Cold tolerance of the montane Sierra leaf beetle, *Chrysomela aeneicollis*

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Abstract

Small ectothermic animals living at high altitude in temperate latitudes are vulnerable to lethal cold throughout the year. Here we investigated the cold tolerance of the leaf beetle *Chrysomela aeneicollis* living at high elevation in California's Sierra Nevada mountains. These insects spend over half their life cycle overwintering, and may therefore be vulnerable to winter cold, and prior studies have demonstrated that survival is reduced by exposure to summertime cold. We identify overwintering microhabitat of this insect, describe cold tolerance strategies in all life stages, and use microclimate data to determine the importance of snow cover and microhabitat buffering for overwinter survival. Cold tolerance varies among life history stages and is typically correlated with microhabitat temperature: cold hardiness is lowest in chill-susceptible larvae, and highest in freeze-tolerant adults. Hemolymph osmolality is higher in quiescent (overwintering) than summer adults, primarily, but not exclusively, due to elevated hemolymph glycerol. In nature, adult beetles overwinter primarily in leaf litter and suffer high mortality if early, unseasonable cold prevents them from entering this refuge. These data suggest that cold tolerance is tightly linked to life history stage. Thus, population persistence of montane insects may become problematic as climate becomes more unpredictable and climate change uncouples the phenology of cold tolerance and development from the timing of extreme cold events.

Key Words: Chrysomelidae; cryoprotectant; freeze tolerance; egg; larva; pupa; glycerol; alpine; snow cover

1. Introduction

Montane habitats impose a range of environmental stresses on the organisms that inhabit them, including high ultraviolet radiation and insolation that cause high daytime body temperatures in exposed habitats, and low atmospheric pressure that cause desiccation stress and hypoxia (Sømme, 1989). In these high elevation habitats, low environmental temperatures occur year-round and winter temperatures may be especially cold, even at low latitude (Sømme, 1995). Environmental cold may have significant impacts on small ectotherms such as insects, affecting activity and growth, and causing mortality both through the action of cold on cells and molecules, and because of the likelihood of internal ice formation (Harrison et al., 2012). Prolonged exposure of montane insects to sub-zero temperatures may lead to overwintering mortality and sub-lethal reduction of fitness characters, like growth and reproductive output (Sinclair et al., 2003). Unfortunately, the physiology of cold tolerance in montane insects has been incompletely explored (e.g. Kohshima, 1984; Sinclair and Chown, 2005; Sømme, 1989; Vrba et al., 2012; Wharton, 2011; Zettel and Zettel, 1994), especially in the mountains of the Americas (but see Edwards, 1986; Ring, 1982; Sømme et al., 1996).

Low temperatures experienced by montane insects may be particularly influenced by the duration and depth of snow cover. Paradoxically, extreme low temperatures and freeze-thaw cycles are more common in montane habitats during climatically-warm winters, which often lack significant snowfall relative to cooler, wetter winters, during which accumulated snow cover provides significant insulation to overwintering insects (e.g. Sinclair, 2001b). Interactions between snow cover and extreme low temperature can be especially important in spring and autumn, when early accumulation or late melt of snow cover can extend the period during which

environmental temperatures are buffered. In contrast, a reduced snow pack can expose overwintering organisms to extreme low temperatures in the spring and autumn, at times and/or life stages when the insect's cold tolerance may be inadequate for survival through the episode (Williams et al., 2015). Predicted winter climate change, with modified temperature and precipitation regimes, may therefore have a substantial impact on montane insects by modifying both the occurrence and intensity of cold exposure (Gu et al., 2008; Williams et al., 2015). Thus, understanding mechanisms of cold tolerance, and how it varies through the life history of an animal, is essential for understanding the potential impacts of climate change on organisms in montane environments.

Cold-hardy insects primarily use one of two strategies to survive low temperatures; they either withstand the formation of internal ice (freeze tolerance), or depress their supercooling point (SCP, the temperature at which they freeze) to remain unfrozen at low temperatures (freeze avoidance). Many insects, however, are killed by cold at temperatures where they remain unfrozen, and these are termed chill-susceptible (Lee, 2010). The biochemistry associated with insect cold tolerance is fairly well-described. Briefly, low molecular weight cryoprotectants (for example, polyols such as glycerol or sugars such as trehalose) can protect cells and membranes against the osmotic dehydration associated with freeze tolerance or colligatively depress the SCP in freeze avoidant insects. Improvements in cold tolerance have been associated with even small (10-100 mM) increases in the concentration of cryoprotectants (Lee et al., 1987), although the mechanisms of this protection are less well-understood (see MacMillan et al., 2015, for a possible relationship with water balance in the cold). Thermal hysteresis agents (including antifreeze proteins and glycolipids) inhibit recrystallization in freeze-tolerant species, or retard

nucleation in freeze-avoidant insects (Walters et al., 2011; Zachariassen and Kristiansen, 2000). Finally, control of ice nucleation is a key difference between strategies, with freeze tolerant species generally initiating ice formation at consistently high (above -10 °C) sub-zero temperatures (Sinclair et al., 2009).

Most species of temperate insects have a specific life history stage in which they overwinter. Not surprisingly, insects are usually most cold tolerant during these overwintering stages, developing cold tolerance during the autumn (or during diapause development) and losing it upon resumption of activity in the spring (Lee, 2010). Not all overwintering insects must develop extreme cold tolerance. For example, larvae of acorn weevils have limited cold tolerance, but overwinter successfully in Southern Canada by relying on the buffering effect of being buried in the soil (Udaka and Sinclair, 2014). This is in contrast to species that are exposed to near-ambient temperatures in unprotected, above-snow, wintering microsites, like goldenrod gall flies, which have evolved robust strategies of freeze tolerance (Irwin and Lee, 2003). The high degree of plasticity of cold tolerance among different life history stages and among species suggests energetic and evolutionary trade-offs in cold tolerance strategy and capacity (see also Sinclair, 1999; Sinclair and Chown, 2010; Voituron et al., 2002; Zachariassen, 1985). Thus, understanding how cold tolerance changes among life stages and seasons is critical to understanding the constraints on cold tolerance. Unfortunately, there are surprisingly few studies of cold tolerance in montane insects that investigate cold tolerance of all life history stages, even those that provide year-round comparisons (e.g. Ramløv, 1999; Ramløv et al., 1992; Sinclair, 1997).

In this study, we investigate cold tolerance in the leaf beetle *Chrysomela aeneicollis*, an ideal species in which to investigate cold tolerance of montane species. This beetle lives on willows along streams, lakes, and bogs throughout Western North America (Brown, 1956; Dellicour et al., 2014); at the southern edge of their range in the southern Sierra Nevada Mountains of Eastern California at high elevation (2800-3200 m). Sierra willow beetles overwinter as adults, emerging from winter diapause in mid-May and completing a single cycle of reproduction and larval development during the summer. Adults mate and females lay eggs in June, eggs hatch and larvae mature through three instars in July and August, pupating in August and September. Newly-eclosed adults feed in August and September, before entering a winter quiescence (or possibly diapause) in early October (Smiley and Rank, 1986). Beetles living in high elevation habitats in the Sierra Nevada are challenged by exposure to potentially-lethal cold temperatures throughout the year. We have observed: 1) cold mortality in adults emerging from diapause after a single night time cold exposure to temperatures between -8 and -10 °C in June (Bruce, 2005; Dahlhoff and Rank, 2007); 2) cold-induced mortality in first- and second-instar larvae during exposure to temperatures between -4 and -6 °C in July (McMillan et al., 2005; Smiley and Rank, 1986); and 3) high mortality of pupae due to a single early frost in October (present study). Furthermore, long-term field observations of Sierra willow beetle populations show that abundance declines precipitously after extremely cold winters. For example, there was virtually no snowpack at most mid- and low-elevation sites in 2007, and beetle populations declined by 80 % in the subsequent year, especially at mid- and low elevation localities (Smiley, Dahlhoff and Rank, unpublished observations). Thus, it appears that cold tolerance may be important year-round in *C. aeneicollis.*

