1   **Cold tolerance of mountain stoneflies (Plecoptera: Nemouridae) from the high Rocky**
2   **Mountains**
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31   **Running head:** Cold tolerance of mountain stoneflies
32

ABSTRACT

36

37 How aquatic insects cope with cold temperatures is poorly understood. This is particularly true

38 for high-elevation species that often experience a seasonal risk of freezing. In the Rocky

39 Mountains, nemourid stoneflies (Plecoptera: Nemouridae) are a major component of mountain

40 stream biodiversity and are typically found in streams fed by glaciers and snowfields, which due

41 to climate change, are rapidly receding. Predicting the effects of climate change on mountain

42 stoneflies is difficult because their thermal physiology is largely unknown. We investigated cold

43 tolerance of several alpine stoneflies (*Lednia tumana*, *Lednia tetonica*, and *Zapada* spp.) from

44 the Rocky Mountains, USA. We measured the supercooling point (SCP) and tolerance to ice

45 enclosure of late-instar nymphs collected from a range of thermal regimes. SCPs varied among

46 species and populations, with the lowest SCP measured for nymphs from an alpine pond, which

47 is much more likely to freeze solid in winter than flowing streams. We also show that *L. tumana*

48 cannot survive being enclosed in ice, even for short periods of time (less than three hours) at

49 relatively mild temperatures (-0.5 °C). Our results indicate that high-elevation stoneflies at

50 greater risk of freezing may have correspondingly lower SCPs, and despite their common

51 association with glacial meltwater, they appear to be living near their lower thermal limits.

INTRODUCTION

Freshwater habitats in cold regions typically experience winter ice cover and freezing temperatures that can be injurious or lethal to aquatic insects (Danks 2008). Resident species may avoid extreme cold by moving to warmer or more thermally stable microclimates or by modifying local conditions (e.g., through case-making; Danks 1971). Others withstand cold conditions *in situ* through supercooling—maintaining internal water in liquid form below its freezing point—or freeze tolerance (Danks 2008, Sinclair et al. 2015, Zachariassen 1985). Organisms living in wet environments are likely to encounter external ice when their body temperatures are already at or near freezing. Thus, inoculative freezing—in which contact with external ice induces the formation of internal ice—is likely the main driver of organismal freezing in these habitats (Frisbie & Lee Jr. 1997).

At high elevations, aquatic habitats are typically distributed across a mosaic of streams and ponds that are fed by an array of hydrological sources (e.g., glaciers, perennial snowfields, subterranean ice; Hotaling et al. 2017) and remain snow-covered for much of the year (Lencioni 2004). Mountain streams appear to not freeze solid because of their steep gradients and year-round flow, with minimum temperatures often close to 0 °C in the main channel (Giersch et al. 2017, Shah et al. 2017, Tronstad et al. in press). Resident taxa are therefore predicted to experience minimum temperatures of ~0 °C. This prediction rests on the assumption that overwintering nymphs remain in the unfrozen primary stream channel throughout the winter season. However, because of the inherent variation in aquatic habitats, nymphs inhabiting different regions of a stream (e.g., littoral zone, bed sediments) may experience subfreezing temperatures, even when the rest of the stream remains unfrozen (Lencioni 2004). Alpine ponds, in contrast, have little or no flow and are often shallow, making them susceptible to freezing

75   solid (Wissinger et al. 2016). As melt-induced flows are reduced in the future under climate

76   change (Huss & Hock 2018), headwater streams will become shallower with less flow velocity,

77   raising the risk of freezing in all seasons for resident organisms.

