

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

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4 Scott Hotaling^{1,*}, Alisha A. Shah^{2,*}, Michael E. Dillon³, J. Joseph Giersch⁴, Lusha M. Tronstad⁵,
5 Debra S. Finn⁶, H. Arthur Woods⁷, and Joanna L. Kelley⁸

6
7 **Affiliations:**

8 ¹ School of Biological Sciences, Washington State University, Pullman, WA, USA

9 ² Division of Biological Sciences, University of Montana, Missoula, MT, USA

10 ³ Department of Zoology and Physiology and Program in Ecology, University of Wyoming,
11 Laramie, WY, USA; michael.dillon@uwyo.edu

12 ⁴ U.S. Geological Survey, Northern Rocky Mountain Science Center, West Glacier, MT, USA;
13 jgiersch@usgs.gov

14 ⁵ Wyoming Natural Diversity Database, University of Wyoming, Laramie, WY, USA;
15 tronstad@uwyo.edu

16 ⁶ Department of Biology, Missouri State University, Springfield, MO, USA;
17 DFinn@MissouriState.edu

18 ⁷ Division of Biological Sciences, University of Montana, Missoula, MT, USA;
19 art.woods@umontana.edu

20 ⁸ School of Biological Sciences, Washington State University, Pullman, WA, USA;
21 joanna.l.kelley@wsu.edu

22 * Contributed equally

23
24 **Correspondence:**

25 Scott Hotaling, School of Biological Sciences, Washington State University, Pullman, WA,
26 99164; Email: scott.hotaling@wsu.edu; Phone: (828) 507-9950; ORCID: 0000-0002-5965-0986

27
28 Alisha A. Shah, Division of Biological Sciences, University of Montana, Missoula, MT, 59812,
29 USA, Email: alisha.shah@mso.umt.edu; Phone: (512) 694-7532; ORCID: 0000-0002-8454-7905

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31 **Running head:** Cold tolerance of mountain stoneflies

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ABSTRACT

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.

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INTRODUCTION

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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).

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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

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.

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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 ($T_{\text{MIN}} < 3\text{ }^{\circ}\text{C}$), low C ($< 10\text{ }\mu\text{S cm}^{-1}$), 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 ($T_{\text{MIN}} > 3 \text{ }^{\circ}\text{C}$), moderate C (10-50 μS
168 cm^{-1}), and a moderately stable stream channel ($\text{PI} = 18\text{-}25$). We categorized streams as icy seeps
169 if they were consistently low (e.g., $T_{\text{MEAN}} \leq 3 \text{ }^{\circ}\text{C}$) 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 ($> 25 \mu\text{S cm}^{-1}$).

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173 Measuring supercooling points

174 In the laboratory, nymphs were held at $3 \text{ }^{\circ}\text{C}$ 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, $\pm 0.6 \text{ }^{\circ}\text{C}$ 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 $\sim 0.5\text{-}1 \text{ }^{\circ}\text{C}$ 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.

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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 °C 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 °C (freezing, $N = 8$), 0.1 °C
196 (near freezing, $N = 13$), or control (5 °C, $N = 6$). 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.

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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.

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RESULTS

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Environmental variation and habitat types

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Supercooling points

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The sites sampled in this study represented four habitat types (Table 1). Tetonica Pond was the only site that lacked steep, flowing water, and thus was the only ‘pond’ we included. We also sampled two glacier-fed streams (Cloudveil Dome, Skillet Glacier outlet), two icy seeps (Wind Cave, Mount Saint John), and one snowmelt-fed stream (Lunch Creek). Over the focal 24-hour period, minimum temperature was lowest at Cloudveil Dome ($T_{\text{MIN}} = 1.8 \text{ }^{\circ}\text{C}$) and highest at Lunch Creek ($T_{\text{MIN}} = 4.2 \text{ }^{\circ}\text{C}$; Table 1). Mean temperature followed the same pattern with Cloudveil Dome the coldest ($T_{\text{MEAN}} = 2.0 \text{ }^{\circ}\text{C}$) and Lunch Creek the warmest ($T_{\text{MEAN}} = 6.2 \text{ }^{\circ}\text{C}$; Table 1). For all sites except Wind Cave, seasonal snow cover persisted well into July (Table S1). For Lunch Creek, Wind Cave, and Skillet Glacier, full-year thermographs indicate that the main stream channel remains unfrozen year-round (Figure S2), but whether this lack of freezing extends to the streambed and margins is unknown.