2. Material and Methods

2.1 Study animals

We hand-collected *Chrysomela aeneicollis* adults and larvae from willow (primarily the Sierra willow *Salix orestera*) during the summer at sites around 3200 m elevation from three study drainages in the Eastern Sierra Nevada mountains of California, which have been well-described (e.g. Dahlhoff et al., 2008; Dahlhoff and Rank, 2000; Rank, 1992a): Big Pine Creek (BPC), Bishop Creek (BC) and Rock Creek (RC). We did not examine the well-described polymorphism at the glycolytic enzyme phosphoglucose isomerase (PGI) described in earlier studies (Dahlhoff and Rank, 2007; Rank, 1992a; Wheat and Hill, 2014), and preliminary experiments did not indicate significant differences in supercooling points of larvae or adults among drainages, so individuals from different drainages were pooled for all experiments. Eggs and first instar larvae were collected as full clutches by plucking the whole leaf on which the clutch was laid; no more than two individuals per clutch were used in any experiment. All other life stages were collected as individuals from willow leaves and returned in an insulated backpack within 12 h to the Owens Valley Laboratory of University of California's White Mountain Research Center (Bishop, CA), where all experiments were conducted. Beetles were maintained in groups of approximately 100 individuals in 5 litre plastic containers at 20°C, 12h D: 4°C, 12h N (20 min ramp-up and –down time) inside an incubator (Percival, Perry, IA, USA). Beetles were fed on a diet of fresh *Salix orestera* collected from 3000 m in Bishop Creek every 2-3 days. After laboratory manipulation, individuals used for later biochemical analysis were placed in 0.6 mL microcentrifuge tubes and frozen at -80 °C.

Summer adults, eggs and first instar larvae were collected and frozen for future analyses between July and August 2011. Second instar larvae, third instar larvae, pupae and newly-eclosed adults were collected and frozen between July and August 2012. Quiescent beetles were obtained either as field-collected pupae or newly-eclosed adult beetles and shipped to University of Western Ontario. Adult beetles were reared in 40×30×40 cm clear plastic cages with a mesh top for ventilation and were provided with *Salix amygdaloides* for food. *Salix amygdaloides* is similar to *S. orestera* in that it has a high salicylate content that is preferred by *C. aeneicollis* (Rank, 1992b). In all cases, willow was changed 2-3 times per week, misted with water daily, and water provided *ad libitum* via cotton-stoppered vials of water. The plastic cages were kept in a walk-in controlled-climate chamber in the Biotron Institute for Experimental Climate Change Research (University of Western Ontario) at 20°C, 12h D: 4°C, 12h N. Quiescence was induced in newly-eclosed adult beetles brought to the University of Western Ontario by low temperature acclimation (4 °C in complete darkness for at least two weeks) starting in September. Overwintering adult beetles were kept in the dark at 0.5 °C in 30 mL translucent plastic cups (Solo, Lake Forest, IL, USA), with a small piece of moist paper towel. Adults with reduced movement in response to stimulus at room temperature with blunt forceps were classified as 'quiescent'. Willow branches and/or paper towels were changed and moistened weekly.

2.2 Overwintering site and field observations

To determine the overwintering microsite of *C. aeneicollis*, we enclosed an entire 1 m tall *S. orestera* plant, plus several potential overwintering microhabitats (a 30 cm pile of rocks and a 1.5 m long, 10 cm diameter rotting pine (*Pinus murrayana*) branch) in a large, chemically-untreated mosquito net (Mombasa Outback double travel net, REI, Sumner, WA) with the edges

partially buried and held down with stones. This experimental plot was located at 3200 m elevation in the Green Lake sub-drainage of Bishop Creek (37°10′41” N; 118° 33′5” W). In mid-September 2011, we placed ca. 200 freshly collected beetles (a mix of larvae, pupae and newly-emerged adults) inside the net (approximately ten beetles were already on the plant). We collected samples at the beginning of November, when no beetles were visible on the leaves of willow plants. We separately collected the net, the dried leaves still in the willow canopy, the soil beneath the rock pile, the leaf litter above, under (<0.1 m), near (between 0.5 m and 1 m) and away (>1 m) from the base of the plant, and the loose soil under, near and away from the base of the plant, down to 20 cm depth. The log was broken up and searched for beetles on site. The bags were returned to the laboratory at White Mountain Research Station, where they were kept in the warm, lighted lab and hand-searched for live beetles for several days. An unseasonable early snow occurred in October 2011 caused significant mortality of juveniles. Before and after that event, beetles were censused at, and collected from, a number of sites in each drainage area, and mortality rates calculated.

2.3 Cold tolerance

We determined cold tolerance strategy, supercooling point (SCP, the temperature at which ice formation begins), and median and lower lethal temperatures for acute cold exposures (highest temperatures that kill 50 % [LT_{50}] and 100 % [LLT] of individuals, respectively) for eggs, all three larval instars, pupae, and adults. We also measured survival of prolonged cold exposure in quiescent adults. Cold exposures were conducted with individuals placed in 0.6 mL microcentrifuge tubes in contact with a 36 AWG type-T thermocouple interfaced to a computer via a TC-08 thermocouple interface (Pico Technology, Cambridge, UK). First-instar larvae and

194 eggs were affixed to the thermocouple using a thin layer of silicone grease; other stages were
195 held in place in the microcentrifuge tube with a piece of cotton wool. Microcentrifuge tubes were
196 placed in wells drilled in an aluminium block cooled by methanol circulated from a Lauda
197 Proline 855C refrigerated circulator (Lauda, Würzburg, Germany).

198

199 To determine the cold tolerance strategy, we used data from the LT_{50} and SCP experiments to
200 determine whether individual animals froze and whether they survived that internal ice formation
201 (see also Crosthwaite et al., 2011; Sinclair and Chown, 2005). If individuals were killed by cold
202 when freezing did not occur, that stage was considered chill susceptible; if only individuals that
203 froze were killed at a given temperature, we deemed them freeze avoidant; and if individuals that
204 experienced internal ice formation survived, we considered them freeze tolerant.

205

206 To measure supercooling points, individuals were equilibrated at 0 °C for 10 min, then cooled
207 from 0 to -30 °C at 0.5 °C·min^{-1}. The SCP was the lowest temperature recorded before the
208 exotherm associated with internal ice formation (e.g. Figure 1). LLT and LT_{50} were assessed
209 according to the approach of Sinclair & Chown (2005). Briefly, eight beetles at a time were held
210 at 0°C for an equilibration period of 10 min before being cooled at 0.1 °C·min^{-1} to a test
211 temperature, where they were held for 1 h, prior to being rewarmed at 0.5 °C·min^{-1} to 0 °C. Four
212 to six test temperatures were used for each life stage, with 0 °C at the upper temperature end, and
213 temperatures evenly spaced to -40 (eggs), -9, -10, -12 (1st, 2nd and 3rd instar larvae, respectively),
214 -20 (pupae), -15, and -30 °C (summer and quiescent adults, respectively). After rewarming,
215 beetles were placed individually in the wells of a 6-well tissue culture plate at +4 °C with a moist
216 paper towel, and survival assessed. Adults and larvae- coordinated movement after 24 h; eggs-

successful hatching; pupae- response to mechanical stimulus. A handling control was also used, wherein individuals were placed in the wells of 6-well tissue culture plate at +4 °C with a moist paper towel and survival monitored after 24 h.

