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## Differences in Thermal Tolerance Between Two Thermally Isolated and Genetically Indistinct Populations of *Paragnetina media* (Walker) (Plecoptera: Perlodidae)

Bridget C. O'Leary<sup>1</sup>, David C. Houghton<sup>1\*</sup>, and Jeffrey Van Zant<sup>1</sup>

### Abstract

The critical thermal maximum (CTM) of *Paragnetina media* (Walker) (Plecoptera: Perlodidae) was studied at two sites of the Big Sable River in northwestern Lower Michigan during summer 2013. The sites were separated by ~8 km and differed in temperature by ~1°C in the early spring to ~5°C in mid-summer. Individual *P. media* specimens from the warm site had consistently higher CTM when acclimated to the mean temperature of the two sites for 3 days prior to experimental trials during May, June, and July. When acclimated for an additional 3 days to a higher or lower temperature, this thermal disadvantage disappeared. Groups of individuals from both sites simultaneously acclimated to both site temperatures for 3 days exhibited similar CTMs, except that cold site specimens acclimated to the cold temperature had a lower CTM than the other treatments. Sequencing of the CO1 gene revealed that nearly 75% of specimens shared a single haplotype, which was found in both warm and cold site individuals. Our results suggest that both long term and short term thermal history can influence thermal tolerance within populations of the same species that do not appear genetically distinct.

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Temperature is widely recognized as one of the most important variables influencing the distribution, life history, and ecology of aquatic organisms, nearly all of which are exothermic (Caissie 2006, Haidekker and Hering 2008, Dallas and Rivers-Moore 2012). Many anthropogenic activities increase the temperature of freshwater ecosystems (LeBlanc et al. 1997, Rutherford et al. 1997, Lessard and Hayes 2003, Daufresne et al. 2004, Hoffman and Sgro 2011). This concern necessitates studies that determine high temperature tolerances of freshwater organisms, especially in light of the current documented decline of such organisms (Ricciardi and Rasmussen 1999, DeWalt et al. 2005, Lynse et al. 2008, Houghton and Holzenthal 2010).

The high temperature tolerance of aquatic organisms is typically defined non-lethally, through the determination of the critical thermal maximum, or CTM (Cowles and Bogert 1944). CTM is the temperature at which a predetermined behavioral endpoint is reached in the laboratory after an experimental increase in temperature. For aquatic invertebrates, this endpoint usually involves a loss of equilibrium or grip on the substrate (Dallas and Rivers-Moore 2012).

Several studies have suggested that thermal history has a significant effect on thermal tolerance. For example, species exhibit higher CTM after acclimation to higher temperatures in the laboratory (Ernst et al. 1984, Moulton et al. 1993, Jumbam et al. 2008, Kumlu and Turkmen 2010, Galbraith et al. 2012). Species that live in colder environments or that are present during colder

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periods of the year have lower CTM than those that are exposed to warmer environments (Garten and Gentry 1976, Moulton et al. 1993, Hopkin et al. 2006, Nyamukondiwa and Terblanche 2010). No study, however, has specifically assessed the effects of thermal habitat differences between two populations of a single species living in close geographic proximity. The question is an important one, because if a population living in a warmer environment has higher thermal tolerance, this difference may be due to its specific genetic ability to adapt to warmer environments. Conversely, higher thermal tolerance may be the result of acclimatizing to such an environment. Such questions of 'nature versus nurture' are fundamental to biology (Pigliucci 2001).

*Paragnetina media* (Walker) (Plecoptera: Perlodidae), or the embossed stonefly, is found in trout streams throughout the eastern U.S. All immature life stages are predatory, feeding on smaller invertebrates such as baetid mayflies, hypsopsychid caddisflies, and chironomid midges (Tarter and Krumholz 1971). The species has been determined to be semivoltine in both Michigan and Kentucky streams, with adult emergence throughout the summer (Lehmkuhl 1970, Tarter and Krumholz 1971). Heiman and Knight (1972) studied the amount of time it took to kill half of the specimens in an experimental trial ( $LT_{50}$ ) of a western Lower Michigan population of *P. media* and found that thermal tolerance was higher during the summer than the winter and that flowing water in experimental trials increased thermal tolerance.

