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1 # Autumn shifts in cold tolerance metabolites in overwintering adult mountain pine beetles

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3

4 *Abstract:*

5 The mountain pine beetle, *Dendroctonus ponderosae* (Coleoptera: Curculionidae) is a
6 major forest pest of pines in western North America. Beetles typically undergo a one-year life
7 cycle with larval cold hardening in preparation for overwintering. Two-year life cycle beetles
8 have been observed but not closely studied. This study tracks cold-hardening and preparation for
9 overwintering by adult MPB in their natal galleries. Adults were collected *in situ* between
10 September and December (2016) for a total of nine time points during 91 days. Concentrations of
11 41 metabolites in these pooled samples were assessed using quantitative NMR. Levels of
12 glycerol and proline increased significantly with lowering temperature during the autumn. Newly
13 eclosed mountain pine beetles prepare for winter by generating the same cold-tolerance
14 compounds found in larvae, but high on-site mortality suggested that two-year life cycle adults
15 have a less efficacious acclimation process. This is the first documentation of cold acclimation
16 metabolite production in overwintering new adult beetles and is evidence of physiological
17 plasticity that would allow evolution by natural selection of alternate life cycles (shortened or
18 lengthened) under a changing climate or during expansion into new geoclimatic areas.

19 *Introduction:*

20 The mountain pine beetle, *Dendroctonus ponderosae* (Coleoptera: Curculionidae), is an
21 irruptive forest insect native to western North America (Bracewell et al. 2017). In the past 20
22 years, mountain pine beetle outbreaks of unprecedented size killed much of the mature pine in
23 British Columbia (Hrinkevich and Lewis 2011) and expanded beyond their historical range over

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24 the Rocky Mountains to Alberta and into the north of British Columbia (Cullingham et al. 2011;
25 Janes et al. 2014). Cold winters – greater than two weeks at –40 – are thought to have limited
26 the scope of previous outbreaks by killing off mountain pine beetle brood by freezing (Safranyik
27 and Linton 1998; Safranyik and Carroll 2006). Climate change has led to a warming trend in the
28 past 30 years, reducing the length and frequency of reaching and sustaining this temperature
29 threshold. This trend is particularly the case in the early- and late-winter season when
30 overwintering insects would normally be the most vulnerable due to a lack of overwintering
31 metabolites, therefore increasing mountain pine beetle winter survival rates (Bentz et al. 2010;
32 Goodsman et al. 2018).

33 Most mountain pine beetles have a univoltine lifecycle, meaning they mature over the
34 course of a single year (Bentz and Powell 2014; Six and Bracewell 2015). Life cycle
35 development is dictated by climatic factors; adults lay eggs in the late summer to early fall,
36 allowing for partial larval development prior to freezing winter temperatures. Mountain pine
37 beetles are freeze intolerant and will experience mortality if water within their soft tissue
38 crystalizes (Bentz and Mullins 1991). For this reason, mountain pine beetles generate
39 cryoprotectants in the autumn, especially glycerol, to reduce their super cooling point and protect
40 against the formation of ice crystals (Régnière and Bentz 2007; Robert et al. 2016; Bleiker et al.
41 2017; Fraser et al. 2017). Early instar larvae (instars 1 and 2) are marginally less cold tolerant
42 than late-instars (instars 3 and 4), with the first three instars generating similar proportions of
43 glycerol in relation to bodyweight, and the final instar producing slightly more (Logan et al.
44 1995; Safranyik and Carroll 2006). Larvae also void their guts in preparation for cooler
45 temperatures in order to reduce the number of internal ice nucleation surfaces (Keeling et al.

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46 2013). Pupation and maturation follow in the spring and beetles fly in the summer to find new
47 hosts and repeat the life cycle.

48 Mountain pine beetle phenology is known to be most responsive to temperature changes,
49 with hormonal regulation and photo period playing little part in cold acclimation (Régnière and
50 Bentz 2007). Photoperiod, historically thought to have little effect on cold hardening, may be
51 involved to some degree as new evidence suggests that mountain pine beetle larvae respond
52 negatively to light, even when located in a sub-cortical environment (Wertman et al. 2018).
53 Reliance on temperature occasionally results in phenological delays causing an extension of the
54 life cycle beyond one year. Such larvae overwinter, pupate later in the summer, and overwinter a
55 second time as new adults (Amman 1973). Larval cold hardening and cryoprotectant generation
56 in insects is mainly understood (Storey and Storey 1983), with mountain pine beetle-specific
57 studies of cryoprotectant production (Régnière and Bentz 2007), RNA transcript generation
58 (Robert et al. 2016; Fraser et al. 2017), and proteomic data (Bonnett et al. 2012) suggesting some
59 of the mechanisms and pathways of larvae winter survival. While overwintering new adults are
60 occasionally recorded in the literature (DeLeon et al. 1934; Amman 1973), and have been
61 observed as an early-May flight during the height of the outbreak in central BC (DPWH,
62 personal communication), they represent a less common life strategy and the precise mechanisms
63 of overwintering are unknown. In this study we recorded the production of metabolites in newly
64 eclosed adult mountain pines beetle *in situ* from late-fall to the coldest day of the winter in order
65 to quantify their cold acclimation process.