To determine survival of prolonged periods of internal ice formation by quiescent adults, four quiescent adults were exposed to -8 °C for each of 1, 4, 6, or 12 h. Individuals were cooled at 0.1 °C·min^{-1} (as above), and survival assessed after 24 h at 4 °C. A control group of four individuals were held at -4 °C (a temperature at which they do not freeze) for 12 h, and survival assessed after 24 h.

We compared supercooling points among life stages and seasons using a one-way ANOVA followed by Tukey's *post hoc* tests in R version 2.15.1 (R Core Team, 2012). We fitted generalized linear models to survival data (binomial error, logit link; using R) and used this to calculate the LT_{50} (the temperature at which 50 % of beetles were killed), as well as the LT_5 and LT_{95}. We used a lack of overlap of these intervals to identify differences in LT_{50} among life stages and seasons.

2.4 Cryoprotectants

We collected a representative sample from each life stage in 2011 and 2012 to determine the identity and concentration of cryoprotectants. Gas chromatography (GC) was used to identify low molecular-weight cryoprotectants present in homogenized whole individuals. Because glycerol was the dominant cryoprotectant, its concentration was measured in a higher-throughput

spectrophotometric assay (Crosthwaite et al., 2011). Haemolymph osmolality and thermal hysteresis activity were measured using nanolitre osmometry (cf. Sinclair and Chown, 2002). Individual beetles were killed in liquid nitrogen vapour, weighed (fresh mass), dried (70 °C, 4 d) and reweighed (dry mass), and total water content determined as the difference between fresh and dry mass.

Cryoprotectants were identified by gas chromatography of their alditol acetate derivatives, using methods derived from Blakeney et al. (1983) and described by Crosthwaite et al. (2011). Briefly, individuals were homogenized in distilled, deionized water (100 µL for individuals <20 mg, 200 µL for individuals >20 mg), heated for 20 min at 100 °C to denature proteins and centrifuged at 25000 × *g*. Lipids were removed from the supernatant by removing the aqueous layer after addition of sulphuric acid (0.1 M, 0.7 mL) and then hexane (2 mL). An aliquot of this aqueous layer (0.1 mL) was then neutralized with ammonium hydroxide and an internal standard (50 µL xylitol, 1 mg/mL in 1 M ammonia) added. Monosaccharides were reduced to polyhydric alcohols by incubation at 40 °C (90 min) after the addition of 1 mL 2% w/v sodium borohydride in DMSO. Excess sodium borohydride was decomposed after cooling to room temperature by the addition of 0.1 mL glacial acetic acid. The reduced sugars were then acetylated by addition of 0.2 mL 1-methylimidazole (as a catalyst) followed by acetic anhydride (2 mL) for 10 min at room temperature. After addition of distilled deionised water to decompose excess acetic anhydride, the sample was mixed with dichloromethane (1 mL) and an aliquot of the dichloromethane layer dried under nitrogen and re-suspended in 0.2 mL dichloromethane to concentrate the sample. Samples not analysed immediately were stored at -20 °C.

Derivitised samples were identified by capillary gas chromatography with flame ionization detection (Agilent 7890, Agilent Technologies, Santa Clara, CA, USA) using a CP-Sil 88, WCOT fused silica, 25 m x 0.25 mm i.d. column (Varian, Palo Alto, CA, USA) with nitrogen as a carrier gas, an injector temperature of 250 °C and an FID temperature of 300 °C. Samples (1 µL) were injected in splitless mode at an initial column temperature of 140 °C, and eluted as follows: 140 °C held for 5 minutes, followed by a ramp to 230 °C at 4 °C/min, 15 minutes isothermal at 230 °C and a final ramp to 240 °C at 10 °C/min. The final temperature was held for 6.5 minutes. Compounds were identified by comparison of retention times with a mix of known standards, and quantified by comparison to a calibration curve constructed for authentic standards prepared by the same procedure as above.

To measure glycerol content spectrophotometrically (see also Crosthwaite et al., 2011), whole beetles were homogenized in 0.05% v/v Tween 20 in water and incubated with free glycerol reagent (Sigma Aldrich, Inc., St Louis, MO, USA) at room temperature (22 °C) for five minutes. Following incubation, absorbance at 540 nm was read on a microplate spectrophotometer (SpectraMax 340PC, Molecular Devices, Sunnyvale, CA, USA) and glycerol content calculated by comparison with a standard curve. Glycerol content per individual was corrected to mass and average water content for the life stage (measured in additional individuals) to estimate concentration, assuming equal intracellular and extracellular concentration. Glucose concentration was measured spectrophotometrically using methods described by Marshall and Sinclair (2010). Haemolymph osmolality and thermal hysteresis were measured using a nanolitre osmometer (Clifton Technical Physics, Hartford, NY, USA), according to the methods of Sinclair & Chown (2002). The abdomen of adult beetles was pierced with a clean dissection pin,

and the resulting droplet of hemolymph was stored frozen at -80 °C under type B immersion oil (cat. no. 16484, Cargille Laboratories, Cedar Grove, NJ, USA) to minimize oxidation and evaporation. We determined the melting point (and therefore the osmolality) of the hemolymph, the difference between the melting and freezing point (thermal hysteresis) and observed ice crystal morphology (angular ice crystals, or those with distinctly flat edges are indicative of antifreeze protein activity (Zachariassen and Kristiansen, 2000)).

We compared glycerol content and water content among life stages and seasons in R using an ANCOVA with body mass as a covariate followed by a Tukey's *post-hoc* test. We compared (estimated) glycerol concentration, osmolality, and thermal hysteresis among life stages and/or seasons using Welch's t-tests, or ANOVA, followed by a Tukey's *post hoc* test, also in R.

2.5 Microclimate temperature recordings

Microclimate temperature data were collected at 30 min intervals year-round at six sites (Table 1) in the Sierra Nevada Mountains using HOBO Pendant UA-002-08 data loggers (Onset Computer Corporation, Bourne, MA, USA). The data loggers were deployed in sets of three on the same willow plant: mid-willow (shielded inside a white plastic cup hanging upside down ca. 1.2 m above the ground, indicative of the canopy under-leaf temperatures the beetles experience when feeding; note that the willows may collapse under the weight of snow in the winter, reducing the height above the ground considerably), at the base of the willow stem, and buried ca. 5 cm in the soil next to the roots of the willow. The mid-willow data loggers were deployed during the summer of 2000 and the base-of-the-willow and soil loggers were deployed in the

summer of 2009. To assess the relationship between recorded logger temperatures and snowpack, we analysed data from a snow sensor used by the California Department of Water Resources at 3108 meters in the Bishop Creek catchment, using data available to the public (California Data Exchange Center; ca.gov), and compared that data with those from a pendent logger deployed at the base of the willow, about 500 meters from the weather station.

Using these microhabitat temperature data and published observations of phenology for each life stage (e.g. Rank, 1994) we defined the span of dates that could plausibly be experienced by each life stage, and constrained our analysis of each stage's cold tolerance to those periods. Within each period, for each life stage, we identified cold events that crossed the LT_{50} threshold for each life stage during the time of the year that they are present using R code based on Marshall and Sinclair (2012b) and Sinclair (2001a).

3. Results

3.1 Field observations

Fifty-one live adult beetles were recovered from the overwintering cage in November. The majority of the beetles were present in the soil below the leaf litter (ca. 20 cm depth): 22 were found within 0.5 m of the base of the plant, 6 at 0.5-1 m, and 8 >1 m from the base of the plant. Fourteen beetles were found in leaf litter directly at the base of the plant, whereas no beetles were present in leaf litter away from the plant. One beetle was observed on the above-ground portion of the willow, none were beneath stones or logs.