Across all species, populations, and habitats, the mean SCP was $-6.5 \pm 1.7 \text{ }^{\circ}\text{C}$ (Table 1). Larger nymphs had higher SCPs than smaller nymphs ($F_{(1,101)} = 7.14$, $P = 0.009$; Figure S3); we therefore included body length as a covariate in our statistical models. We found significant differences in SCPs among species ($F_{(2,109)} = 3.25$, $P = 0.043$; Figure 2A), populations ($F_{(7,95)} = 4.528$, P , ANOVA < 0.001), and habitats ($F_{(4,98)} = 4.556$, $P = 0.002$). Within a single species, *L. tetonica*, the SCP varied significantly among habitats ($F_{(3,62)} = 10.4$, $P < 0.001$; Figure 2B). Individuals from Tetonica Pond had the lowest mean SCP overall ($-7.5 \pm 1.3^{\circ}\text{C}$, Table 1) and those from glacier-fed streams had the highest (Table 1).

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Tolerance to ice enclosure

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DISCUSSION

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No *L. tumana* nymphs ($N = 0$ of 8) in the freezing treatment (-0.5 °C) survived being entirely enclosed in ice. In the near-freezing treatment (0.1 °C), ~84% of nymphs ($N = 7$ of 8) survived. A thin layer of ice formed on the surface of the well during the treatment but the remainder of the streamwater beneath it containing each nymph did not freeze. All nymphs survived the 5 °C control treatment ($N = 6$ of 6).

Stoneflies are a major component of aquatic biodiversity in mountain ecosystems yet little is known of their ability to cope with low temperatures, particularly as it relates to potential freezing stress. In this study, we measured supercooling points (SCPs) and tolerance to ice enclosure for high-elevation stonefly nymphs in the Rocky Mountains to link their physiological traits to the thermal regimes they experience. Our SCP estimates aligned with those described for other aquatic insects (-3.3° to -7.4 °C; Moore & Lee Jr 1991) and varied by species, population, and habitat. Because SCPs are typically higher for aquatic insects in summer versus winter, the values observed in our study likely reflect maximum dry SCPs for our focal species (Moore & Lee Jr 1991). Within *L. tetonica*, the alpine pond population exhibited lower SCPs than those from icy seeps (and the lowest mean SCP of any population, -7.5 ± 1.3 °C), which in turn had lower SCPs than those from glacier-fed streams. Based on our results, we cannot disentangle the mechanism driving these patterns of cold tolerance (i.e. plasticity or local adaptation), but a correlation between thermal regime and SCP appears to exist. Although hydrologically 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., $T_{\text{MEAN}} > 5^{\circ}\text{C}$), *L. tumana*

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.

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TABLES

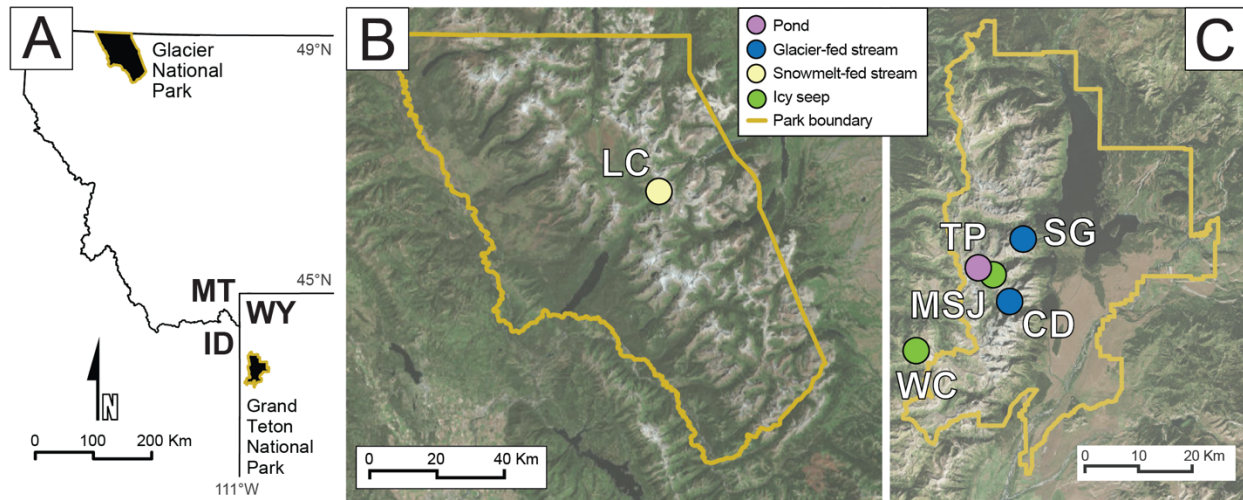
423 Table 1. Environmental variation and habitat types included in this study. T_{MIN} and T_{MEAN} : the
424 minimum and mean temperatures observed for each site, respectively. C: specific conductivity
425 ($\mu\text{S cm}^{-1}$); PI: Pfanckuch Index, a measure of stream channel stability (higher values 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)	<i>L. tumana</i>	Snowmelt	4.2	6.2	40.8	25	-5.9 ± 2.0
Wind Cave (WC)	<i>Zapada</i> spp.	Icy seep	2.6	2.8	101.1	18	-7.2 ± 2.0
Mt. Saint John (MSJ)	<i>L. tetonica</i>	Icy seep	2.4	3.0	25.0	34	-5.8 ± 0.7
Cloudveil Dome (CD)	<i>L. tetonica</i>	Glacier-fed	1.8	2.0	4.1	32	-7.0 ± 0.9
Skillet Glacier (SG)	<i>L. tetonica</i>	Glacier-fed	2.7	4.4	3.1	34	-5.2 ± 1.1
Tetonica Pond ^a (TP)	<i>L. tetonica</i>	Pond	2.6	3.1	29.3	n/a	-7.5 ± 1.3