66 *Materials and Methods:*

67 Beetle collection began in late-summer (17 Sept 2016) at the Lucerne campground
68 (Robson Provincial Park, British Columbia, 52°50'59.48"N, 118°34'21.84"W, 1125m). This site

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69 is located very near to the Great Divide, within the Rocky Mountain range (Figure 1). The
70 infested stand consisted mostly of lodgepole pine (*Pinus contorta)* with evidence of recent
71 mountain pine beetle attack dating from several previous years, including the most recent
72 summer. Sampling continued weekly (25 Sept, 2 Oct, 11 Oct) and then bi-weekly (23 Oct, 6
73 Nov, 20 Nov, 3 Dec, 16 Dec) for nine total collection days during a 91-day period spanning
74 almost the entire autumn of 2016. Brood galleries were exposed using a draw knife to remove
75 bark from the tree. A minimum of 40 new adult beetles were collected from separate galleries in
76 five randomly selected trees during each collection event (eight beetles per tree). For the first
77 eight sampling days, beetles were confirmed to be living (by observing movement) prior to
78 collection. Temperatures below –30□ on the final day of sampling precluded this step as beetles
79 were too cold for movement. Ice crystals were observed within sampled galleries on the final
80 sampling day. Beetles were placed in either 2 mL or 1.5 mL dry snap-capped microcentrifuge
81 tubes and flash frozen in the field using liquid nitrogen and then transported to the lab and stored
82 at –80°C until metabolite processing. Three HOBO U23 Pro v2 data loggers (HOBOware, Onset
83 Computer Corporation) were placed at separate locations at the field site to track temperature.
84 Loggers were moved from their original positions after the eighth sampling date due to sanitation
85 logging on the site but were relocated near to their original positions within the sampling area.
86 Pine beetle larvae were quickly thawed on ice. Approximately 1g of larval tissue was
87 transferred to a mortar and pestle with 3 mL of chloroform:methanol (1:2 v/v) The larvae were
88 then ground for 3 min and the extract was transferred to a 4 dram vial. The mortar was rinsed
89 with 2 mL of a chloroform:methanol (1:2 v/v) mixture for the complete recovery of the extract.
90 The entire extract was filtered using vacuum filtration. The residue was transferred into a 15 mL
91 sterile screw-capped plastic centrifuge tube and 5 mL chloroform:methanol (1:2 v/v) mixture

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was added to the residue and was shaken at 250 rpm for 30 min at ambient temperature on a shaker. This extract was filtered again using vacuum filtration, combined with the first filtrate in a 13.5 mL Teflon lined screw-capped glass vial and the combined filtrate was transferred to a 50 mL sterile screw-capped plastic centrifuge tube. To this filtrate one quarter of the total volume of the filtrate 0.88% KCl was added. The tube was vortexed for 1.5 min and placed aside for 10 min for phase separation of an upper aqueous layer and lower organic layer. The tube was then centrifuged for 30 minutes at 3000 rpm. The upper aqueous layer (water-soluble metabolites) was transferred into a 15 mL sterile screw-capped plastic centrifuge tube and 2.5 mL HPLC water was added to the water-soluble metabolite extract and flash frozen in liquid nitrogen. This sterile screw-capped plastic centrifuge tube was lyophilized with frozen water-soluble metabolites for 24 h and the resultant freeze-dried powder of was divided into 15 mg aliquots for NMR analysis.

A single 15 mg aliquot of the lyophilized water-soluble extract from pine beetle larvae was taken in 1.5 mL snap-capped microcentrifuge tube. To this powder, 570 μL of water was added. The sample was sonicated for 15 min in a bath sonicator. To this sample, 60 μL of reconstitution buffer (585 mM phosphate buffer with 11.67 mM DSS) and 70 μL of D2O were added. The solution was vortexed for 1 min and centrifuged at 10,000 rpm for 15 min at ambient temperature. The clear supernatant was transferred into an NMR tube for NMR analysis.