An unseasonal hard frost (-14 °C) during October 6-8 2011 occurred when larvae, pupae and adults were still present on willow leaves. The day after this event, adult beetles were still observed, but no live pupae or larvae were observed across the full range of drainages and elevations occupied by *C. aeneicollis* (see Table S2).

3.2 Cold tolerance

All quiescent adult beetles and 99 % of summer-collected adult beetles survived internal ice formation for one hour and are therefore classified as freeze tolerant. By contrast, pupae and eggs were killed by freezing, but not by cold exposure above the SCP, suggesting that they are freeze avoidant. All three larval stages were killed by cold exposure at temperatures that did not initiate internal ice formation, and are therefore chill-susceptible (Table 2).

Eggs had the lowest SCP, which was significantly lower than that of the three larval instars (which did not differ significantly from one another), while adults had the highest SCP (Table 3). SCPs of adults did not differ significantly between summer-collected and quiescent individuals (Table 3). When frozen, 18 of 24 pupae showed double exotherms (-13.6 ± 3.2 °C and -17.5 ± 0.8 °C; Figure 1), while the remaining six had only a single exotherm at -11.1 ±2.8 °C, which did not differ significantly from the higher of the double exotherms (Table 3). Dissections showed that double-exotherm pupae contained tissue moulted from the 3rd instar larva that had not completely dried. Single-exotherm pupae were later in development, and the desiccated remains of the 3rd instar cuticle no longer frozen. Early-development pupae that experienced a single exotherm were killed (n= 18), so we infer that the first exotherm represents freezing of the

developing pupal tissue, and that this first SCP is a valid measure of lethal temperature for all individuals.

The LT_{50} of eggs was lower than other life stages, and there was a high variance in the LT_{50} of pupae, with a 7 °C difference between LT_5 and LT_{95}. Larvae had a relatively high LT_{50} (ranging from -3.3 to -8.7 °C), which did not differ significantly among instars (Table 3). The LT_{50} of quiescent adults (LT_{50}: -15 °C) were significantly more cold-tolerant than summer adults (LT_{50}: -9.2 °C; Table 3), although the estimated LT_{50} for the former is lower than the lowest temperature survived by any individual quiescent beetle (-10 °C), which we use as an estimate of low temperature tolerance for subsequent considerations. Although quiescent adults survived for one hour after the initiation of ice formation when held at -8°C, survival decreased rapidly, with only 6 of 8 individuals surviving four hours of freezing, 2 of 8 after 6 h and no beetles survived 12 h of ice formation.

3.3 Cryoprotectants

Hemolymph osmolality of quiescent beetles (877 ± 5.5 mOsm) was more than double that of summer-collected beetles (380 ± 3.3 mOsm; t_8=77.4, $p<0.001$). Very low levels of thermal hysteresis activity were observed in adult hemolymph in both summer (0.08 ± 0.004 °C) and winter (0.22 ± 0.05 °C; t_8=2.9, $p=0.020$). Growing ice crystals were circular in both summer and quiescent adults. Water content varied from 68 to 86 %, was lowest in adult beetles and eggs, intermediate in the three larval instars, and highest in pupae (Figure 2).

373 Glycerol, rhamnose, fructose and glucose were the main sugars and polyhydroxy alcohols
374 identified by gas chromatography in extracts of whole individuals (Figure 3). Gas
375 chromatography suggested that the quantity of glycerol and glucose changed significantly from
376 summer to winter (Figure 3, n=1/season). However, spectrophotometric measurement of glucose
377 concentration revealed no significant difference between quiescent (1.4 ± 0.18 mM/L) and
378 summer-collected (3.0 ± 0.81 mM/L) beetles (t_4 = 1.948, p=0.123). Glycerol content and
379 estimated concentration was significantly higher in quiescent beetles than in all other life stages
380 (Figure 2).

381

382 *3.4 Microclimate temperatures*

383 Microclimate varied by altitude, season and among years, and was affected by snow cover
384 (Figure 4; Supplementary Material Table S1, Figures S1, S2). When significant snow cover (~6
385 cm) was present (e.g. a few days in October 2011, plus Feb-April 2012 for one study locality),
386 air temperatures at the top of the soil were close to 0 °C. However, when snow was scant or
387 absent, surface temperatures were well below or above zero. Minimum microclimate
388 temperatures were lowest in the willow canopy, with -24.8 °C recorded at the mid-willow logger
389 on 12 January 2007 at 11.55pm at the 2905 m "40 bog" site in Big Pine Creek. Temperatures at
390 the base of the willow were more buffered (minimum of -13.3 °C at 3353 m on 5 October 2009
391 at 6.00 am), and never dropped below -5.9 °C (8.30 am, 6 December 2009 at the 2773m site) in
392 the soil. There was considerable variation in temperature, and minimum temperatures below 0 °C
393 were recorded at the mid willow loggers almost year-round (Figure 4). Although minimum
394 temperatures at the mid-willow reached very low temperatures, we did not record any instances
395 of minimum temperatures exceeding the tolerance of the life stage(s) of *C. aeneicollis* most

likely to be present, assuming that quiescent beetles overwinter in the soil (see above; Figure 5). Microclimate temperatures at all microsites were strongly influenced by snow cover. During the winter, snow cover buffered temperatures to close to 0 °C at most locations during the extent of this study (Figure 4).

4. Discussion

Pre-adult life stages of *Chrysomela aeneicollis* are relatively intolerant to cold, and we report mortality of pupae in the field after an early autumn cold event. Such direct reports of unseasonable cold-related mortality events are rare but have been documented in other beetles, such as the North American bark beetle *Dendroctonus ponderosae* (Creeden et al., 2014). We also show that freeze-tolerant adults overwinter in the leaf litter and soil, where they are buffered from climate extremes. However, we recorded several instances of mid-willow temperatures below the beetle's LT_{50}, and the lowest soil temperatures we recorded were at the lowest-elevation site, suggesting that the buffering effect of snow cover (which is less reliable at low elevations) is important for overwintering survival. This supports the hypothesis that local low altitude extinctions of sub-populations of *C. aeneicollis* may have been caused by cold winter temperatures at low elevations in years with little snow cover (Rank, 1994).

It is not usually possible to predict the cold tolerance strategy adopted by insects; many montane insects are freeze tolerant (Sinclair and Chown, 2005; Sømme, 1989; Wharton, 2011), but there is often little phylogenetic signal within groups (Sinclair and Chown, 2010). Among chrysomelid beetles, the Colorado Potato Beetle (*Leptinotarsa decemlineata*), which overwinters in the soil as

418 a diapausing adult (Lee et al., 1994), is freeze-avoidant, but there are also several species that are
419 freeze tolerant (e.g. Zachariassen et al., 2008) and chill-susceptible (e.g. Watanabe and Tanaka,
420 1998, 1999). Glycerol is accumulated as a cryoprotectant by at least one freeze-tolerant
421 chrysomelid, *Melasoma collaris* (Gehrken and Southon, 1997), and myo-inositol by the chill-
422 susceptible species (Watanabe and Tanaka, 1998, 1999). Thus, while there is a diversity of cold
423 tolerance strategies in the Chrysomelidae, freeze tolerance and glycerol accumulation in adult *C.*
424 *aeneicollis* is consistent with other cold-hardy species in the family.

425

426 The lowest temperature that adult *C. aeneicollis* survived in our experiments was -10 °C, and
427 there was low survival of longer periods frozen, with 75% mortality after 6 h at -8 °C. This may
428 reflect a reliance on the buffered habitat to prevent long exposure to cold in the field. Alternately,
429 our laboratory protocol may not have maximized the cold tolerance capabilities of this species.
430 Freeze tolerance can change during the winter season (e.g. Sinclair, 1997; Storey and Storey,
431 1983), and cold tolerance may be plastic during rapid cold-hardening (e.g. Lee et al., 2006) or in
432 response to repeated cold stress (Marshall and Sinclair, 2012a). Further experiments on the
433 plasticity of cold tolerance, or on beetles field-collected during winter are necessary to determine
434 whether we observed maximal cold tolerance for overwintering adults in this species.