431 ^a Named by the authors. Does not reflect official conventions.

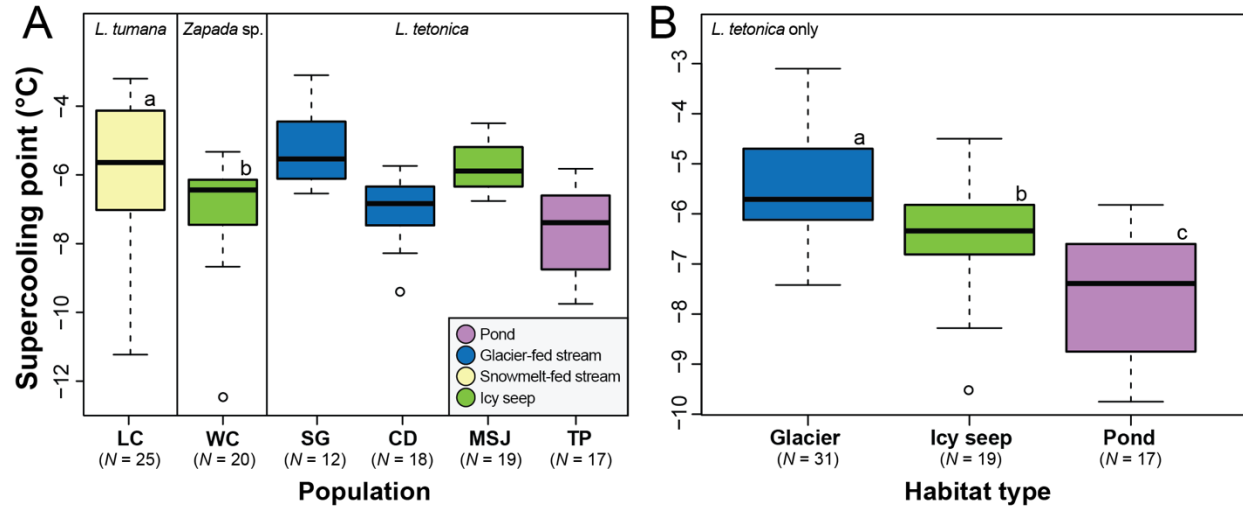
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FIGURES



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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.



439

440 Figure 2. Box-and-whisker plots of the supercooling point (SCP) for alpine stonefly nymphs in
441 the Rocky Mountains grouped by (A) species and population and (B) habitat type for *Lednia*
442 *tetonica* only. In each plot, groups of lower-case letters are significantly different at Tukey's $P <$
443 0.05 (see Table S2 for detailed statistics). Dark lines represent median values in the data. Upper
444 and lower bounds of the box are the upper and lower quartiles, respectively. Outliers are
445 indicated by open circles. Population acronyms: Lunch Creek (LC), Wind Cave (WC), Skillet
446 Glacier (SG), Cloudveil Dome (CD), Mount Saint John (MSJ), and Tetonica Pond (TP).