All 1H-NMR spectra were collected on a 700 MHz Avance III (Bruker) spectrometer equipped with a 5 mm HCN Z-gradient pulsed-field gradient (PFG) cryoprobe. 1H-NMR spectra were acquired at 25°C using the first transient of the NOESY pre-saturation pulse sequence (noesy1dpr), chosen for its high degree of quantitative accuracy. All FID's (free induction decays) were zero-filled to 250 K data points. The singlet produced by the DSS methyl groups

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115 was used as an internal standard for chemical shift referencing (set to 0 ppm) and for
116 quantification all 1H-NMR spectra were processed and analyzed using the online Bayesil
117 software package. Bayesil allows for qualitative and quantitative analysis of an NMR spectrum
118 by automatically and semi-automatically fitting spectral signatures from an internal database to
119 the spectrum. Specifically, the spectral fitting for metabolites was completed using the standard
120 serum metabolite library. Typically, all visible peaks were assigned. Most of the visible peaks
121 are annotated with a compound name. This fitting procedure provides absolute concentration
122 accuracy of 90% or better. Each spectrum was further inspected by an NMR spectroscopist to
123 minimize compound misidentification and mis-quantification.

124 A one-way ANOVA was used to determine significance between measured metabolites,
125 with a post-hoc Tukey's honestly significant different test used for pair-wise comparison of
126 metabolite mean concentration between timepoints [adjusted p-value (FDR) = 0.05] following
127 statistically significant ($p < 0.05$) ANOVA results. Both measures were conducted using
128 MetaboAnalystR (Chong and Xia 2018). Concentration values and temperature measurements
129 were visualized in Microsoft Excel (2016). Correlation of glycerol, proline, and trehalose to
130 temperature values was performed in Rstudio (R version 3.4.4) using Pearson's product-moment
131 correlation.

132 *Results:*

133 Temperature at the study site initially decreased towards freezing but warmed and
134 remained at or above freezing during the day during October and November. Temperatures
135 dropped rapidly during early December and remained below –20 □ for approximately two
136 weeks, reaching the coldest temperature of the winter (–30.1□) on the final sampling day, 16
137 December 2016 (Figure 2). A spring collection was attempted when the site was again accessible

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138 following snow melt on 26 May 2017, but no living new adults could be found in a search of the
139 area, including thorough investigation of trees previously sampled.

140 We detected 41 metabolites in overwintering adults (full list provided in supplemental 1).
141 A one-way ANOVA with a post-hoc Tukey's HSD test for pairwise comparison showed 27 of
142 these metabolites differed significantly at one or more of the time points taken during the study
143 (Figure 3, but see also supplemental 2). Of these 27 significant measures, three metabolites –
144 glycerol, trehalose, and proline – became highly elevated and are likely biologically significant
145 in addition to being statistically significant (Figure 4). Glycerol concentrations were highest in
146 beetles at the end of the sampling period, reaching 468.91 μg/mg of body weight (SE ± 49.74).
147 Trehalose levels increased into the month of October but decreased in December, reaching a
148 peak of 180.16 μg/mg (SE ± 13.67) on 6 Nov. Proline levels increased until the middle of the
149 sampling period and remained stable for the final four sampling days, reaching a final
150 concentration of 46.02 μg/mg (SE ± 2.93) on the final sampling day in mid-December. Glycerol
151 ($p = 0.002$) and proline ($p= 0.016$) both had a significant strong positive correlation to decreasing
152 temperatures while trehalose had a non-significant correlation (Figure 5).

153 *Discussion:*

154 We found that new adult mountain pine beetles form their own metabolic antifreeze
155 compounds in response to autumn temperature cues. The most responsive metabolites to
156 increasing cold in adults were glycerol, trehalose, and proline, for all of which there is also
157 previous transcriptomic and proteomic evidence of biosynthesis during larval cold hardening
158 (Bonnett et al. 2012; Robert et al. 2016; Fraser et al. 2017). Mountain pine beetle larvae typically
159 only survive winter temperatures if insulated below the snow line, or if temperatures within the
160 bark do not fall below approximately –40□ (Safranyik and Carroll 2006; Bleiker et al. 2017).

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161 Studies that have referenced the presence of newly eclosed adult mountain pine beetles and that
162 tracked emergence rates have indicated that new adults have higher winter mortality rates
163 compared to their larval counterparts, but did not investigate potential mechanisms for this
164 reduced success (DeLeon et al. 1934; Amman 1973). We observed high mortality at our site
165 which we postulate is linked to several factors including site temperature regime, differing
166 physiology compared to larvae, and below-bark conditions.