435

436 Our data logger temperatures suggest that, with the exception of pupae late in the season, pre-
437 adult stages of *C. aeneicollis* are not prepared to encounter sub-zero temperatures, and prior
438 studies show that they suffer high mortality when they do so (McMillan et al., 2005).
439 *Chrysomela aeneicollis* eggs were freeze avoidant, (in keeping with most other insect eggs;
440 Sinclair and Chown, 2010), and were able to tolerate brief extremes of temperature, likely

441 because of their small size and resistance to ice nucleation. Eggs are unlikely to encounter
442 extremely low temperatures, and although we did not explore tolerance to extended cold
443 exposure, we expect that eggs would have limited tolerance for this. Larvae were chill-
444 susceptible and had very limited cold tolerance. This is not surprising in a univoltine species, but
445 does potentially leave the species vulnerable to late summer cold snaps – for example, summer
446 temperatures below -5 °C have been recorded at Lake Louise, Alberta, at the North end of the *C.*
447 *aeneicollis* range (Environment Canada; climate.weather.gc.ca), and could account for the
448 northern range limit for this species (Brown, 1956). Indeed, in the Sierra, a single night-time
449 exposure to -6 °C in summer caused significant mortality (McMillan et al., 2005).

450

451 Almost all pupae in which ice formed were killed, suggesting that they are freeze-avoidant. This
452 is borne out by the mortality event we observed in the field, where overnight minima of -7 to -14
453 °C coincided with mortality of all pupae observed at multiple sites. This event occurred at a time
454 when the majority of beetles had emerged as adults (and were therefore freeze tolerant). The
455 double exotherms we observed in some pupae in the laboratory likely derive from the freshly
456 moulted exuvium in the early stages of pupation. Double exotherms are occasionally observed in
457 freeze tolerant species (e.g. Sformo et al., 2009; Sinclair et al., 2009), and are thought to
458 represent freezing of independent compartments. In the present context, we hypothesise that the
459 pupating beetle and the exuvium freeze separately; because the first exotherm was always
460 accompanied by mortality, we propose that it represents freezing of the developing beetle. We
461 did observe survival of a small number of pupae after freezing, and hypothesise that these may
462 have been pharate adults, which we would expect to be freeze tolerant. The three different

responses of ‘pupae’ we observed emphasise the importance of accounting for developmental stage when determining insect cold tolerance, especially during pupation.

In keeping with many other insects (Lee, 2010), glycerol appears to be the main polyol cryoprotectant associated with freeze tolerance in *C. aeneicollis*, although the accumulation (in the region of tens of mM) is relatively low compared to other cold-tolerant insects (e.g. Crosthwaite et al., 2011; Kukal et al., 1988; Zachariassen, 1980). We detected hints of thermal hysteresis activity in the hemolymph, suggesting that hemolymph antifreeze proteins play only a small role in freeze tolerance in this species. Hemolymph osmolality nearly doubled between summer and winter, although glycerol accumulation alone was not sufficient to explain this osmolality increase. It is unlikely that the increased osmolality is due to inorganic ions (Zachariassen et al., 2004). However, accumulation of the free amino acid proline has been implicated in freeze tolerance for several insects (Koštál et al., 2012; Koštál et al., 2011; Ramløv, 1999) and plants (Nanjo et al., 1999), and could be a candidate to explain the additional hemolymph osmolytes during winter.

Chrysomela aeneicollis adults are cold-hardy enough to survive winter temperatures in buffered habitats, but our field observations and laboratory data suggest that late-instar larvae and pupae are susceptible to cold snaps in late summer and early autumn. This could be a significant selective pressure determining the developmental period of this species. In the context of climate change, extreme events such as these are expected to increase in frequency, particularly in conjunction with decreased precipitation (and therefore reduced snow cover buffering) in California (Jayko and Millar, 2001; Pierce et al., 2008; Stewart et al., 2004). This means that

even if mean temperatures are overall warmer, the poor survival of extreme events by pre-adult life stages will likely reduce the possibility of changes in voltinism in response to extended growing season. In turn, this will potentially exacerbate the impacts of energetic drain caused by increased fall temperatures (Sinclair, in press; Williams et al., 2012). Thus, interactions between development stage and cold hardiness have the potential to determine the effects of climate change on univoltine montane species such as *C. aeneicollis*, the magnitude of which may be determined by the intensity of metabolic suppression and extent of overwinter energetic drain. There are well-established metabolism-related genetic polymorphisms in this species (Dahlhoff et al., 2008; Dahlhoff and Rank, 2007; Rank et al., 2007; Wheat and Hill, 2014), and investigating the influence of these on diapause and overwinter energetics may be crucial for understanding the species' responses to climate change.

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678 **Figure Captions**

679 **Figure 1** – Single (A) and double (B) exotherms observed in pupae of *C. aeneicollis*.

680 **Figure 2** – Gravimetric water content (A), glycerol content (B) and estimated hemolymph
681 glycerol concentration (see text for details of estimation) in all life stages of *C. aeneicollis*.
682 'First', 'Second' and 'Third' refer to larval instars. Mean ± SEM presented; values with differing
683 letters are significantly different within a panel.

684 **Figure 3 –** Sample gas chromatography traces of polyols and carbohydrates in (A) summer and
685 (B) quiescent adult *C. aeneicollis* beetles. Carbohydrates were analysed as their acetylated polyol
686 derivatives (see text for details). RP: Reagent peak.

687 **Figure 4** – Sample microclimate temperatures from different microhabitats (A-C) and elevations
688 (D-F) inhabited by *C. aeneicollis* beetles in the Big Pine Creek (BPC) catchment. Three different
689 microsites (A: within the willow canopy; B: in leaf litter at the base of the willow; and C: c. 3 cm
690 below the soil surface) at 3353 m. Temperatures within the willow canopy, where beetles are
691 active during the day in summer, are also shown for willows at 3215, 2905 and 2773 m are
692 shown (see also Table 1); note that the willows may collapse under the weight of snow, reducing
693 the height above the ground in the winter. Horizontal dashed line indicates 0 °C. Periods with
694 little daily variance are likely due to temperatures being buffered by snow cover (see also
695 Supplementary Material Figure S1).

696 **Figure 5** – Mean minimum temperature at three microhabitats for (A) 3553 m and (B) 2770 m
697 sites in the Big Pine Creek catchment over the duration of each life stage of the beetle *C.*
698 *aeneicollis* (median lethal temperatures shown for comparison with the exception of quiescent

699 adults, for which the lowest temperature for which survival was observed (-10 °C) is plotted
700 without error bars, see text for details). See Table 1 for timespan of temperature measurements.
701

Table 1. Microclimate temperature monitoring sites in the Big Pine Creek drainage of the Sierra Nevada Mountains, California. Microclimate temperatures were recorded every 30 min at c. 1.2 m height in the willow canopy (2005-2011), and at the base of the stem and buried c. 5 cm in the soil (2009-2011). All logger data recordings completed in June of 2012 unless noted.

Site	Altitude	Coordinates	Initiation of mid-willow logging	Initiation of other logging
Sam Mac Meadow	3353 m	37.119°N 118.506°W	July 2005	June 2009
Upper Site	3215 m	37.124°N 118.502°W	June 2000	June 2009
40 Bog	2905 m	37.132°N 118.473°W	August 2000	June 2009*
26 Bog	2773 m	37.137°N 118.463°W	August 2000	June 2009

*Recording ended 2011 at base of willow at this site.