78   Stoneflies (Order Plecoptera) inhabit every continent except Antarctica and, when

79   present, often represent a substantial portion of aquatic biodiversity (DeWalt et al. 2015). After

80   hatching, stoneflies follow a two-stage life cycle with development through multiple instars as

81   aquatic, larval nymphs followed by emergence as winged, terrestrial adults. Whether

82   development occurs in a single year or spans multiple seasons, eggs and larvae of high-elevation

83   stoneflies are exposed to near freezing temperatures. Cold tolerance of stoneflies and of aquatic

84   insects broadly is poorly known, particularly for non-adult life stages (i.e., eggs or nymphs;

85   Danks 2008) and species living at high latitudes and elevations—where taxa are most likely to

86   experience freezing. Aquatic insects generally employ an array of physiological and biochemical

87   strategies to mitigate cold stress (reviewed by Lencioni et al. 2004). These include the production

88   of cryoprotectants to lower the temperature of internal ice formation (Duman et al. 2015),

89   cryoprotective dehydration where body water is lost to lower the amount available for freezing

90   (e.g., Gehrken & Sømme 1987), and the ability to resist anoxic conditions induced by ice

91   enclosure (e.g., Brittain & Nagell 1981). Eggs of the stonefly *Arcynopteryx compacta*

92   (Perlodidae) from the mountains of southern Norway avoid freezing by supercooling to -31 °C, a

93   trait mediated by the loss of approximately two-thirds of the eggs' water content (Gehrken 1989,

94   Gehrken & Sømme 1987). Nymphs of another high-latitude stonefly, *Nemoura arctica*

95   (Nemouridae), survive freezing to well below 0 °C by preventing intracellular ice formation

96   through the production of a xylomannan-based glycolipid, which inhibits inoculative freezing

97   through the inactivation of ice nucleating agents (Walters et al. 2009, 2011). However, it is

4

98   unlikely that glycolipid production represents a singular 'magic bullet' that confers freeze

99   tolerance in stoneflies, or aquatic insects broadly, given the diversity of known freeze protecting

100 molecules (Toxopeus & Sinclair 2018). Like *N. arctica*, high-alpine nemourids in North America

101 appear to overwinter as early-instar nymphs (Figure S1) and may be exposed to a similar risk of

102 freezing. How they cope with such risks, however, has not been investigated.

103   In this study, we investigated cold tolerance across populations of late-instar nymphs

104 from at least three species [*Lednia tumana* (Ricker 1952), *Lednia tetonica* Baumann & Call

105 2010, and *Zapada* spp.; Nemouridae)] in the northern Rocky Mountains, USA. For all

106 populations, we measured the dry supercooling point (SCP)—the temperature at which an

107 organism releases latent heat indicative of ice formation (Sinclair et al. 2015)—in the absence of

108 ice. Enclosure in ice may also be a major risk for mountain stoneflies, especially in slower

109 flowing shallow streams. Ice-enclosure can cause mortality through inoculative freezing,

110 hypoxia, mechanical damage, or a combination of factors (Conradi-Larsen & Sømme 1973). One

111 nemourid, *N. arctica*, can survive ice enclosure (Walters et al. 2009), but how widespread this

112 ability is among stoneflies is unknown. Thus, for one population from Lunch Creek, we also

113 tested whether nymphs could survive ice enclosure. Given the perennial and fast-flowing

114 conditions of alpine streams, we expected winter conditions to be largely constant with

115 temperatures near 0 °C and likelihood of freezing to generally be low. Therefore, we did not

116 expect stonefly SCPs to vary among stream-inhabiting populations. Conversely, because high

117 elevation pond-dwelling stoneflies likely experience a greater risk of freezing, we expected

118 resident nymphs to exhibit lower SCPs. Furthermore, given that *N. arctica* nymphs can survive

119 being enclosed in ice and that streams containing *Lednia* are commonly near 0 °C even in

120 summer (Figure S2), we expected *L. tumana* nymphs to survive ice enclosure. Our study

121 represents a preliminary step toward understanding how alpine stonefly cold tolerance varies at

122 broad taxonomic and environmental scales—i.e., across species, populations, and habitats—

123 setting the stage for future, more targeted studies of the group.

