Freeze tolerance in larvae of the winter-active *Diamesa mendotae* Muttkowski (Diptera: Chironomidae)

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INTRODUCTION

There are two commonly recognized cold-hardiness strategies used by insects for surviving subzero temperatures: freeze avoidance (or intolerance) and freeze tolerance (Salt, 1961; Baust & Rojas, 1985). Most cold-hardiness studies ignore the aquatic stages of aquatic insects presumably because these insects are buffered in an aqueous environment and face less extreme conditions. However, many lotic aquatic habitats also freeze or become supercooled resulting in exposure to ice-crystal formation (i.e. frazil ice) within the supercooled fluid. Previous studies have estimated that *D. mendotae* adults have a mean SCP of ~20°C and mortality occurs near their SCP (Bouchard et al., in press), indicating that this species is freeze intolerant. In contrast to adults, the few cold hardiness studies involving larvae of other chironomid species suggest that this life stage uses freeze tolerance as a strategy for survival at subzero temperatures. In the present study, we estimated the SCPs of larvae, pupae, and adults of *D. mendotae* and the lower lethal temperatures (LLT) for larvae. To our knowledge, this is first time SCPs are reported for aquatic pupae and the first time SCPs and LLTs are reported for larvae in the subfamily Diamesinae.

Fig. 1: Trout Brook

METHODS

Collection of test specimens. Larvae, pupae, and adults of *D. mendotae* were collected on 17, 23, and 26 February 2005 from Trout Brook (Minnesota, USA) (Fig. 1). Fourth-instar larvae and pupae were collected in riffles using a D-frame dip net and picked from detritus in the laboratory. Adults were located on the snow alongside the stream and scooped into individual 3.7-ml snap-top vials with a small amount of snow.

Determination of SCPs. Specimens were attached to thermocouples and cooled at a rate of ~1°C min^{-1} in a -80°C freezer to at least -30°C. Temperatures were recorded at 1-s intervals with a multichannel datalogger. The SCPs were estimated as the lowest temperature reached before the release of the latent heat of fusion resulting from the freezing of the insect's body fluids.

Measurement of Larval Phase. Larval maturity was estimated by placing larvae into one of three categories: 1) without well-defined mesothoracic leg sheaths (L1), 2) well-defined mesothoracic leg sheaths widely separated ventrally (L2), and 3) thorax greatly swollen with mesothoracic leg sheaths meeting or nearly meeting ventrally (L3) (Fig. 2).

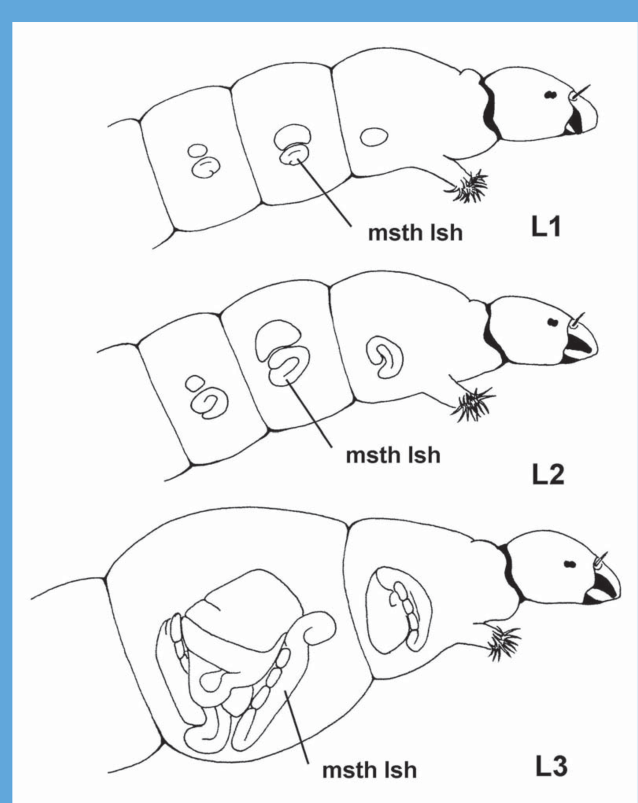

Fig. 2: *Diamesa mendotae* larval developmental phases (msth lsh = mesothoracic leg sheaths).