Table 2. Mortality of each life stage of *Chrysomela aeneicollis* after 1 h cold exposure with and without internal ice formation, yielding cold tolerance strategy. These data represent an aggregate of all experiments in which ice formation was recorded in some beetles.

Life Stage	Died without internal ice formation	Died after internal ice formation	N	Cold tolerance strategy
Egg	0	72	72	Freeze Avoidant
Larvae				
First Instar	44	44	44	Chill Susceptible
Second Instar	40	40	40	Chill Susceptible
Third Instar	57	57	57	Chill Susceptible
Pupa	0	61	66	Freeze Avoidant
Adult				
Quiescent	0	0	30	Freeze Tolerant
Summer-collected	0	3	264	Freeze Tolerant

711 **Table 3**. Mean (±SEM) supercooling point and median lethal temperature (LT_{50}) for each life
712 stage of *Chrysomela aeneicollis*. Similar superscript letters indicate no significant difference
713 between the supercooling points ($p<0.001$); sample size for SCP is in parentheses. The LT_5 and
714 LT_{95} are given in parentheses after LT_{50}; different superscript letters indicate values for which
715 this range does not overlap. Note that the lowest temperature survived by an individual quiescent
716 beetle was -10 °C, and we use that estimate in subsequent analyses.

Life stage	Supercooling point (°C)	LT50 (LT5, LT95)
Egg	-23.4 ± 0.18 ^{b} (47)	-20.1 (-19.3, -20.8) ^{s}
First Instar Larva	-10.6 ± 0.47 ^{c} (22)	-3.2 (-0.3, -6.8) ^{q}
Second Instar Larva	-10.8 ± 0.68 ^{c} (16)	-6.2 (-1.5, -11.0) ^{pq}
Third Instar Larva	-8.6 ± 0.62 ^{c} (32)	-8.7(-8.3, -9.1) ^{p}
Pupa		-11.4 (-4.5, -18.2) ^{pqr}
Single Exotherm	-11.1 ± 1.14 ^{cd} (6)	
Double exotherm		
First Exotherm	-13.6 ± 0.05 ^{de} (18)	
Second Exotherm	-17.5 ± 0.75 ^{e} (18)	
Summer Adult	-4.9 ± 0.05 ^{a} (95)	-9.2 (-8.8, -9.6) ^{p}
Quiescent Adult	-5.3 ± 0.11 ^{a} (28)	-15.0 (-14.4, -15.6) ^{r}