124

125   **MATERIALS AND METHODS**

126   Sampling and environmental data collection

127   For SCP measurements, we collected late-instar stonefly nymphs from six populations in

128 the Rocky Mountains between 29 July – 6 August 2018 (Figure 1; Tables 1, S1). To test whether

129 high-elevation nemourids could survive being enclosed in ice, we collected additional late-instar

130 *L. tumana* nymphs from Lunch Creek in Glacier National Park, MT, on 17 August 2019.

131 Nymphs were transferred in 1 L Whirl-Pak bags (Nasco) filled with streamwater from the

132 backcountry to the laboratory in ice-filled coolers. Transport duration depended on distance from

133 a trailhead, ranging from a few hours (Lunch Creek) to more than 24 hours (Mount Saint John).

134 Populations were sampled from two Rocky Mountain sub-ranges centered around Glacier and

135 Grand Teton National Parks (Figure 1). Due to the remote, rugged terrain and high seasonal

136 snowfall in the Rocky Mountains, we targeted our specimen collections to occur in late summer,

137 soon after most streams in our study area became free of seasonal snow. We identified specimens

138 based on morphology and collection localities following previous studies (e.g., Giersch et al.

139 2017). Unlike *Lednia*, multiple *Zapada* species can be present in the same stream, and therefore,

140 we cannot exclude the presence of more than one species in the Wind Cave population (Table 1).

141   We collected environmental data at each site to characterize the temperatures nymphs

142 experience in the wild and to categorize sites by habitat type (e.g., glacier-fed, snowmelt-fed,

143 groundwater-fed, or a high-elevation pond; see below). We recorded temperature hourly with

144   HOBO loggers (Onset Computer Corporation) placed within ~5 cm of the main channel

145   streambed. Lengths of logger deployments ranged from less than 24 hours (Mount Saint John,

146   Tetonica Pond) to a full calendar year (Lunch Creek, Skillet Glacier, Wind Cave). Using these

147   data, we constructed 24-hour thermographs for each site on a representative day in late July. Our

148   focal 24-hour period was 31 July 2019 for all sites except Lunch Creek (31 July 2014) and Wind

149   Cave (28 July 2019). For the focal period, we measured the lowest (T_{MIN}) and mean (T_{MEAN})

150   temperatures. We also measured specific conductivity (C) with a YSI Professional ProPlus

151   multiparameter probe, which was calibrated at the trailhead before each trip. Specific

152   conductivity reflects ionic concentration in the water and is elevated in water that interacts with

153   inorganic debris (e.g., streams fed by debris-covered rock glaciers). We assessed stream channel

154   stability via a modified version of the Pfankuch Index (PI, Peckarsky et al. 2014). We estimated

155   when sites became ice-free in 2018 using satellite imagery (Copernicus Sentinel 2).

156   We classified sites into one of four habitat types based on a combination of primary

157   hydrological source, environmental variation, geomorphology, and field surveys. Habitat types

158   included: streams fed by a surface glacier (e.g., "glacier-fed"), streams fed by a perennial

159   snowfield ("snowmelt-fed"), streams emanating from subterranean ice (e.g., rock glaciers, "icy

160   seep"; Hotaling et al. 2019, Tronstad et al. in press), and slow-flowing, alpine ponds ("pond"),

161   identified by their low-angle profile and appearance of standing water. To discern among the

162   three stream types, we relied on geographic data paired with the environmental signature of

163   primary hydrological sources—glaciers, snowfields, or subterranean ice masses. Glacier-fed

164   streams were identified by four criteria: named glaciers feeding them, low minimum

165   temperatures ($T_{MIN} < 3\ °C$), low C ($< 10\ \mu S\ cm^{-1}$), and high PI (> 25, indicating more unstable

166   stream channels). Streams were classified as snowmelt-fed if a permanent snowfield was visibly

167 feeding them, they exhibited higher minimum temperatures ($T_{MIN} > 3$ °C), moderate C (10-50 μS

168 cm^{-1}), and a moderately stable stream channel (PI = 18-25). We categorized streams as icy seeps

169 if they were consistently low (e.g., $T_{MEAN} \leq 3$ °C) yet had no named glaciers or significant

