.0965	0.8014
AprMed	-0.1428	0.6485	0.2003	-0.1078
DecMin	-0.2560	-0.2438	0.3024	-0.0808
DecMed	-0.2819	-0.0708	0.2201	-0.2550
JanMeanMin	-0.2823	-0.1696	0.1852	0.0354
FebMeanMin	-0.2819	-0.0075	-0.3329	-0.0164
MarMeanMin	-0.2834	0.1683	-0.2096	-0.1135
AprMeanMin	-0.2031	0.5241	0.2202	0.2134
DecMeanMin	-0.2808	-0.1108	0.2664	-0.1912

Table S1.3) Proportion of variance explained by the top 10 loadings in a principle component analysis of measures of winter cold across 28 sites in the Sierra Nevada Mountain range between 2010-2019.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10
Standard deviation	3.26	1.27	1.05	0.82	0.55	0.45	0.41	0.27	0.26	0.24
Proportion of Variance	0.71	0.11	0.07	0.05	0.02	0.01	0.01	0.01	0.004	0.004
Cumulative Proportion	0.71	0.82	0.89	0.94	0.96	0.97	0.98	0.99	0.99	1

Table S1.4) Evaluating generalized non-linear model fit of metabolic rate temperature curves of *Chrysomela aeneicollis.* Model 6 was selected due to lowest AIC score and that it can better handle negative temperature data without modification. O_2: O_2 consumption rate in $\mu L \cdot hr^{-1}$; S: overall scaling factor; M: body mass coefficient; t: temperature; T_s: thermal scaling factor; L_{met}: minimum life supporting metabolic rate

Model number	Model	AIC	Log-likelihood	df	p-value
1	$0_2=S*M^{-0.25}*e^{Ts*t}+L_{met}$	-52.165	31.083	5	S:<.0001 T_s:<.0001 L_{met}:0.446
2	$0_2=S*M^{-0.35}*e^{Ts*t}+L_{met}$	-49.892	29.946	5	S:<.0001 T_s :<.0001 L_{met}:0.446
3	$0_2=S*e^{Ts*t}+L_{met}$	-58.644	34.322	5	S:<.0001 T_s:<.0001 L_{met}:0.681
4	$0_2=S*M^{-0.35}*(t+10)^{Ts}+L_{met}$	-55.994	32.997	5	S:<.0001 T_s:<.0001 L_{met}:0.221
5	$0_2=S*(t+10)^{Ts}+L_{met}$	-58.800	34.400	5	S:<.0001 T_s:<.0001 L_{met}:0.446
6	$0_2=S*e^{Ts*t}$	-60.441	34.220	4	S:<.0001 T_s:<.0001
7	$0_2=S*(t+10)^{Ts}$	-60.024	34.012	4	S:<.0001 T_s:<.0001

Table S1.5) Range of temperatures experienced in experimentally manipulated snowy or dry plots at Rock Creek Lodge (37°28'09.1"N 118°43'22.5"W, 2826m elevation) between 7th November 2015 and 15th May 2016. Temperatures (°C) were measured with iButton data loggers (±0.53°C). Values are mean ± SD.

Measure	Snowy Plot	Dry Plot
Mean temperature (± SD)	1.38 ± 1.09	-1.58 ± 1.45
Minimum temperature	0.57	-9.03
Mean monthly minimum temperature	0.81±0.26	-5.21±3.09
Maximum temperature	11.12	9.61
Mean monthly maximum temperature	3.83±5.14	2.21±3.51

Table S1.6) Measures of lipid stores and most abundant cryoprotectants of *Chrysomela aeneicollis* from a field snow-manipulation experiment . Triacylglycerides, Glycerol, Sorbitol, and Glucose measurements from 7th November 2015 (Pre-winter, n=8) and 15th May 2016 (Post-winter, n=20/treatment), from beetles that overwintered in a snowy or dry plot.

	Pre-winter	**Post-winter**	
Measure		**Snowy Plot**	**Dry Plot**
Triacylglycerides (mg)	0.67±0.06	0.08±0.02**	0.27±0.04**
Free glycerol (µg)	149.32±31.35	113.39±21.11	267.50±15.01**
Sorbitol (µg)	16.09±3.31	22.38±2.55	20.95±3.07
Glucose (µg)	14.20±3.25	7.78±0.81**	8.45±0.72**