METHODS (Contined)

Effect of short-term exposure to subzero temperatures on larval mortality. Ten larvae each were placed in test tubes, cooled to -5, -10, -15, -20, -25, and -30°C at a rate of ~0.3-0.4°C min^{-1} in a -80°C freezer, and removed 1 min after the target temperature had been achieved. To correct for non-treatment mortality, an additional 10 larvae were added to another test tube and maintained at 6°C as a control. Each temperature was tested at least three times. After testing, larvae were maintained on wet filter paper at 6°C (Fig. 3) and survivorship was assessed as the number of larvae active or moving when prodded with forceps 24 h post treatment. The cumulative proportion of individuals freezing (CPIF; proportion of individuals freezing at or above each 1°C step) was calculated and plotted against the mean proportion of mortality from the six treatments.

Analysis of data. Differences among the SCPs for the three larval phases, pupae, and adults were tested for differences using an ANOVA after applying an $x^{0.25}$ transformation as recommended by the Box-Cox procedure. An ANOVA on the arcsine-square-root transformed proportion of mortality was performed to test for significant differences in mortality among temperature treatments for the experiments on short-term exposure to subzero temperatures. When significant differences were identified ($P < 0.05$), Tukey's Studentized Range Test was used to separate the means. Probit analysis was used to estimate the LLT_{50} and LLT_{99}.

Fig. 3: *Diamesa mendotae* larvae

Table 1: SCPs for *Diamesa mendotae* life stages.

Stage	n	Mean SCP (°C) ± SE	Range (°C)
Larvae	83	-7.42 ± 0.26	-15.64, -2.28
L1	23	-6.57 ± 0.23	-9.67, -3.60
L2	32	-6.90 ± 0.32	-11.93, -2.28
L3	27	-8.85 ± 0.60	-15.64, -4.43
Pupae	24	-9.14 ± 0.58	-15.65, -4.26
Adults	46	-19.72 ± 0.58	-25.83, -10.45

RESULTS

SCPs. Significant differences were found in the mean SCP among phases of development stages ($F = 114.54$; $df = 4, 147$; $P < 0.0001$) (Fig. 4). The mean SCPs for immature stages were relatively high with a steady decrease in the mean as the insect matures and then a large decrease in SCP in the adults (Table 1; Fig. 4).

Effect of short-term exposure to subzero temperatures on larval mortality. Significant differences in the proportion of larval mortality among subzero temperatures were observed ($F = 40.90$; $df = 5, 19$; $P < 0.0001$) (Fig. 5). A significant increase in mortality of ~61% occurred when the temperature dropped from -15 to -20ºC. In contrast, larval mortality was not significantly different at temperatures above -15ºC and remained below ~22%, despite a mean SCP of only -7.4ºC. The larval LLT_{50} and LLT_{99} (+/- 95% FL) were -17.4°C (-18.7, -16.2) and -25.4°C (-30.3, -23.0), respectively. The LLT_{99} occurred ~10ºC below the minimum SCP recorded for *D. mendotae* larvae (-15.6ºC).

DISCUSSION

SCPs. The determination of SCPs for different developmental stages of *D. mendotae* indicates this insect begins to decreases its mean SCP late in the larval stage into the pupal stage (Fig. 4). The mean SCP drops abruptly from -9.1 to -19.7ºC between the pupal and adult stages; however, it is not clear at which stage this large decrease in the SCP occurs. The lower mean and minimum SCP values through development suggest that there could be physiological preparation late in the 4th instar and pupa for the transition to increased supercooling capabilities in the adult stage.