We tested thermal tolerance of *P. media* in the Big Sable River. The river originates in Lake County in northwestern Lower Michigan and flows westerly for approximately 80 km into Hamlin Lake and then into Lake Michigan. It is a cold-water (mean July temperature  $<20^{\circ}\text{C}$ ) trout stream for much of its length, supporting naturally-reproducing populations of brook [*Salvelinus fontinalis* (Mitchill)] and brown (*Salmo trutta* L) trout (Tonello 2006). For approximately 10 km of length, however, water temperatures during the summer have approached  $24^{\circ}\text{C}$  and cannot support trout year-round. This change is natural, brought about by the river passing through 4 km of the Bear Swamp. Downstream of the swamp, cold-water upwelling and input from small tributaries decrease water temperatures and the river can again support trout year-round (Tonello 2006). Since populations of *P. media* exist throughout most of the Big Sable continuum, the river provided a unique opportunity to test thermal tolerance differences between populations of a single species living in close geographic proximity but in thermally different habitats.

Our study had several objectives. First, we compared the CTMs of *P. media* at two thermally isolated sites to determine inherent differences in thermal tolerance. Second, we tested different laboratory acclimation protocols on the two populations to assess their adaptability to different thermal regimes. Third, we sequenced the CO1 gene to make a preliminary assessment of population structure.

## Materials and Methods

**Study site.** We collected *P. media* from two locations of the Big Sable. The first location, designated 'warm site' ( $\text{N}44^{\circ}05'$ ,  $\text{W}86^{\circ}07'$ ), was ~5 km downstream of the Bear Swamp. The second, designated 'cold site' ( $\text{N}44^{\circ}07'$ ,  $\text{W}86^{\circ}12'$ ), was ~8 km downstream of the warm site. The two sites were chosen based on their geographic proximity and their previously documented difference in temperature (Tonello 2006). Both sites had largely intact riparian zones, but were wide enough to allow for substantial sunlight penetration (Fig. 1).

Stream temperature was measured using a single Onset Hobo Water Temp Pro V2 temperature probe ([www.onsetcomp.com](http://www.onsetcomp.com)) deployed at each site. Probes were placed near the center of the river channel, approximately 46 cm below low flow depth. They recorded water temperatures hourly from mid-May to the beginning of August, our approximate study period.

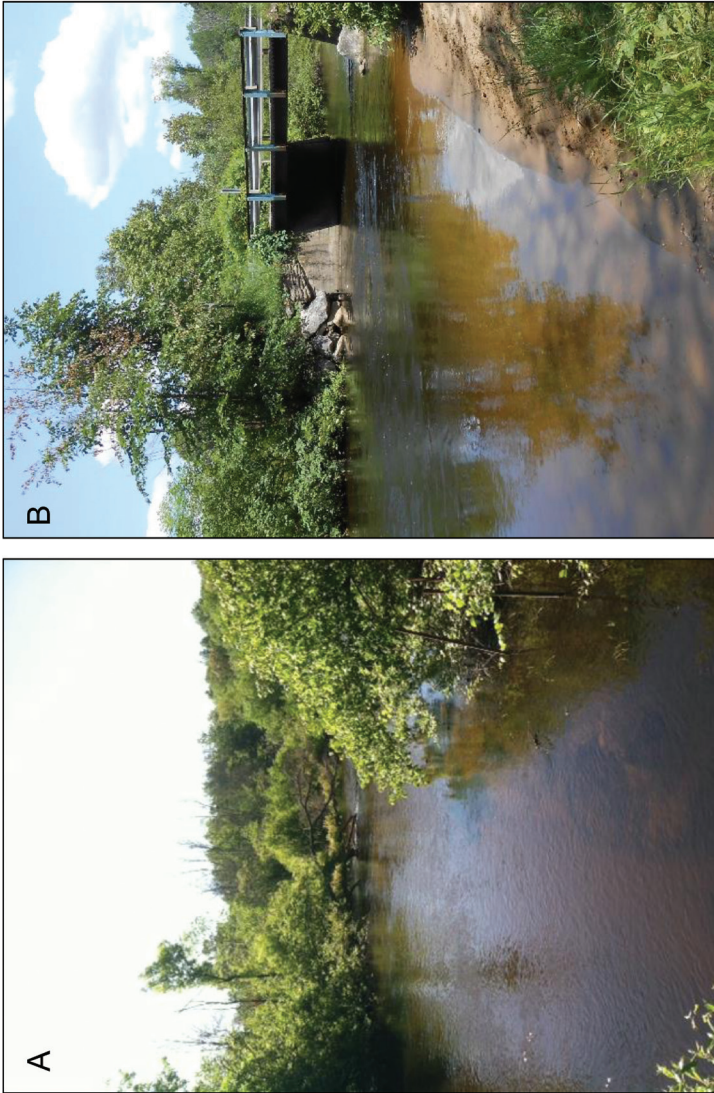


Figure 1. Photographs of the Big Sable River at our warm site (A) and cold site (B) near the temperature probes.