167 Glycerol is a known cryoprotectant in many insects, including other *Dendroctonus* spp.,
168 and has been documented in mountain pine beetle larvae (Miller and Werner 1987; Bentz and
169 Mullins 1991; Régnière and Bentz 2007; Wang et al. 2016). It is a relatively inert compound that
170 can be maintained at high concentration without interfering with other cellular processes or
171 enzymatic reactions (Leather et al. 1995). It is also nontoxic, so insects experience few fitness
172 trade-offs when generating this compound, and it can be converted into glycogen when
173 temperatures begin to warm (Leather et al. 1995; Storey and Storey 2012; Fraser et al. 2017).
174 Our previous studies have shown that larvae increase their capacity to generate glycerol in
175 correlation to temperature similar to what we have observed here with adults (Bonnett et al.
176 2012; Robert et al. 2016; Fraser et al. 2017) (Figure 4a). Recent cold acclimation metabolite
177 work has shown that mountain pine beetle larvae produce a concentration of glycerol an order of
178 magnitude more per mg of tissue compared to the new adults profiled in our study (Batista pers.
179 comm.). This lower concentration of glycerol may have reduced the new adult beetles' ability to
180 supercool and thereby increased mortality rates.

181 Trehalose is a major sugar constituent of insect haemolymph that acts as a mobile energy
182 source for cellular respiration (Leather et al. 1995; Thompson 2003). We observed increasing
183 levels of trehalose in the mid- to late-fall, but no continual increase as temperatures grew cooler

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184 (Figure 4b). In European populations of *Ips typographus* (Coleoptera: Curculionidae), trehalose
185 undergoes a similar increase through October in response to cold temperatures (Koštál et al.
186 2011). Trehalose can also act as a cryoprotectant, stabilizing proteins at cold temperatures and
187 keeping cellular membranes intact (Feng et al. 2016). Increasing the durability of cellular
188 membranes would reduce cellular damage in the event of changing osmotic pressure or ice
189 crystal formation. Trehalose has also be linked to changing dietary cues for insects (Thompson
190 2003), meaning changing trehalose levels in the blood might lead to a reduction or cessation of
191 feeding behavior as temperatures cool, helping with voiding of the gut prior to onset of winter.

192 Proline is known to be a cryoprotectant in both plant and yeast cells (Pemberton et al.
193 2012) but is not well-documented as a cryoprotectant in insects. The increase of proline levels in
194 response to temperature within the new adults suggests a connection to cold acclimation in
195 mountain pine beetles (Figure 4c). In the red flat bark beetle, *Cujucus clavipes* (Coleoptera:
196 Cucujidae), proline and alanine are thought to work together with trehalose to slow the freezing
197 process (Carrasco et al. 2012). Alanine was detected in the new adults, though it did not correlate
198 with temperature over the duration of the study (supplementary Table 1). It is possible that new
199 mountain pine beetle adults use proline to decrease their supercooling point, though further study
200 would be needed to confirm this possibility. Proline is also metabolized along with carbohydrates
201 during flight (Gäde and Auerswald 2002; Teulier et al. 2016). Proline generation may serve a
202 dual purpose where beetles use the amino acid as a cryoprotectant in the winter and then
203 metabolize remaining proline as a flight fuel for dispersal.

204 On-site phenology drivers and additional metabolic demands on new adults are likely
205 contributing factors to the level of mortality observed in the field. While field temperatures
206 initially dropped at a steady rate from September to early October, they rewarmed between mid-

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207 October and November (Figure 3). The extended period of warmer weather may have confused
208 the cues that normally trigger the beetles to produce cryoprotectants. New adults feed on fungal
209 associates after eclosing but prior to emergence from under the bark (Safranyik and Carroll
210 2006); based on our observations of bark and phloem conditions, adequate food resources were
211 available to beetles on the study site. Adults beetles do, however, have different metabolic
212 demands compared to larvae. They must develop fat reserves to support flight and also maintain
213 gonadal tissue for reproduction (Gäde and Auerswald 2002; Six and Bracewell 2015). It should
214 be noted that adult females partition their metabolic resources to at least some extent – for
215 instance, they not generate vitellogenin until they come into contact with a host tree following
216 dispersal flight (Pitt et al. 2014). Larvae have not yet developed these tissues beyond imaginal
217 disks, and may thus have more resources to allocate to cold hardiness.