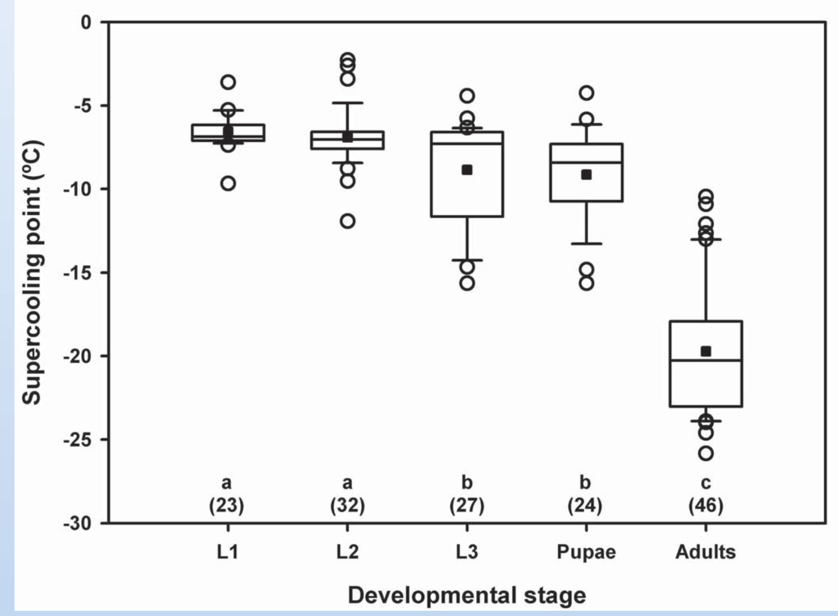

Fig. 4: Effect of developmental stage on the supercooling point (SCP) of *Diamesa mendotae*.

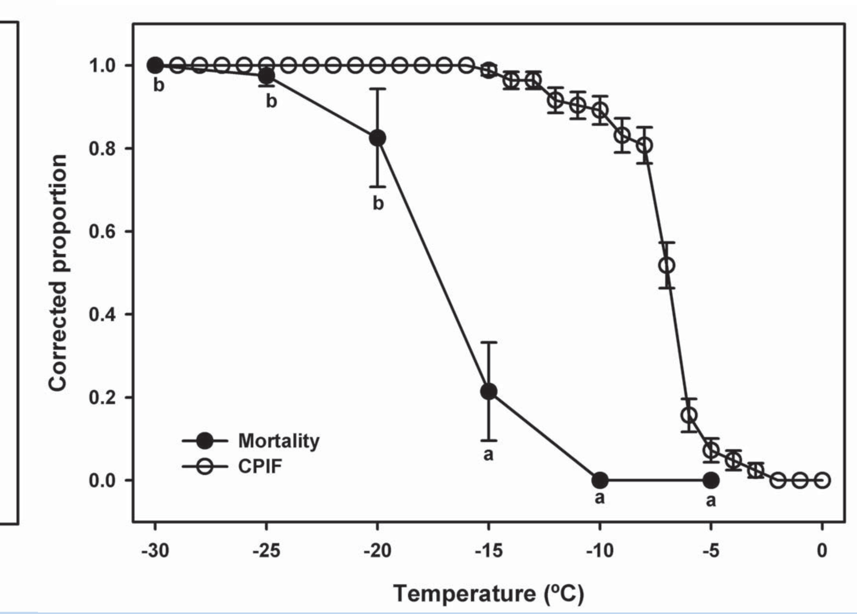

Fig. 5: Corrected mean proportion of mortality (± SE) (closed circles) and cumulative proportion (± SE) of *Diamesa mendotae* larvae freezing (open circles, CPIF) after short-term exposure (1 min) to subzero temperatures.

DISCUSSION (Continued)

Evidence for larval freeze tolerance. Comparison of the proportion of mortality and CPIF curves (Fig. 5), indicated that mortality occurred well below larval SCP temperatures. The LLT_{99} for *D. mendotae* larvae occurred at ~10ºC below the temperature at which the CPIF curve reached 100%, and therefore we conclude that *D. mendotae* larvae are freeze tolerant and this pattern is not an artifact of the methodology (e.g. short-term exposure). The ancestral Diamesinae may have been freeze tolerant; therefore in *D. mendotae* larvae this is possibly an evolutionarily conserved trait. Although specimens in this study were collected from a thermally-buffered habitat, implications of larval freeze tolerance include:

- **Survival in unpredictable conditions.** The ability to tolerate ice inoculation may allow *D. mendotae* larvae to remain active in habitats undergoing short periods of freezing temperatures.
- **Diapause during long cold periods.** Freeze tolerance and the ability to overwinter by diapausing as larvae could allow survival in habitats that freeze for long periods of time.

Larva to adult: changing cold-hardiness strategies. In *D. mendotae*, shifts from one strategy to another between larvae and adults may be a result of the different environments these life stages inhabit. For adults, the ability to greatly lower their SCP allows them to be active in subzero conditions, a requirement for mating and ovipositing during the winter (Carrillo et al., 2004). Freeze tolerance may be facultative in the larvae and permit survival to short-term exposures to ice crystals or diapause for longer periods, increasing the range of habitats this species occupies.

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