**Collecting and laboratory acclimation.** Late instar ( $\geq 3/4$  of maximum size) specimens were collected by hand in batches of  $\sim 100$  from each site during 4 collecting trips in May, June, and July (Fig. 2). Specimens were abundant at both sites and typically collected in  $\sim 1$  h by 2–4 people within 100 m of the temperature probes. Specimens were readily identifiable in the field by the unique markings on their head and pronotum. We did not collect any other perlotid stoneflies at this site.

In the laboratory, all specimens were housed without food in Frigid Units Living Stream™ environments ([www.frigidunits.com](http://www.frigidunits.com)), set to ambient photoperiod. Specimens were segregated by stream site into flow-through containers in groups of 20–30 within the Living Stream environment. Living Stream water was composed of  $\sim 20\%$  stream water and  $\sim 80\%$  unchlorinated well water. Any specimens that had visible injuries (e.g., missing appendages) during the acclimation process, or appeared weak and unable to cling to the substrate, were not used in experimental trials.

In the May experiment, all specimens were acclimated to  $12^\circ\text{C}$  for 3 days prior to experimental trials. This temperature approximated the mean between the two stream sites (Fig. 2). After CTM was determined for specimens at the two sites, remaining untested specimens were acclimated to  $17^\circ\text{C}$ —a standard acclimation temperature in the literature (Dallas and Rivers-Moore 2012)—for an additional 3 days before another round of experimental trials. In the June experiment, this process was reversed: all specimens were acclimated to  $17^\circ\text{C}$  for 3 days and then the remaining acclimated to  $12^\circ\text{C}$  for another 3 days. In the first July experiment, all specimens were acclimated only to  $17^\circ\text{C}$  for three days, again approximating the mean temperature between the two sites. In the second July experiment, groups of both warm site and cold site specimens were simultaneously acclimated to  $18$  and  $22^\circ\text{C}$ —the temperatures of the cold and warm sites—in separate Living Stream environments.

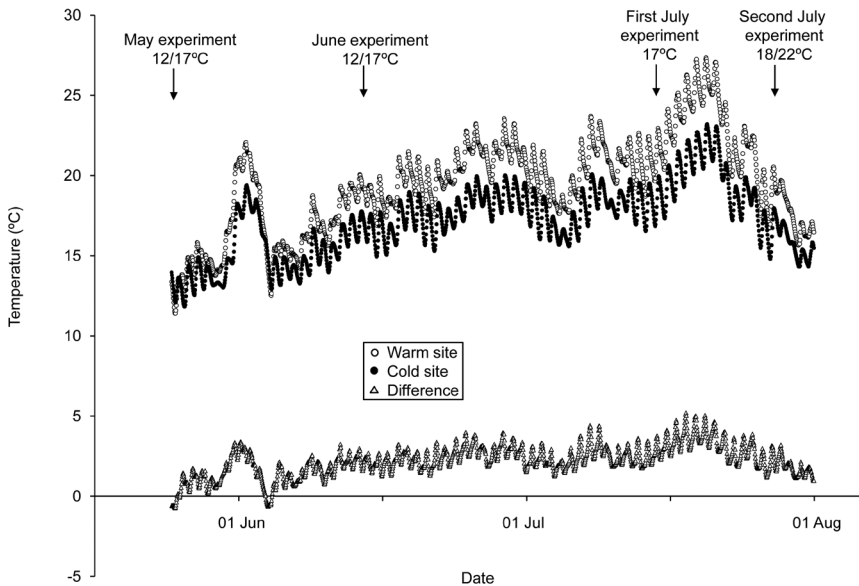


Figure 2. Big Sable water temperatures and the difference (warm site temperature minus cold site temperature) between them, as recorded throughout our summer 2013 study period. Arrows indicate dates of our experimental trials and corresponding acclimation temperatures (Figures 3 and 4).

