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Validation of CT_{max} Protocols Using Cased and Uncased *Pycnopsyche guttifer* (Trichoptera: Limnephilidae) Larvae

David C. Houghton^{1*}, Ashley C. Logan¹, and Angelica J. Pytel¹

Abstract

The critical thermal maximum (CT_{max}) of a northern Lower Michigan population of *Pycnopsyche guttifer* was determined using four rates of temperature increase (0.10, 0.33, 0.50, and 0.70°C per minute), and two case states (intact and removed). Across all temperature increase rates, larvae removed from their cases had a significantly lower mean CT_{max} than those remaining in their cases, suggesting that the case can increase the larva's ability to tolerate thermal stress, possibly due to respiratory advantages. Regardless of case state, mean CT_{max} was significantly lower at the 0.10°C per minute increase rate than the other three rates, likely due to increased exposure time. Our results indicate that CT_{max} studies done using 0.33–0.70°C per minute increase protocols would be comparable with each other, but not with studies using an increase rate of 0.10°C per minute.

Temperature is widely recognized as one of the most important variables influencing the distribution and ecology of aquatic organisms, nearly all of which are exothermic (Reyjol et al. 2001, Caissie 2006, Haidekker and Hering 2008, Dallas and Rivers-Moore 2012). Since many anthropogenic activities increase the temperature of freshwater ecosystems, it is important to accurately determine and predict the thermal tolerance of freshwater organisms to thermal pollution. The critical thermal maximum, CT_{max}, is a non-lethal means of experimentally assessing this thermal tolerance (Cowles and Bogert 1944). In these studies, temperatures are raised at a prescribed rate starting at the acclimation temperature until a predetermined behavioral endpoint is reached. For aquatic invertebrates, this endpoint usually involves a loss of equilibrium or grip on the substrate (Dallas and Rivers-Moore 2012).

Since CT_{max} usually occurs at temperatures higher than organisms encounter in the wild, the primary importance of CT_{max} studies is in the comparison of determined values to those of other organisms (Lutterschmidt and Hutchison 1997, Dallas and Rivers-Moore 2012). Thus, it is necessary to conduct CT_{max} studies so that results are comparable to each other. For example, several studies have demonstrated that CT_{max} values using different laboratory acclimation temperatures may not be comparable to each other, even within the same species (Ernst et al. 1984, Moulton et al. 1993, Galbraith et al. 2012).

Several studies on fish and terrestrial insects have suggested that temperature increase rate can also affect CT_{max} (Becker and Genoway 1979, Elliot and Elliot 1995, Mora and Maya 2006, Rezende et al. 2011, Ribeiro et al. 2012). No comparable studies have been done on aquatic insects. The objective of our study, therefore, was to test several different temperature increase rates on a population of caddisfly. A second objective was to test the influence of portable

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caddisfly cases on CT_{max} . Our study organism, *Pycnopsyche guttifer* (Walker) (Limnephilidae) is a tube case-making caddisfly common in woodland streams throughout lower MI (Houghton et al. 2011). No data on the CT_{max} of *P. guttifer*, or of any other limnephilid caddisfly, have been previously reported.

Materials and Methods

Fifth instar larvae of *P. guttifer* were collected from the Little Manistee River (N44.02°, W85.63°), a third-order woodland stream located in the northwestern portion of the Lower Peninsula of MI. A detailed description of this site can be found in Houghton et al. (2013). Specimens were collected by hand during May 2012, transported back to the laboratory, and housed without food in a Frigid Units Living Stream™ (www.frigidunits.com). Temperature (17.5°C) and photoperiod were set to ambient, although there was some minor deviation in temperature due to mechanical problems (Table 1).

Four different temperature increase rates were tested: 0.10, 0.33, 0.50, and 0.70° C per minute. Two different case states were also tested: intact and removed. For the latter scenario, cases were carefully deconstructed piece-by-piece until larvae evacuated. Each case state and temperature increase protocol had three trials each. The order of the 24 trials was randomized. Sample size for each individual trial was 5 specimens. All trials finished before *P. guttifer* began burying into the substrate for their summer aestivation.