170 perennial snowfields feeding them, a classic "lobe" of a rock glacier was visible (e.g., Mount

171 Saint John), and they exhibited moderate to high C for mountain streams (> 25 μS cm^{-1}).

172

Measuring supercooling points

174   In the laboratory, nymphs were held at 3 °C with no access to food for 12-72 hours

175 (Table S1). For each SCP measurement, we blotted nymphs with Kimwipes (Kimberly Clark)

176 and placed them in dry 1.5 mL microcentrifuge tubes. A 30-gauge T-type thermocouple (2 mm

177 soldered tip) was held in contact with each nymph's abdomen by a piece of cotton wedged into

178 the tube. Thermocouples were attached to 4-channel loggers (UX-120, ± 0.6 °C accuracy, Onset

179 Corporation). Up to 12 microcentrifuge tubes were placed in an aluminum block in contact with

180 a thermoelectric cooler, attached to a heat sink immersed in a glycol bath on the opposite face.

181 We used a custom temperature controller to decrease the temperature at ~0.5-1 °C per minute.

182 We estimated the SCP as the lowest recorded temperature before observing the transient increase

183 in temperature as the body released latent heat and started to freeze. After SCPs were recorded

184 for all nymphs in a given experimental group, we promptly removed them from the experimental

185 bath. Nymphs were clearly dead upon removal from the microcentrifuge tubes after the SCP

186 tests, with no movement for several minutes and no response to probing. We subsequently

187 measured body length to the nearest millimeter for all specimens with a microscope and ocular

188 scale.

189

190   Testing tolerance to ice enclosure

191   We assessed whether late-instar *L. tumana* nymphs could survive being enclosed in ice, a

192   potential environmental risk factor in high-elevation streams and ponds. After collection,

193   nymphs were maintained at 5 ℃ in the laboratory for 72 hours with no access to food. We then

194   placed a single nymph in each well of an ice-cube tray with approximately 22 mL of

195   streamwater. Nymphs were assigned to one of three treatments: -0.5 ℃ (freezing, $N = 8$), 0.1 ℃

196   (near freezing, $N = 13$), or control (5 ℃, $N = 6$). Thermocouples were inserted into the wells to

197   monitor temperature. Once the streamwater reached the desired temperature, we began the

198   treatment which lasted for three hours. Following the experiment, we placed nymphs at 5 °C to

199   recover for seven hours. After the recovery period, we assessed survival by observation and

200   gentle prodding.

201

202   Statistical analyses

203   We performed all analyses in R (R Core Team 2017). We first conducted an ANOVA to

204   assess the effects of body length on SCP across all species. Next, to test if there were differences

205   in SCP across populations, we conducted an ANOVA using population as the predictor variable

206   and SCP as the response variable. A Tukey HSD correction for multiple comparisons was used

207   to compare the mean SCP for each pair of streams. We used the same approach (ANOVA with

208   Tukey HSD correction) to estimate the effect of habitat type (glacier-fed, snowmelt-fed, icy seep,

209   or pond) or species (*L. tumana*, *L. tetonica*, or *Zapada* spp.) on SCP. Finally, we tested for

210   population-specific variation in SCP for *L. tetonica*. We included body length as a covariate in

211   all models to account for body size effects.

212

RESULTS

Environmental variation and habitat types

215   The sites sampled in this study represented four habitat types (Table 1). Tetonica Pond

216   was the only site that lacked steep, flowing water, and thus was the only 'pond' we included. We

217   also sampled two glacier-fed streams (Cloudveil Dome, Skillet Glacier outlet), two icy seeps

218   (Wind Cave, Mount Saint John), and one snowmelt-fed stream (Lunch Creek). Over the focal

219   24-hour period, minimum temperature was lowest at Cloudveil Dome (T_{MIN} = 1.8 °C) and

220   highest at Lunch Creek (T_{MIN} = 4.2 °C; Table 1). Mean temperature followed the same pattern

221   with Cloudveil Dome the coldest (T_{MEAN} = 2.0 °C) and Lunch Creek the warmest (T_{MEAN} = 6.2

222   °C; Table 1). For all sites except Wind Cave, seasonal snow cover persisted well into July (Table

223   S1). For Lunch Creek, Wind Cave, and Skillet Glacier, full-year thermographs indicate that the

224   main stream channel remains unfrozen year-round (Figure S2), but whether this lack of freezing

225   extends to the streambed and margins is unknown.