**Experimental trials.** Trials were conducted using a Julabo MB-13 circulating heater ([www.julabo.com](http://www.julabo.com)) set to 40% external and 60% internal circulation. The device was linked to a computer using Julabo EasyTemp software, allowing for precise programming and logging of temperature protocols. In each trial, specimens were placed into a bath containing Living Stream water, given both natural stream rocks and 1x1 mm latex window screen to use as substrate, and allowed to orient themselves relative to the current for 5 minutes before the temperature was raised. Water temperature began at the acclimation temperature in the Living Stream and was raised by 0.33°C per minute until CTM was reached for all trial specimens (Dallas and Rivers-Moore 2012, Houghton et al. 2014). CTM was defined as the inability to cling to substrate and, thus, being dislodged by the current. Specimens temporarily dislodged by the current or by other specimens were left in the water bath if they were able to re-attach and assume a normal posture on the substrate.

Once CTM was reached for a specimen, the specimen was removed from the water bath and placed into an 850 ml bowl which was floated in the Living Stream to cool specimens back to acclimation temperature over a 30–60 minute period. Once acclimation temperature was reached, specimens were returned to the Living Stream and their survival checked at 48 h.

Each experiment consisted of alternating trials of 5 warm site specimens and 5 cold site specimens, for a total of 5 trials for each treatment. To mitigate the potential effects of outlier trials, global mean CTM was determined for each site by combining all 25 specimens from the 5 trials. In the second July experiment referenced above, 4 treatments were alternated instead of 2 (warm site and cold site acclimation to 18 and 22°C).

**Genetic analysis.** Population structure was assessed using the mtDNA gene cytochrome c oxidase subunit 1 (CO1). During August, 39 specimens were collected from the warm site and 51 from the cold site. Upon collection, they were placed in 1.5ml microcentrifuge tubes containing 80% ethanol. For DNA isolation, heads were removed from each specimen and cut into multiple pieces to optimize tissue lysis. Whole genomic DNA was extracted using Qiagen's Dneasy Blood and Tissue Kit ([www.qiagen.com](http://www.qiagen.com)). Extracted DNA was quantified using a Thermo Scientific Nanodrop 1000 ([www.nanodrop.com](http://www.nanodrop.com)). The polymerase chain reaction (PCR) was used to amplify a 576-bp portion of the CO1 region of the mtDNA using primers LepF1- ATTCAACCAATCATAAAGATATTGG and LepR1 – TAAACTTCTGGATGTCCAAAAAATCA (Hebert et al. 2004). PCR amplifications were conducted using Qiagen TopTaq Master Mix Kit ([www.qiagen.com](http://www.qiagen.com)). Reactions were run in an Applied Biosystems Veriti 96 Well Thermal Cycler ([www.lifetechnologies.com](http://www.lifetechnologies.com)). Amplification reactions were conducted in 25 µL volumes containing 10.0 µL H<sub>2</sub>O, 12.5 µL TopTaq Master Mix, 0.75 µL LepF, 0.75 µL LepR, and 1.0 µL DNA. PCR reactions were: 35 cycles 94°C (30 s) denaturing, 48°C (30 s) annealing, and 72°C (30 s) extension, followed by one 5-min period at 72°C. Product was determined to be present or absent by means of UV light visualization. PCR product was cleaned using QIAquick PCR Purification Kit and sequenced using an ABI 3130 Genetic Analyzer ([www.appliedbiosystems.com](http://www.appliedbiosystems.com)).

MEGA 6.0 (Tamura 2013) and Muscle (Edgar 2004) were used to manipulate and proof sequence data and to create a topology using the maximum parsimony optimality criterion. To search for the best topology, a single specimen of *Paragnetina immarginata* Say (GenBank #JN200677.1) was designated as the outgroup, and a heuristic search was used with 100 random additions and tree-bisection-reconnection (TBR) branch swapping. Robustness and nodal support were evaluated using 1000 bootstrap iterations (Felsenstein 1985).