218 Ice crystals were observed within the galleries on the coldest sampling day. This may
219 have been a contributing factor to the increased mortality observed on the sampling site. Direct
220 contact of ice crystals on the surface of an insect's exoskeleton creates a surface where point
221 nucleation of ice can occur (Elnitsky et al. 2008; Bleiker et al. 2017). New adults are melanized
222 with hardened carapaces and have more surface area compared to larva. Having undergone
223 pupation, new adults also have thin legs that are liable to freeze faster due to their exposure. It is
224 probable that beetles with elevated levels of cryoprotectants still experienced internal ice crystal
225 formation due to the external ice contact. In addition, it is unknown if new adults are capable of
226 voiding their guts as larvae do in preparation for freezing temperatures (Keeling et al. 2013). If
227 they are not capable, new adults would have more internal surfaces for ice crystal formation due
228 to retained food, making them yet more susceptible to freezing.

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229 We found overwintering, newly eclosed mountain pine beetle adults prepare for
230 overwintering by producing three known antifreeze metabolites. Previous research suggests that
231 these are the same three major metabolites produced by larvae during cold acclimation (Bonnett
232 et al. 2012; Robert et al. 2016; Fraser et al. 2017), but it is likely newly eclosed adults produce
233 less of each cryoprotectant. This is the first time that a metabolic mechanism for new adult
234 survival has been documented. While the new adults in our study experienced high mortality,
235 larvae at nearby sites in Jasper National park were found to have survived the winter of 2016-
236 2017. New adult MPB have been most commonly described at high elevation, due to a
237 comparatively late start to spring and early start to cooling autumn temperatures (Amman 1973;
238 Bentz et al. 2016). Beetles that experience this extension in their life cycle are likely most
239 successful in areas with milder winters that do not reach the low freezing temperatures at our
240 study site. In areas where winters are long and have temperatures reaching below –30 □ it is
241 likely that new adult beetles that experience an extended life cycle will not be able successfully
242 overwinter. Our observation of high mortality further supports the original field records of lower
243 overwintering success in new adults (DeLeon et al. 1934; Amman 1973), and suggests both
244 metabolic and physical drivers. This work provides new parameters for modeling of the spread
245 of mountain pine beetles in their expanding geographic range and is evidence of physiological
246 plasticity in this insect that could be susceptible to natural selection for varied life cycle lengths
247 (longer or shorter) as moves into novel, colder regions or as other populations experience more
248 developmental degree days due to a warming climate.

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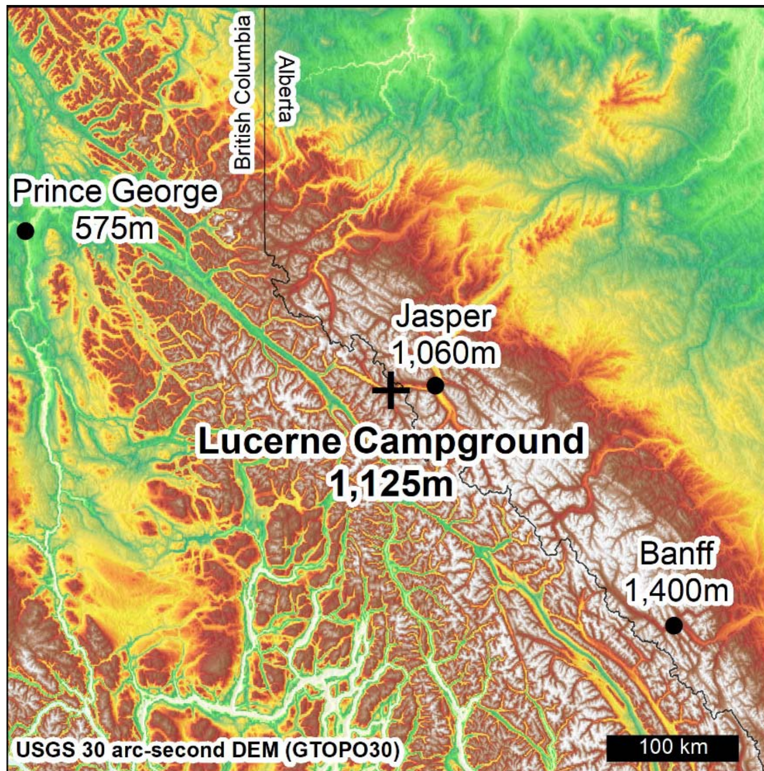
423 

424 Figure 1: Location of the study site (cross) in relation to the closest major urban centers (dots).
425 The sample site itself is located proximal to the Continental Divide of the Americas (latitude
426 52°50'59.48"N, longitude 118°34'21.84"W).