226

227   Supercooling points

228   Across all species, populations, and habitats, the mean SCP was -6.5 ± 1.7 °C (Table 1).

229   Larger nymphs had higher SCPs than smaller nymphs ($F_{(1,101)}$ = 7.14, P = 0.009; Figure S3); we

230   therefore included body length as a covariate in our statistical models. We found significant

231   differences in SCPs among species ($F_{(2,109)}$ = 3.25, P = 0.043; Figure 2A), populations ($F_{(7,95)}$ =

232   4.528, P, ANOVA < 0.001), and habitats ($F_{(4,98)}$ = 4.556, P = 0.002). Within a single species, *L.*

233   *tetonica*, the SCP varied significantly among habitats ($F_{(3,62)}$ = 10.4, P < 0.001; Figure 2B).

234   Individuals from Tetonica Pond had the lowest mean SCP overall (-7.5 ± 1.3°C, Table 1) and

235   those from glacier-fed streams had the highest (Table 1).

236

237   Tolerance to ice enclosure

238   No *L. tumana* nymphs (N = 0 of 8) in the freezing treatment (-0.5 °C) survived being

239   entirely enclosed in ice. In the near-freezing treatment (0.1 °C), ~84% of nymphs (N = 7 of 8)

240   survived. A thin layer of ice formed on the surface of the well during the treatment but the

241   remainder of the streamwater beneath it containing each nymph did not freeze. All nymphs

242   survived the 5 °C control treatment (N = 6 of 6).

243

244   **DISCUSSION**

245   Stoneflies are a major component of aquatic biodiversity in mountain ecosystems yet

246   little is known of their ability to cope with low temperatures, particularly as it relates to potential

247   freezing stress. In this study, we measured supercooling points (SCPs) and tolerance to ice

248   enclosure for high-elevation stonefly nymphs in the Rocky Mountains to link their physiological

249   traits to the thermal regimes they experience. Our SCP estimates aligned with those described for

250   other aquatic insects (-3.3° to -7.4 °C; Moore & Lee Jr 1991) and varied by species, population,

251   and habitat. Because SCPs are typically higher for aquatic insects in summer versus winter, the

252   values observed in our study likely reflect maximum dry SCPs for our focal species (Moore &

253   Lee Jr 1991). Within *L. tetonica*, the alpine pond population exhibited lower SCPs than those

254   from icy seeps (and the lowest mean SCP of any population, -7.5 ± 1.3 °C), which in turn had

255   lower SCPs than those from glacier-fed streams. Based on our results, we cannot disentangle the

256   mechanism driving these patterns of cold tolerance (i.e. plasticity or local adaptation), but a

257   correlation between thermal regime and SCP appears to exist. Although hydrologically

258   connected to steep, fast-flowing streams, the small size, shallowness, and extremely slow flow of

259   Tetonica Pond likely elevates winter freezing risk for resident taxa; alpine ponds in the Rocky

260   Mountains often freeze solid in winter (Wissinger et al. 2016). Snow cover persisted on Tetonica

261   Pond until mid-July in 2018 (Table S1), suggesting it also may have been frozen late into the

262   summer. An alternative explanation for the variation in SCPs we observed across habitats is the

263   potential for differences in gut contents given the range of holding periods (12-72 hours, Table

264   S1). Because gut contents can include efficient ice nucleating agents (and raise SCPs; Danks,

265   2008), we would expect higher SCPs for populations that were held for the shortest amount of

266   time if this was confounding our results. However, Tetonica Pond had one of the shortest holding

267   periods (12 hours) yet the lowest SCP (Tables 1, S1).