717

718

Figures

Fig 1

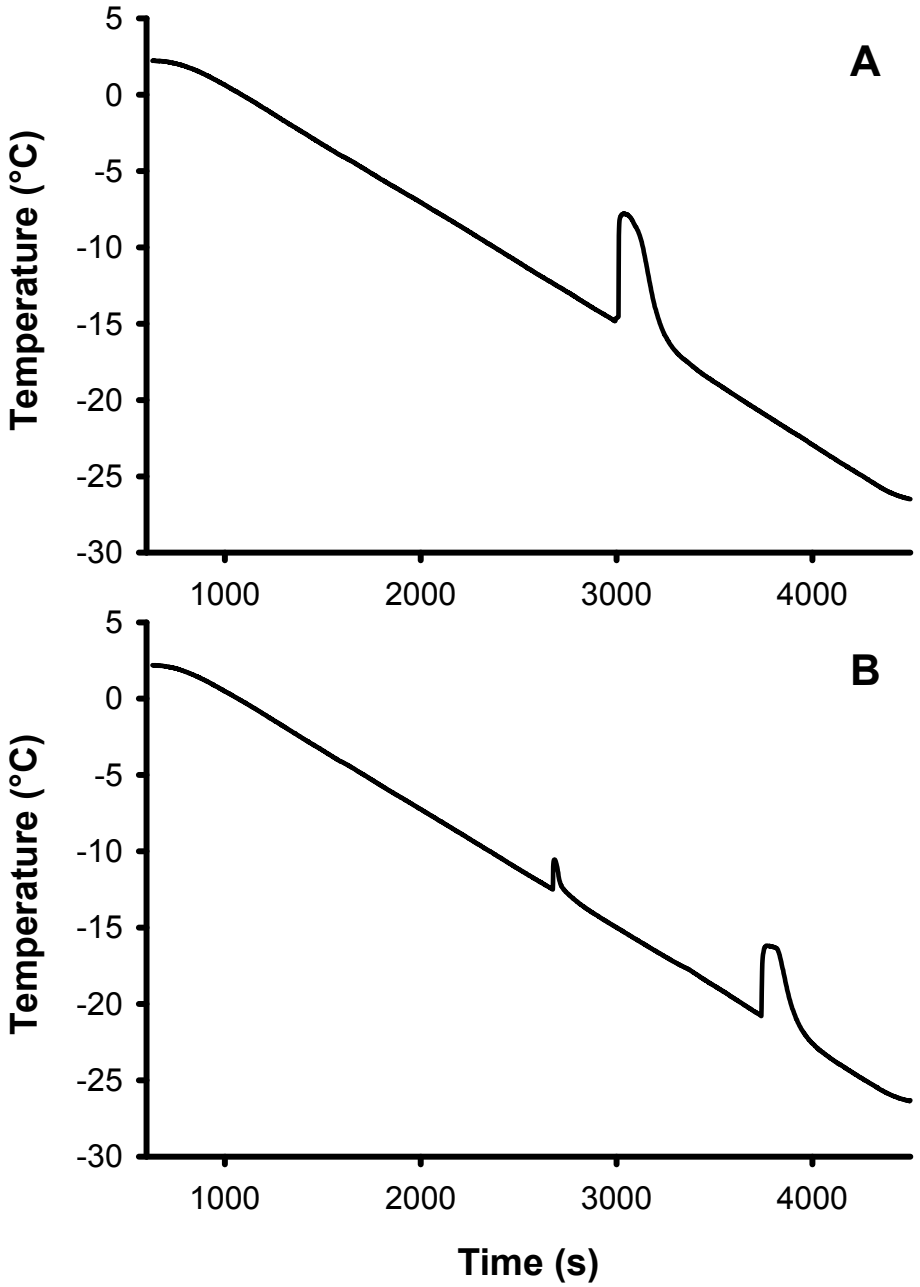


Fig 2

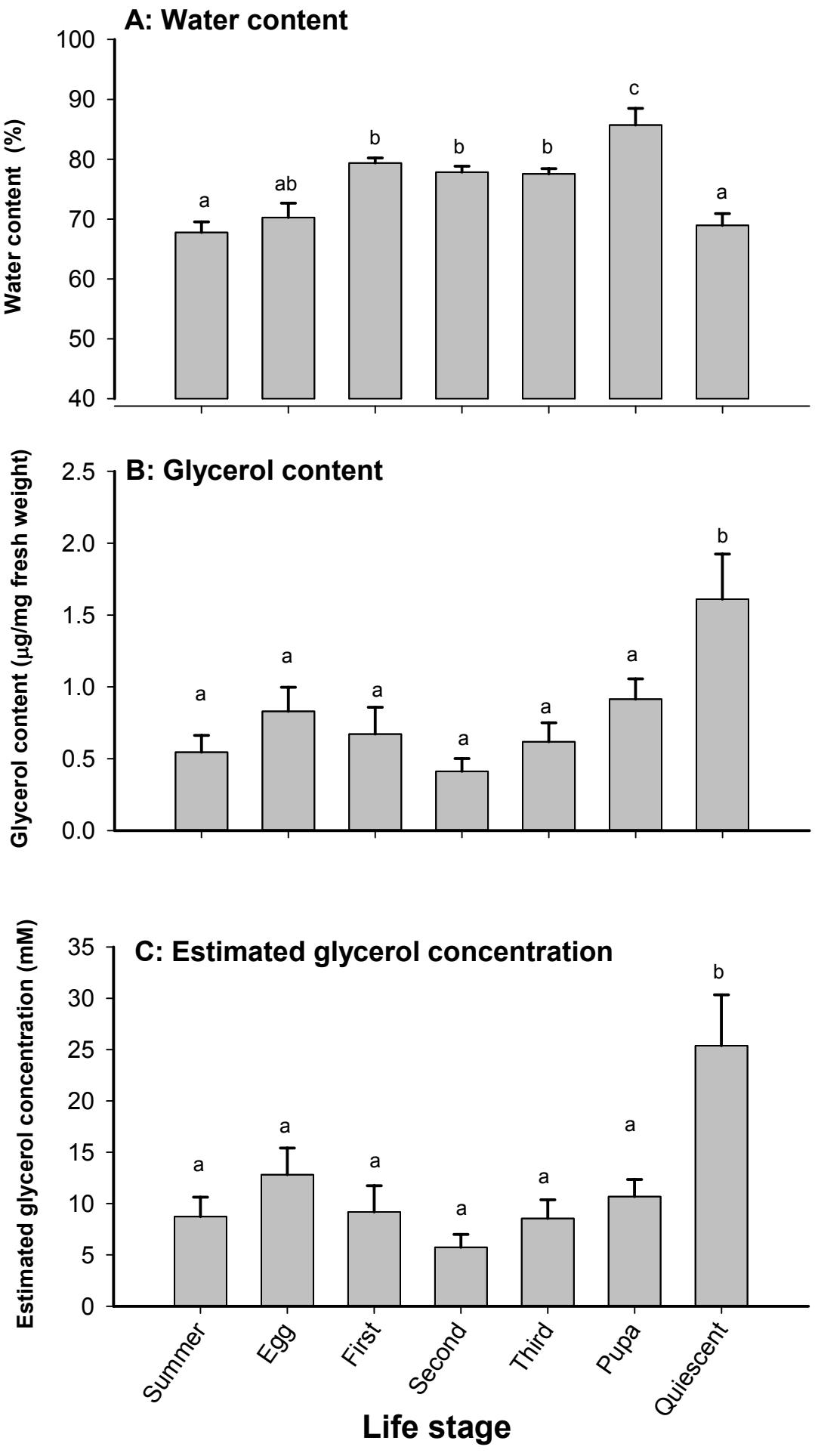

A: Water content
Water content (%)
100
90
80
70
60
50
40
a
ab
b
b
b
c
a
B: Glycerol content
Glycerol content (µg/mg fresh weight)
2.5
2.0
1.5
1.0
0.5
0.0
a
a
a
a
a
a
b
C: Estimated glycerol concentration
Estimated glycerol concentration (mM)
35
30
25
20
15
10
5
0
a
a
a
a
a
a
b
Summer
Egg
First
Second
Third
Pupa
Quiescent
Life stage

Fig 3

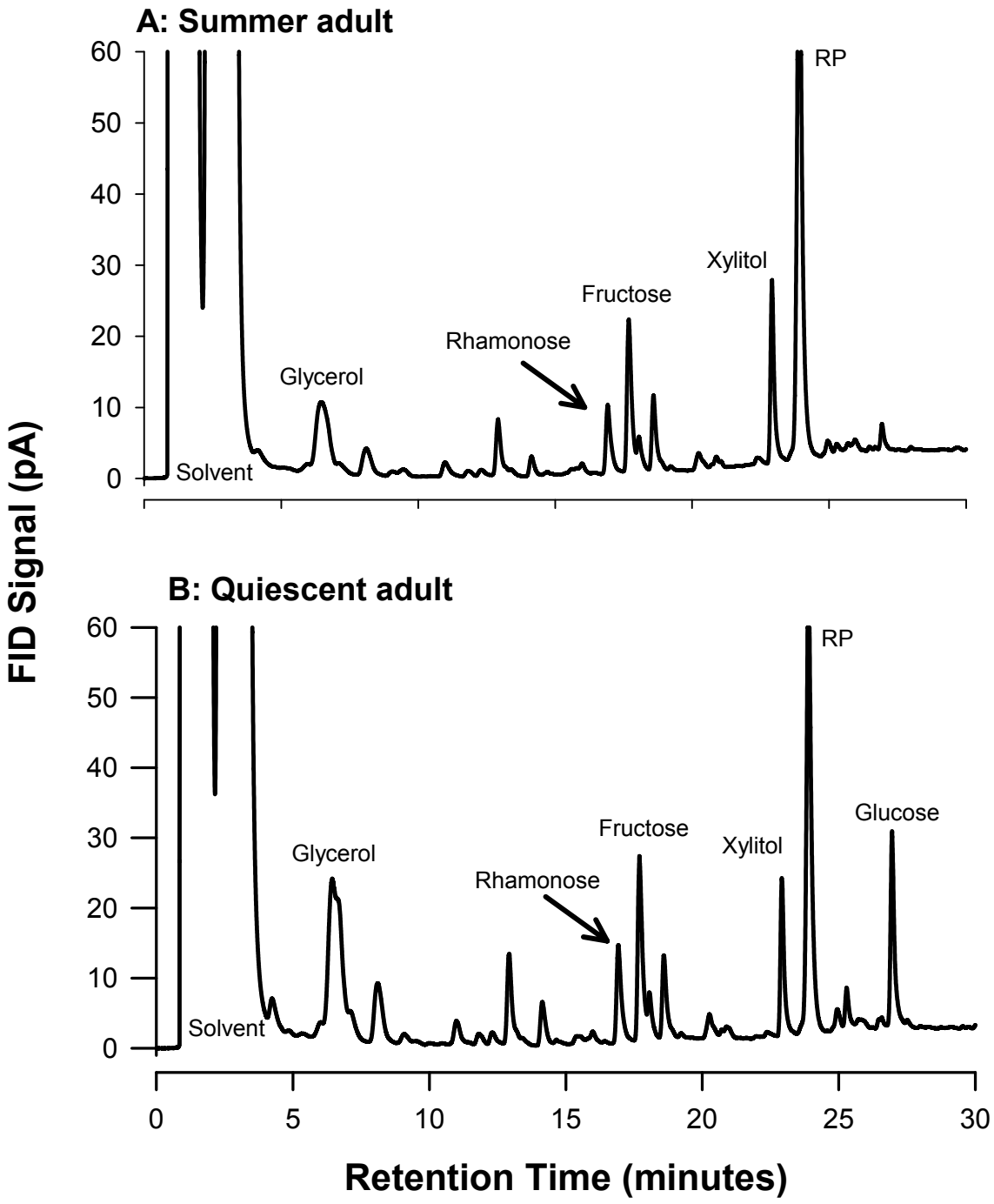

A: Summer adult
B: Quiescent adult
FID Signal (pA)
Retention Time (minutes)
Solvent
Glycerol
Rhamonose
Fructose
Xylitol
Glucose
RP
0
10
20
30
40
50
60
0
5
10
15
20
25
30