## Results

Mean hourly water temperature during our study period was significantly higher at the warm site (19.4°C) than the cold site (17.1°C) (Paired  $T$ -test,  $P < 0.001$ ). Except for two morning measurements, water temperature was higher at the warm site than the cold site on every occasion that it was recorded. Although there were some outlier dates, the magnitude of this difference generally increased throughout the spring and summer—reaching nearly 5°C in mid-July—before decreasing in the late summer (Fig. 2).

In the May experiment, warm site *P. media* specimens had higher mean CTM than did cold site specimens when both were acclimated to 12°C for 3 days (Fig. 3). There was no difference between populations when acclimated to 17°C for an additional 3 days. In June, warm site *P. media* specimens had higher mean CTM when acclimated to 17°C for 3 days, but not when acclimated to 12°C for an additional 3 days. In July, warm site specimens had higher mean CTM than cold site specimens when acclimated to 17°C. Mean CTM for the combined warm site and cold site specimens acclimated to 17°C increased from May to June and from June to July (Fig. 4). In the second July experiment, cold site specimens acclimated to 18°C had the lowest mean CTM. There was no difference in mean CTM between the other 3 treatments (Fig. 5).

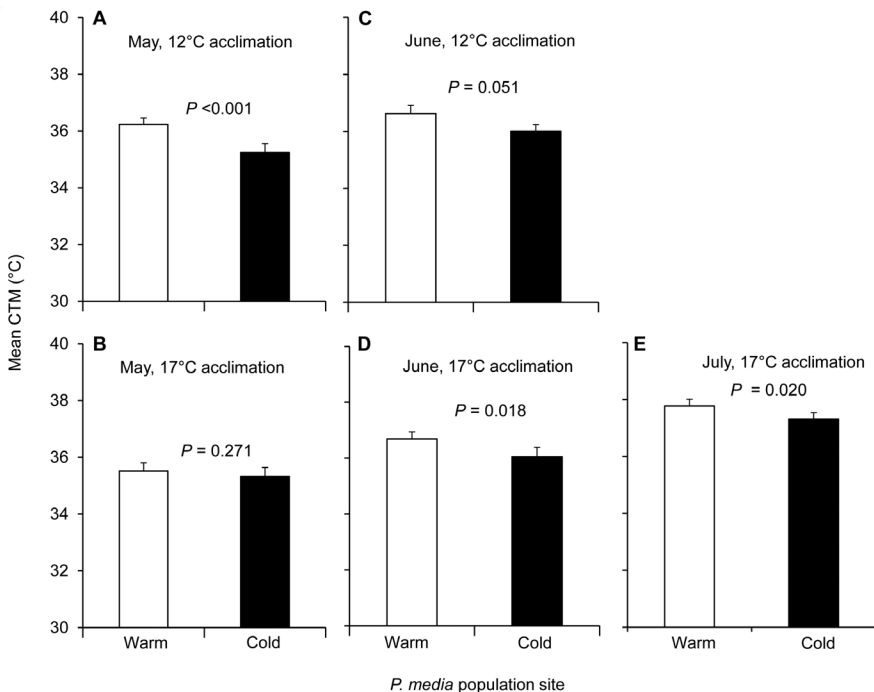


Figure 3. Mean (+SE) CTM for warm site and cold site populations of *Paragnetina media* based on experiments conducted in May, June, and July 2013 under two acclimation temperatures. A: 12°C acclimation in May, B: 17°C acclimation in May, C: 12°C acclimation in June, D: 17°C acclimation in June, E: 17°C acclimation in July. Each  $P$ -value reflects an individual Paired  $T$ -test comparison between mean CTM values of the 25 total specimens from the 5 trials each of warm and cold site.