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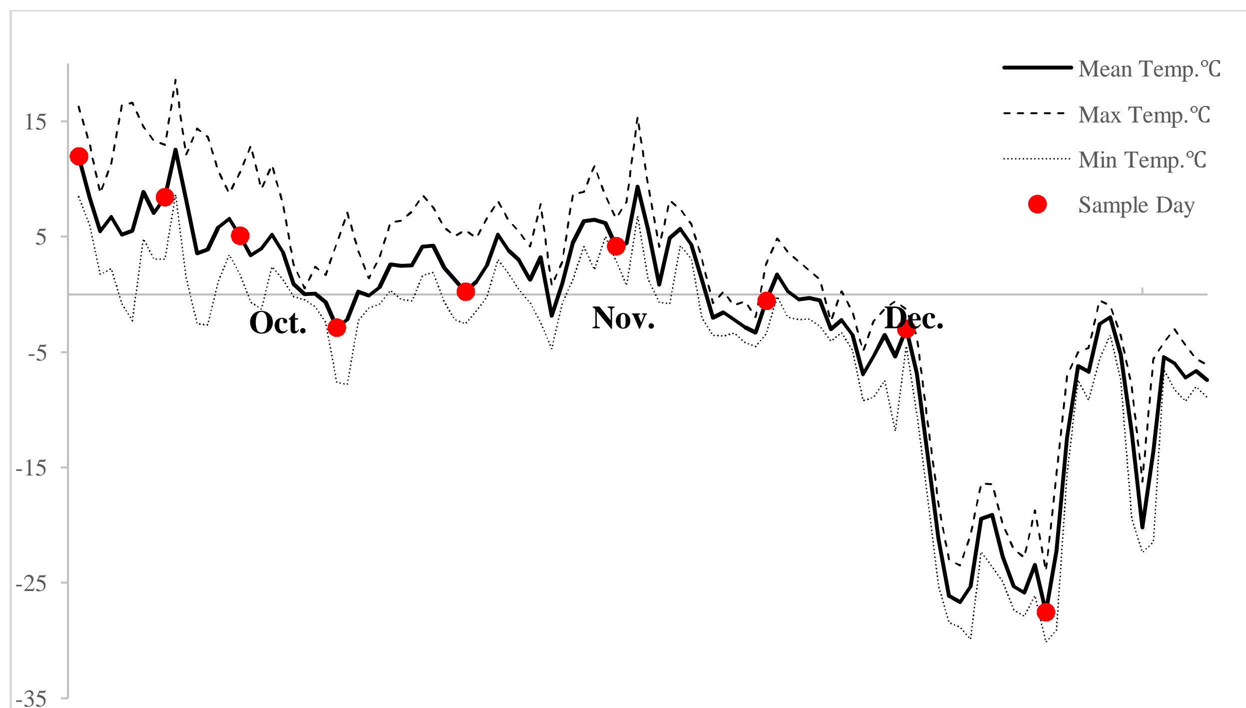

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442 Figure 2: Local temperatures taken from climate data loggers at Lucerne campground in Robson
443 Provincial Park from September to December 2016.

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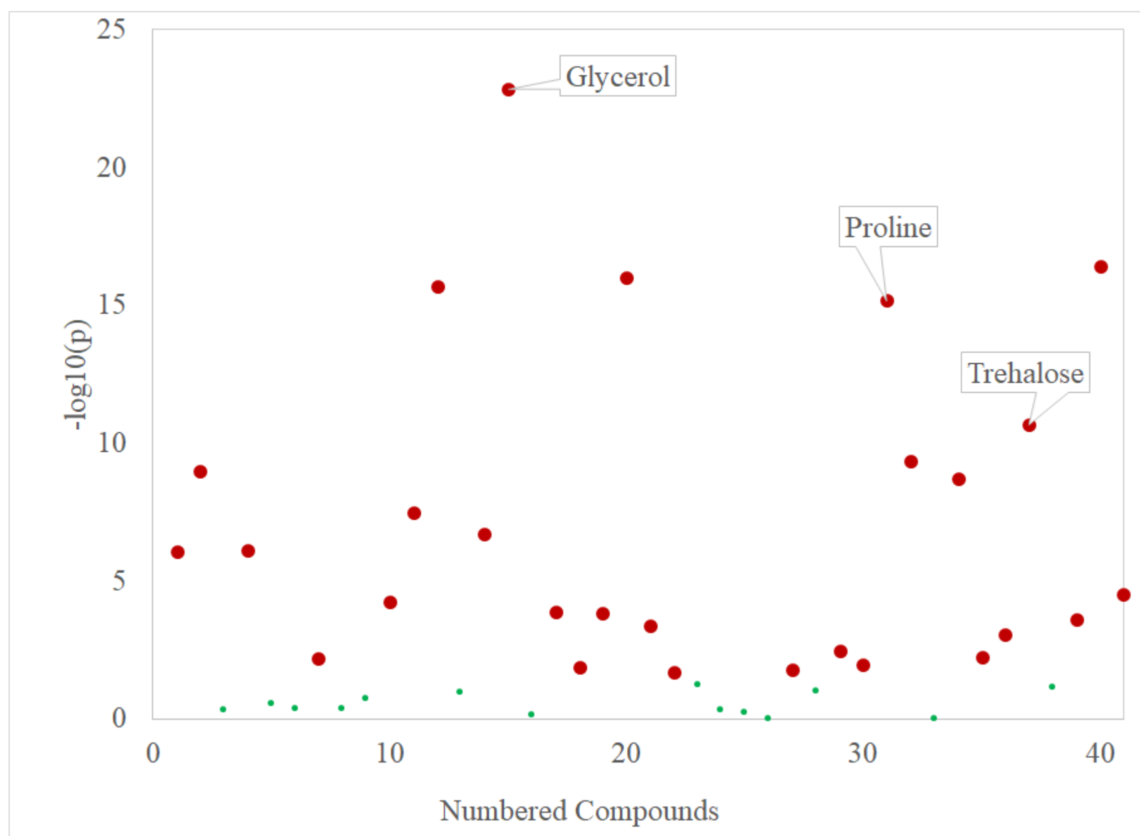