Each trial group was placed in a Julabo MB-13 circulating heated water bath (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 the water bath containing Living Stream water, given 1x1 mm latex window screen to use as substrate, and allowed to orient themselves relative to the current for 2–3 minutes before the temperature was raised. Water temperature began at ambient and was raised until CT_{max} was reached for all trial specimens. CT_{max} was defined as the loss of equilibrium or ability to cling to the substrate. Specimens that were temporarily dislodged by the current or by other specimens were not removed from the water bath if they were able to re-attach and assume a normal posture.

After the trial ended, specimens were placed in 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 every 8 h for 96 h. Voucher specimens were deposited in the Hillsdale College Insect Collection.

In a separate experiment, three trials of each protocol were conducted to clarify the relationships between time, temperature, dissolved oxygen, and percent oxygen saturation. *P. guttifer* larvae were added to the water bath to ensure consistent experimental conditions, but CT_{max} was not determined. Instead, the variables were recorded every minute using a YSI-55 oxygen probe (www.ysi.com) as temperatures increased from 17 to 35°C. These observations were all conducted within a 2-day period to ensure similar beginning experimental conditions.

Results

CT_{max} for cased *P. guttifer* acclimated to 17°C with a 0.33°C per minute temperature increase protocol was 33.7°C. Mean CT_{max} was higher in specimens with intact cases (32.8°C) than those removed from their cases (31.7°C) across all temperature increase protocols. CT_{max} was lower for specimens using the 0.10° C per minute temperature increase protocol (30.3°C) than the other protocols (32.9°C) across both case states (2-way Analysis of Variance with *post-hoc* Tukey test, $F = 27.6, 17.9$; $df = 3, 1$; $P < 0.001$) (Fig. 1). Interactions between

Table 1. Summary data for CT_{max} trials of *P. guttifer*. 'Temperature' and 'dissolved oxygen' were measured in the living stream immediately before the respective trial commenced. n.d. = no data.

Date	Temperature (°C)	Dissolved oxygen (mg/L)	Case status	Increase (°C/Minute)	CT _{max} (°C)
28 May	17.9	8.1	intact	0.50	32.9
29 May	17.9	8.1	removed	0.70	31.2
29 May	17.9	8.4	removed	0.50	31.0
30 May	17.3	8.9	intact	0.33	33.8
30 May	17.3	8.4	removed	0.10	28.3
30 May	17.5	8.5	intact	0.10	30.8
01 June	17.2	8.9	removed	0.70	33.5
02 June	17.3	8.7	intact	0.10	30.7
05 June	17.1	8.4	intact	0.70	34.0
05 June	17.1	8.4	removed	0.50	32.4
05 June	17.1	8.4	removed	0.33	32.6
05 June	17.1	8.4	removed	0.33	33.1
11 June	18.3	7.1	removed	0.10	30.0
11 June	18.3	7.1	removed	0.70	33.7
11 June	18.3	7.1	removed	0.10	30.9
12 June	18.3	6.6	intact	0.10	30.8
12 June	18.3	6.6	removed	0.50	32.6
12 June	18.5	7.5	intact	0.70	33.2
13 June	17.3	8.3	intact	0.33	34.0
13 June	17.3	8.3	intact	0.50	33.4
13 June	17.3	8.3	removed	0.33	33.3
13 June	17.3	n.d.	intact	0.33	33.4
18 June	17.5	8.0	intact	0.70	32.7
18 June	17.5	8.0	intact	0.50	34.2

the factors were not significant ($P = 0.81$). There was no correlation with CT_{max} between trial date ($R^2 = 0.08$), or temperature ($R^2 = 0.03$) or dissolved oxygen level ($R^2 = 0.13$) in the Living Stream immediately before the beginning of trials. Post-trial mortality was <1% within 96 h of the experiment.

Mean dissolved oxygen exhibited a very strong negative linear correlation with temperature for all observed temperature increase protocols (Fig. 2). Mean beginning dissolved oxygen levels were the same for all protocols (1-way Analysis of Variance, $F = 0.2$, $P = 0.86$). Mean ending dissolved oxygen level was lower for the 0.10°C protocol than for the other protocols, even though all protocols ended at the same temperature (1-way Analysis of Variance, $F = 42.3$, $P < 0.001$). Dissolved oxygen saturation continually increased in the 0.70 and 0.50 °C protocols, leveled off towards the end of the 0.33°C protocol, and decreased after an initial increase in the 0.10°C protocol.