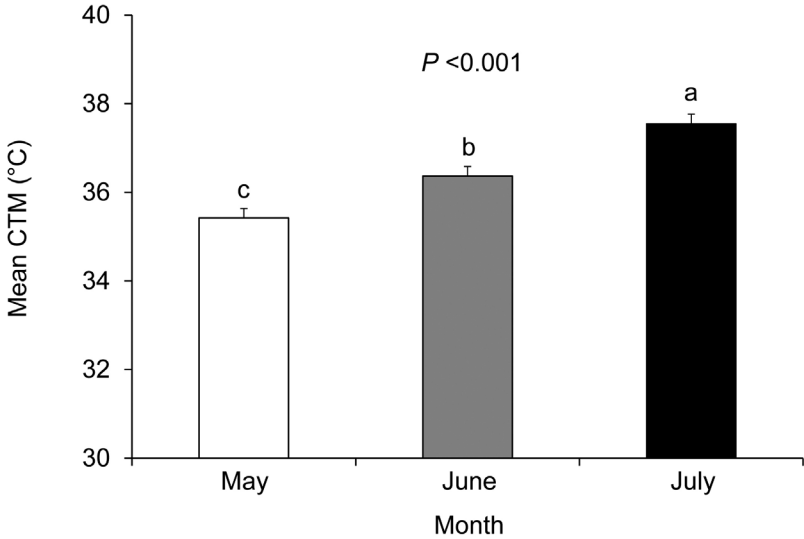


Figure 4. Changes in the combined CTM of warm site and cold site populations of *Paragnetina media* during the three months of our study. All specimens were acclimated to 17°C. Superscript letters denote statistically distinct means (1-way Analysis of Variance with post-hoc Tukey test,  $df = 187$ ,  $F = 9.43$ ).

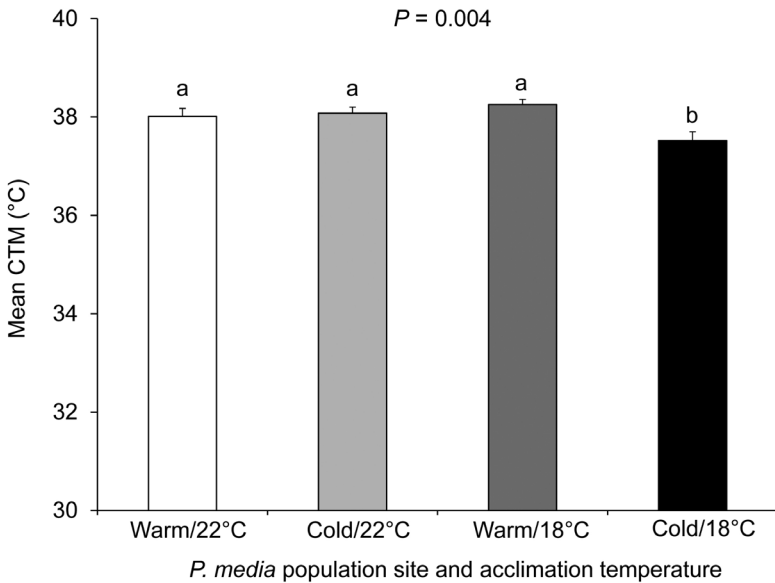


Figure 5. Mean (+SE) CTM for warm site and cold site populations of *Paragnetina media* simultaneously acclimated to 18 and 22°C during July 2013. Superscript letters denote statistically distinct means (1-way Analysis of Variance with post-hoc Tukey test,  $df = 79$ ,  $F = 4.71$ ).



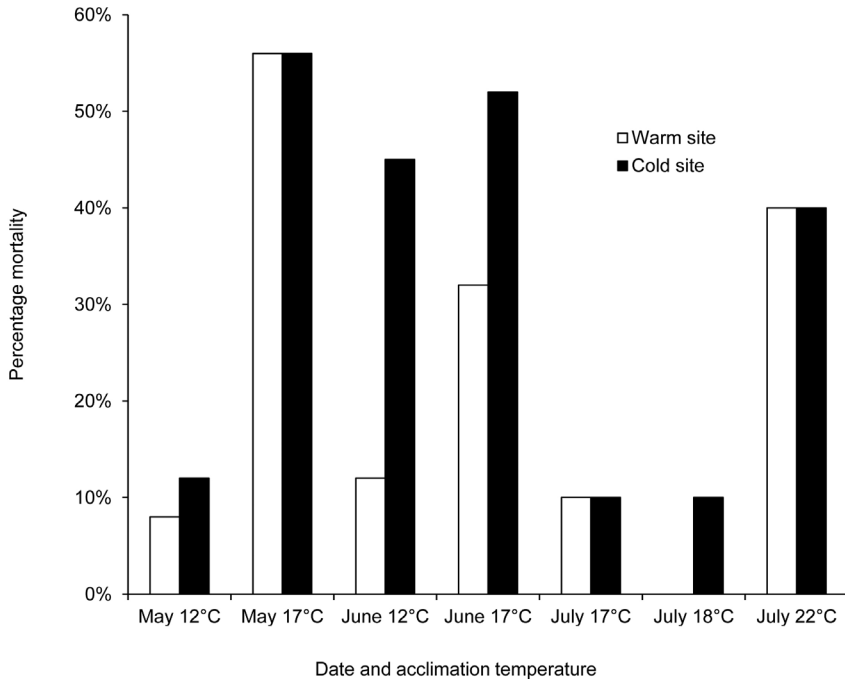


Figure 6. Percentage mortality for cold site and warm site *Paragnetina media* specimens 48 h after CTM experiments for the different experimental conditions.