268   Because we tested SCPs in the absence of ice, it is likely that the inoculative freezing

269   temperatures of our focal species are much higher than the values we report. For example, when

270   in contact with ice, the SCP of *N. arctica* is -1.5 °C, but in its absence, *N. arctica* supercools to -

271   7.8 °C (Walters et al. 2009), a value that aligns with the range we observed (-7.5 to -5.2 °C;

272   Table 1). Our ice enclosure experiment results also support the potential for higher inoculative

273   freezing temperatures for *L. tumana* nymphs in contact with ice. Indeed, nymphs collected in late

274   August did not survive being enclosed in ice, even at temperatures just below freezing (-0.5 °C).

275   It is unclear, however, if the nymphs did not survive due to inoculative freezing or something

276   else (e.g., internal mechanical damage or hypoxia). Nevertheless, a similar result was found for

277   *N. arctica*. In mid-August, less than 20% of *N. arctica* nymphs survived a two-hour exposure to -

278   1.5°C. Yet, by late September when stream temperatures are colder and closer to 0°C, more than

279   80% survived a 7-day exposure to -6.2 °C, suggesting the presence of plasticity in cold tolerance

280   (Walters et al. 2009). Although testing for seasonal plasticity in freeze tolerance was beyond the

281   scope of our study, when typical summer conditions prevail (e.g., $T_{MEAN} > 5°C$), *L. tumana*

12

282   nymphs from Lunch Creek are intolerant of even mild subzero temperatures. But unlike the

283   Chandalar River in Alaska, where *N. arctica* resides and temperatures reach -10 °C at the

284   sediment-water interface in winter, streams in the high Rocky Mountains appear to rarely, if

285   ever, fall below 0 °C. Indeed, for the streams in this study for which we have full year

286   thermographs (Figure S2), only Lunch Creek approached 0 °C at any point during the year. This

287   thermal buffering likely reflects high levels of insulating snow in the region. However, our

288   reported temperatures reflect the thermal conditions of the main stream channel. At stream

289   margins, where flows are slower and less water is available for thermal buffering, temperatures

290   may be quite different.

291   In this study, we showed that SCPs vary among species, populations, and habitat types

292   for stonefly nymphs inhabiting Rocky Mountain headwaters. The population most likely to

293   experience winter freezing stress also exhibited the lowest SCP, suggesting a potential ecological

294   role for SCP in freeze avoidance among alpine stoneflies. Our results, of course, apply only to

295   nymphs, one of three life stages in stoneflies: eggs, nymphs (i.e., larvae), and adults. We focused

296   on nymphs because this stage is the key period when most growth occurs. High-elevation

297   nemourid stoneflies appear to spend at least one winter as larvae (Figure S1), and statistical

298   models indicate that species' vulnerability to climate change can be greatly underestimated when

299   the larval period is overlooked (Levy et al. 2015). With evidence across stonefly species for

300   cryoprotective dehydration greatly lowering the freezing point of eggs (Gehrken & Sømme

301   1987), the ability for nymphs to survive being enclosed in ice (Walters et al. 2009), and adults

302   that emerge in winter (e.g., Bouchard Jr. et al. 2009), it is clear that stoneflies can survive

303   freezing conditions across all life stages. However, the degree to which freeze tolerance is

304   common or rare, and how cold tolerance varies across life stages within species are key questions

305   for future study.

306   As climate change proceeds, glaciers and perennial snowfields are receding, driving

307   reduced mountain streamflow (Hotaling et al. 2017) and habitat reductions for high-elevation

308   aquatic invertebrates (Domisch et al. 2011, Muhlfeld et al. 2020). Loss of meltwater, higher

309   ambient temperatures, and rapid contemporary stream warming (e.g., Niedrist & Füreder 2020)

310   are raising the risks for headwater biodiversity, including many stoneflies that are considered

311   cold-water stenotherms (de Figueroa et al. 2010, Domisch et al. 2011, but see Hotaling et al.