Fig 4

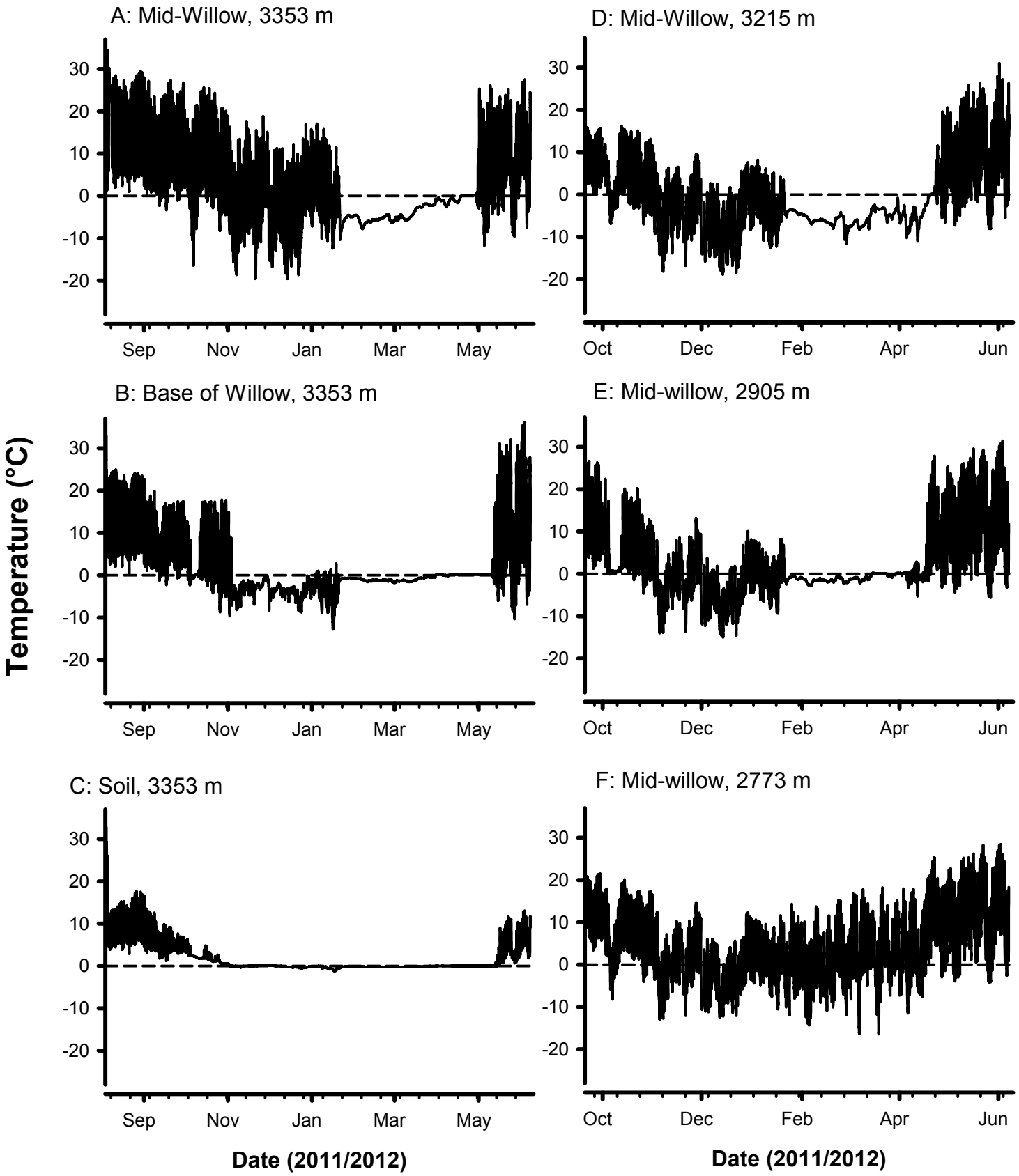
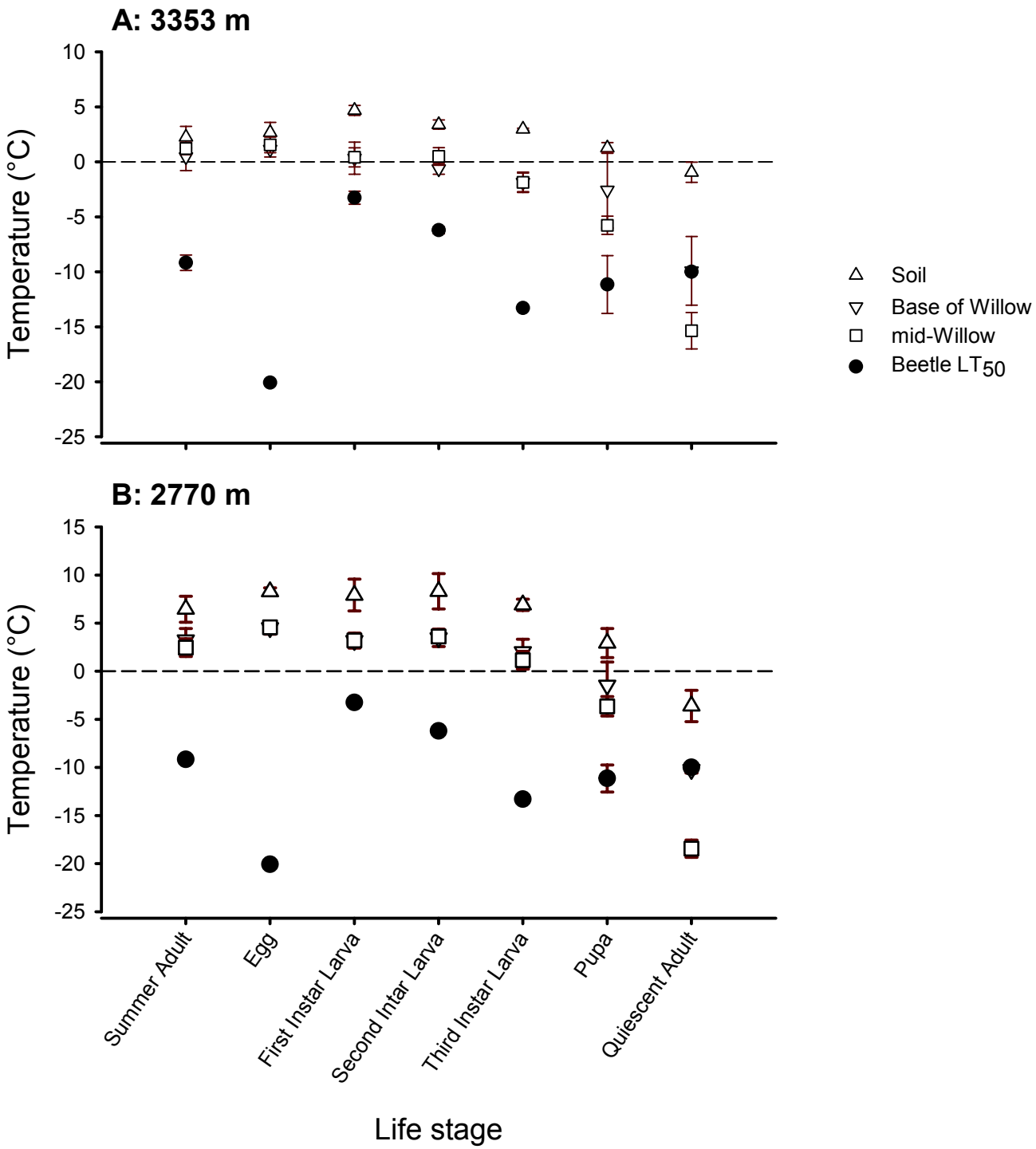

A: Mid-Willow, 3353 m
D: Mid-Willow, 3215 m
B: Base of Willow, 3353 m
E: Mid-willow, 2905 m
C: Soil, 3353 m
F: Mid-willow, 2773 m
Temperature (°C)
30
20
10
0
-10
-20
Sep
Nov
Jan
Mar
May
Oct
Dec
Feb
Apr
Jun
Date (2011/2012)
Date (2011/2012)

Fig 5


A: 3353 m
B: 2770 m
Temperature (°C)
10
5
0
-5
-10
-15
-20
-25
15
Soil
Base of Willow
mid-Willow
Beetle LT50
Summer Adult
Egg
First Instar Larva
Second Intar Larva
Third Instar Larva
Pupa
Quiescent Adult
Life stage

Graphical Abstract