Mean post-trial mortality ranged 10–60%. Specimens from all experiments acclimated for 6 days had higher post-trial mortality than those acclimated for 3 days (Two-sample *T*-test,  $P = 0.03$ ) (Fig. 6). Cold site specimens had slightly higher post-trial mortality than did warm site specimens (Two-sample *T*-test,  $P = 0.04$ ). Mortality during the acclimation period was approximately 25%.

CO1 sequences revealed 13 unique haplotypes. Seven haplotypes were found only at the warm site, 3 were found only at the cold site, and 3 were found at both the warm and cold sites. Nearly 75% of specimens shared a single haplotype found at both sites (Fig. 7).

Of the 574 characters analyzed, 563 were constant and 4 were parsimony informative considering the ingroup. Including the ingroup and outgroup, 477 were constant and 6 were parsimony informative. Maximum parsimony analyses produced 12 trees. The score of the best tree found was 102. Mean nucleotide diversity ( $\pi$ ) across all *P. media* haplotypes was 0.003. Haplotypes from the warm site, cold site, and warm and cold sites were intermixed on the tree topology (Fig. 7).

## Discussion

Heiman and Knight's (1972)  $LT_{50}$  experiment on a *P. media* population ~130 km south of our population measured the amount of time it took to kill half of the specimens in an experimental trial at various acclimation and experimental temperature combinations. Although results varied based on acclimation temperature and other factors, experimental temperatures <33°C typically led

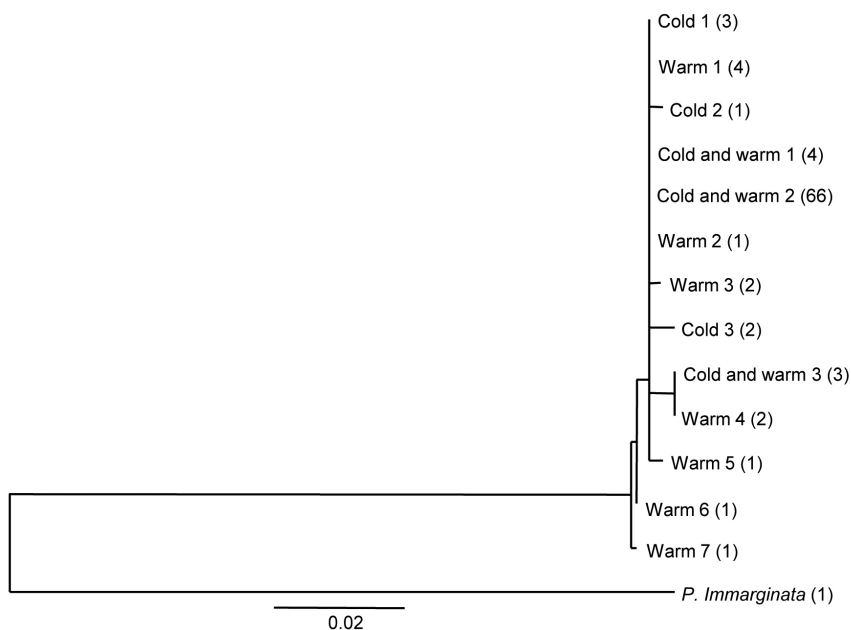


Figure 7. Maximum parsimony topology for the 13 *Paragnetina media* haplotypes based on our warm and cold sites, and our *P. immarginata* outgroup. Number of specimens of each haplotype is in parentheses. The scale bar represents the number of substitutions per site.

to indefinite survival, whereas experimental temperatures  $>38^{\circ}\text{C}$  led to an  $\text{LT}_{50}$  in  $<1$  h. Our CTM values for the various treatments ( $\sim 35$ – $38^{\circ}\text{C}$ ) appear similar to these values. Both studies found an increase in thermal tolerance as stream temperatures increased during the summer.