Discussion

Our determined CT_{max} for *P. guttifer* of 33.7°C based on acclimation to 17°C and a 0.33°C/minute increase protocol—the most frequently used testing conditions in the literature—rated the species as barely 'moderately sensitive' (as defined by CT_{max} = 33–39°C) according to the Dallas and Rivers-Moore (2012) sensitivity classification scheme. The species probably has value as a thermal bioindicator due to its relative stenothermy. Some of its observed value,

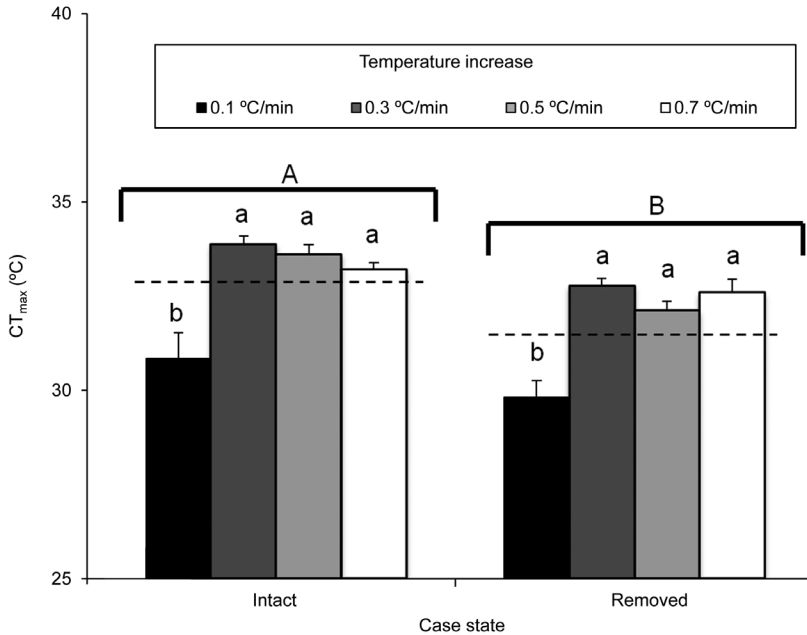


Figure 1. CT_{max} values for *P. guttifer* based on case state and temperature increase protocol. Dashed lines indicate the means for larvae in intact and removed cases, respectively. Superscript letters denote statistically distinct groups (2-way Analysis of Variance with *post-hoc* Tukey test, $F = 27.6, 17.9$; $df = 3, 1$; $P < 0.001$).

however, may come from its specific habitat in our study. The Little Manistee River is one of the coldest and most stable streams in Lower Michigan, only rarely rising above 20°C during the heat of the summer (Tonello 2005, Shoup and Houghton 2013). Our studied population of *P. guttifer* may be relatively intolerant to thermal stress due to its consistently cold environment never exposing it to warm temperatures. Several studies have shown that the thermal history of an organism has a strong influence on its determined CT_{max} (Garten and Gentry 1976, Moulton et al. 1993, Galbraith et al. 2012). Thus, populations of this widely-distributed species in warmer streams may have a more eurythermic response.

Differences in CT_{max} based on case status and temperature increase protocol may be due to changes in both temperature and dissolved oxygen. As observed in our study and elsewhere, oxygen solubility decreases as temperature increases. The metabolic activity of ectothermic organisms, however, increases with an increase in temperature. Thus, oxygen needs increase simultaneously with decrease in oxygen availability. Determining whether CT_{max} is caused by thermal stress, asphyxiation, or a combination of these factors constitutes a difficult challenge in CT_{max} studies (Portner 2001, Portner and Knust 2007).

The lower CT_{max} of specimens using our 0.10°C protocol and the higher CT_{max} result for cased larvae both suggest that low dissolved oxygen levels may affect thermal tolerance. In the case of the latter observation, many tube case-making caddisflies such as *P. guttifer* rely on woody debris as a food