312   2020). Reduced streamflow will lower the thermal buffering capacity of streamwater (Shah et al.

313   2020) and, despite warmer winter temperatures, it will also increase the potential for freezing

314   stress (Williams et al. 2015). This effect will likely be compounded by decreasing levels of

315   insulating snowpack as more winter precipitation falls as rain instead of snow (e.g., Huntington

316   et al. 2004). Specifically, if snowpack accumulates later or melts off earlier, freezing risk in

317   transitional periods between seasons (e.g., November and June in the Northern Hemisphere) may

318   increase. Thus, a greater risk of freezing may represent an overlooked climate change threat to

319   alpine aquatic biodiversity. Although only two stoneflies are known to actively mitigate freezing

320   stress during non-adult stages (Gehrken 1989, Walters et al. 2009), it is unclear if this reflects

321   general stonefly biology or a lack of investigation.

322

331

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422   **TABLES**

423   Table 1. Environmental variation and habitat types included in this study. T_{MIN} and T_{MEAN}: the

424   minimum and mean temperatures observed for each site, respectively. C: specific conductivity

425   (μS cm^{-1}); PI: Pfankuch Index, a measure of stream channel stability (higher values correspond

426   to a less stable streambed); SCP: mean and standard deviation of the supercooling point. The

427   focal 24-hour period for temperature measurement was 31 July 2019 for all sites except Lunch

428   Creek (31 July 2014) and Wind Cave (28 July 2019). All temperatures (T_{MIN}, T_{MEAN}, SCP) are in

429   degrees Celsius. All sites are in Grand Teton National Park except Lunch Creek (Glacier

430   National Park).

Population	Taxon	Type	T_{MIN}	T_{MEAN}	C	PI	SCP
Lunch Creek (LC)	*L. tumana*	Snowmelt	4.2	6.2	40.8	25	-5.9 ± 2.0
Wind Cave (WC)	*Zapada* spp.	Icy seep	2.6	2.8	101.1	18	-7.2 ± 2.0
Mt. Saint John (MSJ)	*L. tetonica*	Icy seep	2.4	3.0	25.0	34	-5.8 ± 0.7
Cloudveil Dome (CD)	*L. tetonica*	Glacier-fed	1.8	2.0	4.1	32	-7.0 ± 0.9
Skillet Glacier (SG)	*L. tetonica*	Glacier-fed	2.7	4.4	3.1	34	-5.2 ± 1.1
Tetonica Pond[a] (TP)	*L. tetonica*	Pond	2.6	3.1	29.3	n/a	-7.5 ± 1.3

431   [a] Named by the authors. Does not reflect official conventions.
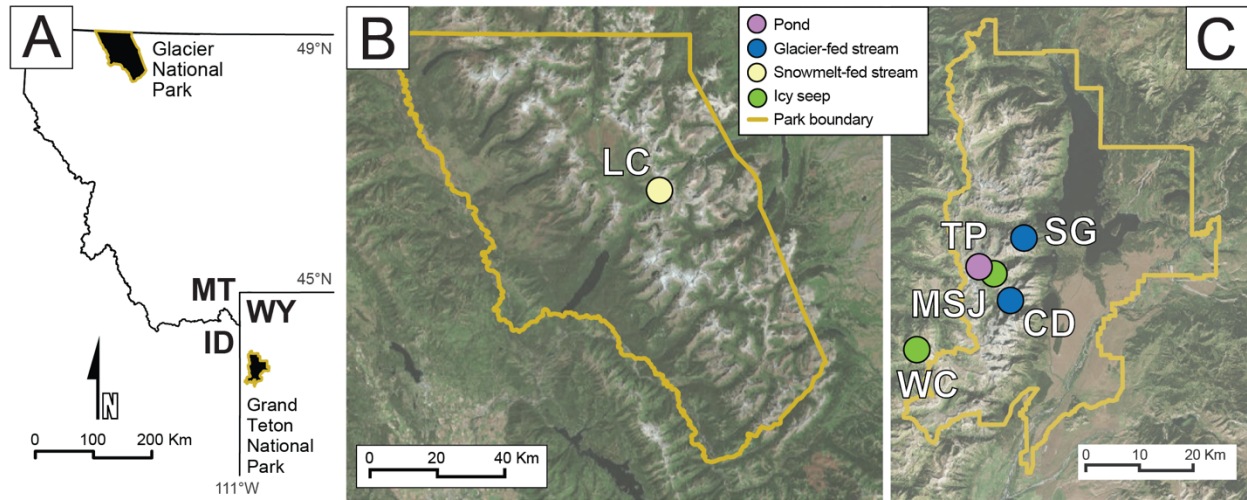
FIGURES