Dallas and Rivers-Moore (2012) proposed a classification scheme of relative thermal tolerance for aquatic insects. Both of our *P. media* populations tested during all experiments would be considered ‘moderately sensitive’ (as defined by  $\text{CTM} = 33$ – $39^{\circ}\text{C}$ ) based on this scheme. Most insects in this scheme were tested under similar conditions as *P. media*, with an acclimation temperature of  $17^{\circ}\text{C}$  and a  $0.33^{\circ}\text{C}$  rate of experimental increase.

Our CTM data indicate that specific habitat influences thermal tolerance in *P. media*. In the May, June, and first July experiments, CTM was always higher in warm site specimens than in cold site specimens after 3 days of laboratory acclimation to the same temperature. The May experiment in particular suggests the importance of long term thermal history in determining CTM, since the warm site was only  $0$ – $2^{\circ}\text{C}$  warmer than the cold site during this time period. Adaptation of the warm site population to warmer temperatures may have carried over from the previous summer in this semivoltine species. Several authors have suggested that long term field acclimatization can yield ‘irreversible’ increases in thermal tolerance (Lagerspetz 2006, Chown and Terblanche 2007, Nyamukondiwa and Terblanche 2010). Further, the combined CTM of both populations continued to increase throughout the summer as stream temperatures increased, despite laboratory acclimation to a single ( $17^{\circ}\text{C}$ ) temperature, providing further evidence of maintained thermal tolerance due to thermal history.

Our data also suggest that recent thermal history can mitigate the effects of habitat. After 6 days of acclimation in the May and June experiments, cold site specimens had the same CTM as warm site specimens, suggesting that cold site specimens had a similar ability to adapt to ambient conditions as warm site specimens if given enough time. In the second July experiment, the cold site specimens acclimated to 18°C had lower CTM than the other treatments probably since they had only been exposed to relatively cold conditions. Warm site specimens—regardless of acclimation temperature—and cold site specimens acclimated to 22°C likely had higher CTM due to recent thermal history, either during laboratory acclimation or in their native habitat. Again, it appears that thermal tolerance is moderated by both long term and short term temperature variation.

Our genetic topology did not reveal any clear distinction between the two populations. Although we did find haplotypes unique to each site, 73% of individuals were of the same haplotype which was found at both sites. Mean nucleotide diversity was low, reflecting close evolutionary relationships between individuals within a haplotype. Such a result is typical for weak flying insects with low vagility (Smith et al. 2006, Young et al. 2013). Specimens and haplotypes were intermixed between sites, suggesting that differences between haplotypes were not related to differences between the warm and cold sites. This result further supports the idea that it is thermal history that is affecting CTM differences between these populations, and not inherent genetic differences between them.

Our high post-trial mortality is a potential cause for concern since it suggests that our CTM endpoint may not have been truly sub lethal (Lutterschmidt and Hutchison 1997). Our determined endpoint, however, has been used for many species of aquatic insects, including perlotids and other stonefly species (Ernst et al. 1984, Poulton et al. 1989, Dallas and Rivers-Moore 2012). We suspect, due to the higher post-trial mortality in specimens acclimated for 6 days instead of 3, that high post-trial mortality actually reflected stress in captivity more than stress of the CTM trials. The higher mortality of cold site specimens was, likewise, probably due to the stress of being acclimated at temperatures higher than that of their recent thermal history. Further, we estimated mortality during the acclimation period at nearly 25%, which is far higher than that of the dozen other aquatic insect species that we have tested (e.g., Shoup and Houghton 2013, Houghton et al. 2014, Houghton and Shoup 2014). Some pre- and post-trial mortality was due to cannibalism of this predatory species. Thus, we are comfortable with our CTM endpoint determination but do not think *P. media* is the ideal species for long term acclimation studies.

Future research should include genetic analysis with hypervariable markers such as microsatellites (Goldstein et al. 1999). Such markers are frequently used to determine population structure and would be better suited to investigate genetic connectivity between sites than the more conserved CO1 gene (Hebert et al. 2003, Kress et al. 2005). Additional sites along the Big Sable continuum should also be tested. Finally, while testing longer acclimation periods would help to separate the relative importance of short term and long term thermal history, housing this species for longer periods of time may require special care to avoid the high mortality.

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