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451 Figure 3: One-way ANOVAs for all metabolites sampled. All points marked in red exhibited
452 significant differences between time points during the study while all points marked in green did
453 not vary significantly during the study (FDR=0.05, n=8 samples per time point).

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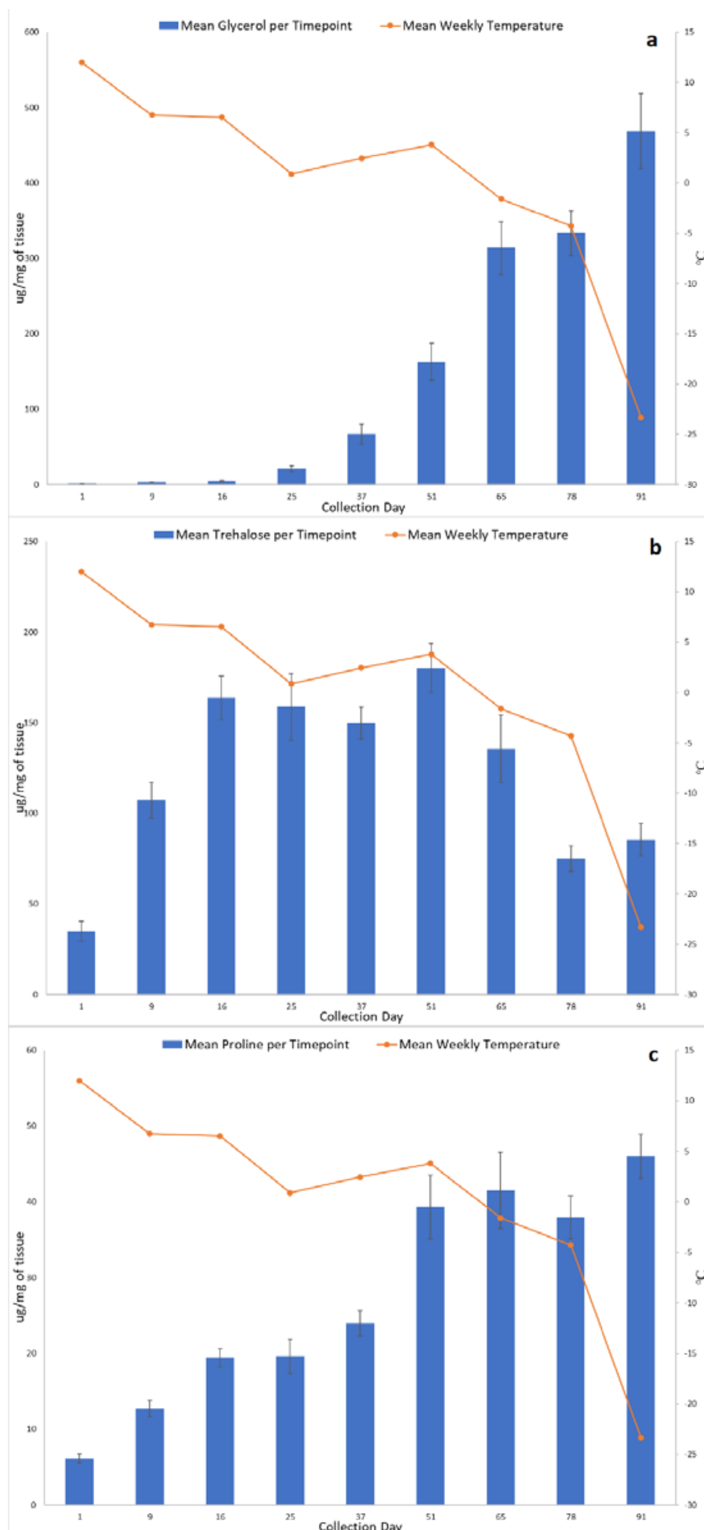