434
435
436   Figure 1. Populations and habitat types included in this study. A) Locations of the focal ranges in

437   the Rocky Mountains, B) Glacier National Park, Montana, USA, and C) Grand Teton National

438   Park and surrounding mountains, Wyoming, USA. See Table 1 for complete population names.
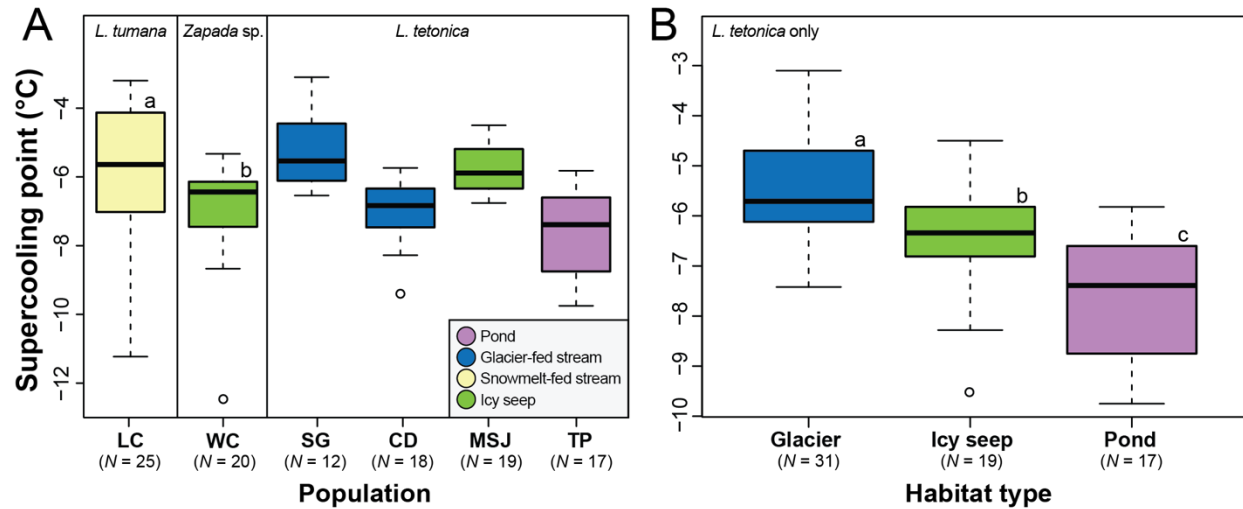
Figure 2. Box-and-whisker plots of the supercooling point (SCP) for alpine stonefly nymphs in the Rocky Mountains grouped by (A) species and population and (B) habitat type for *Lednia tetonica* only. In each plot, groups of lower-case letters are significantly different at Tukey's $P <$ 0.05 (see Table S2 for detailed statistics). Dark lines represent median values in the data. Upper and lower bounds of the box are the upper and lower quartiles, respectively. Outliers are indicated by open circles. Population acronyms: Lunch Creek (LC), Wind Cave (WC), Skillet Glacier (SG), Cloudveil Dome (CD), Mount Saint John (MSJ), and Tetonica Pond (TP).