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460 Figure 4: Mean glycerol (a), trehalose (b), and proline (c) concentrations of new adult beetles
461 (n=8 samples per time point) in relation to ambient site temperature. Error bars show standard
462 error of the mean.

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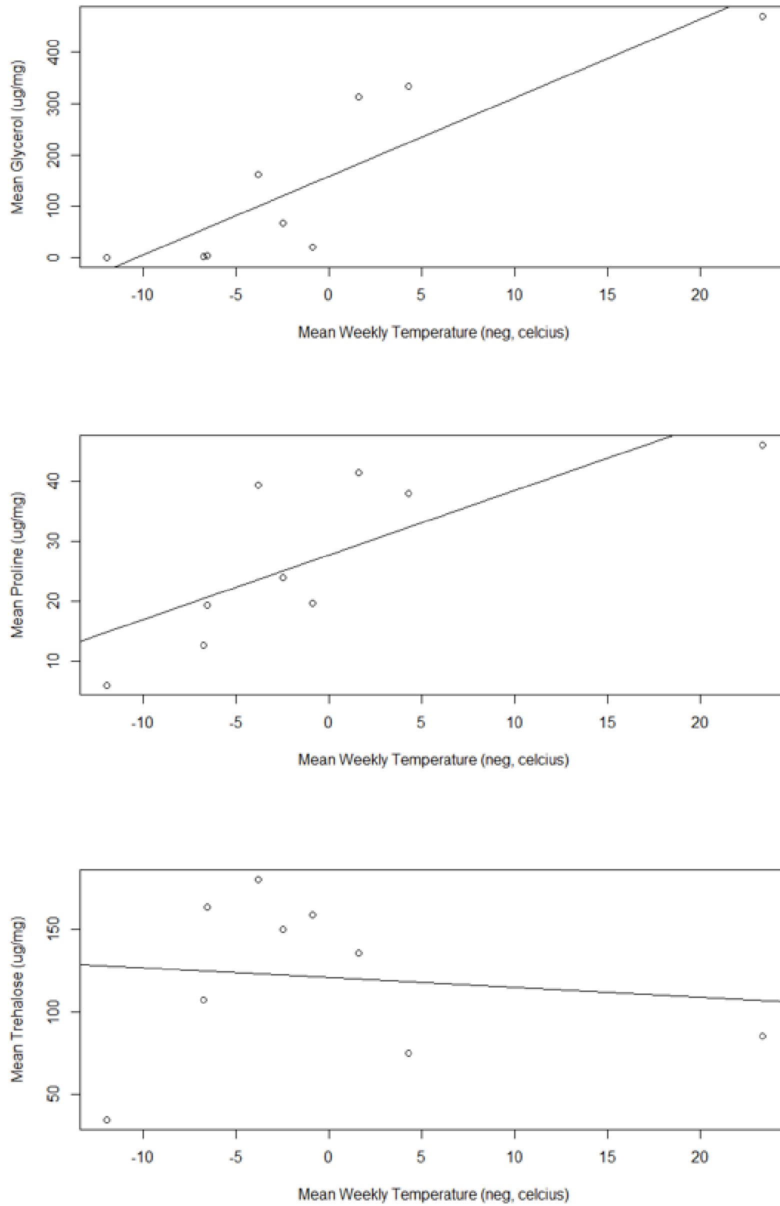

Figure 5: Pearson's product-moment correlation between mean metabolite concentration and negatively transformed temperature (□) over time (n=8 samples per timepoint, µg/mg of tissue). Mean glycerol concentration ($r = 0.873$, $p < 0.01$, $R^2 = 0.7615$) and mean proline concentration ($r = 0.767$, $p < 0.05$, $R^2 = 0.588$) were both significant while mean trehalose concentration ($r = 0.125$, $p > 0.05$, $R^2 = 0.0157$) was non-significant.