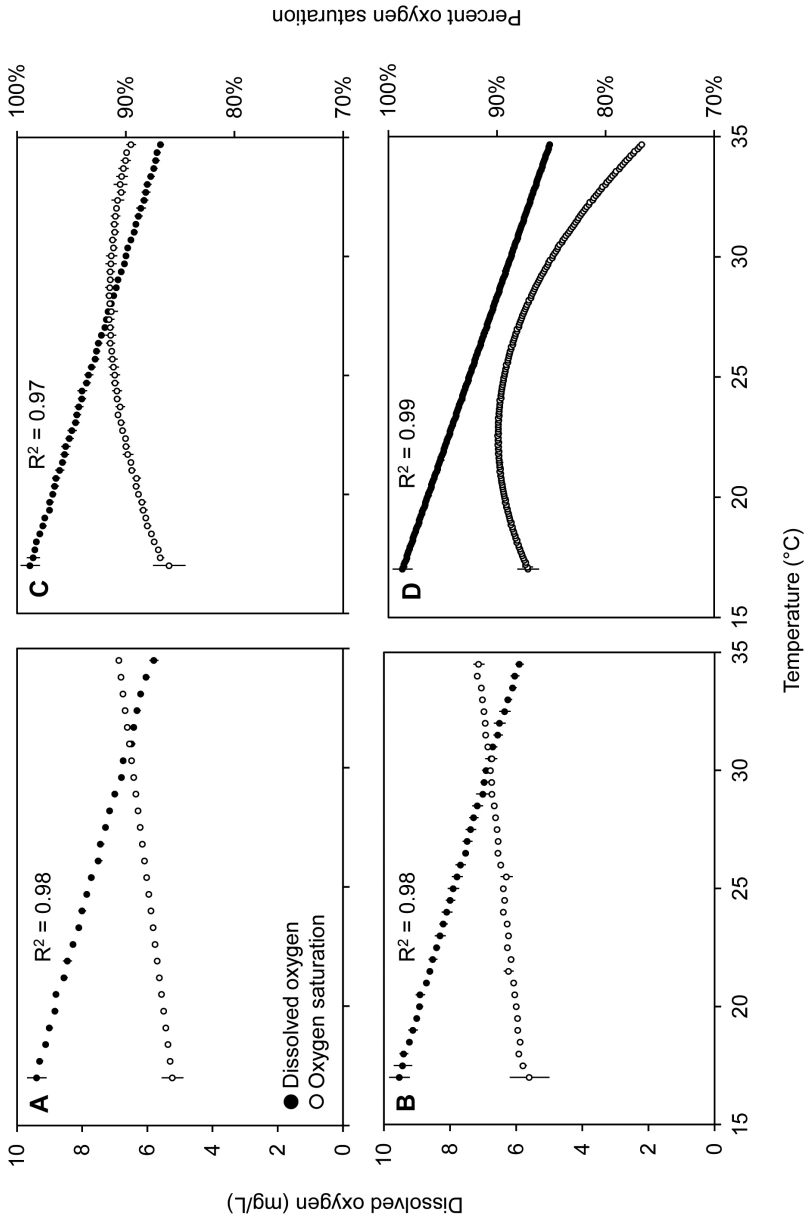


Figure 2. Correlations between mean (\pm SE) dissolved oxygen and mean (\pm SE) percent oxygen saturation with temperature during our experimental trials. A: 0.70 °C/minute, B: 0.50 °C/minute, C: 0.33 °C/minute, D: 0.10 °C/minute temperature increase protocols. Measurements were taken every minute.

source (Wiggins 1996). Thus, they often live in areas of low current, such as the depositional areas of streams where such woody debris accumulates. The tubular case, therefore, may serve a respiratory function — a larva undulating its abdomen inside its case will create a steady micro current of water to bathe the gills (Williams et al. 1987). Thus, the higher CT_{max} of cased larvae in our study may be due to this respiratory advantage in an increasingly deoxygenated experimental environment. Cased larvae also may have had a higher CT_{max} because the case increased their volume, thus taking longer for them to heat up (Ribeiro et al. 2012).

Our results also indicate that the time spent under adverse conditions also has an effect on dissolved oxygen and CT_{max} . Larvae of our 0.10°C protocol likely exhibited a lower CT_{max} than the other protocols because the slow increase exposed them to adverse conditions for a longer (2–3 h) period of time (Rezende et al. 2011, Ribeiro et al. 2012). Likewise, dissolved oxygen levels may also have been affected by the slower temperature increases. The rate of oxygen outgassing in our warming water bath appeared to be slower than the rate of temperature increase in the 0.50 and 0.70°C protocols; thus, saturation continued to increase throughout the trials. In the 0.33°C protocol, the outgassing rate appeared similar to temperature increase rate. In the 0.10°C protocol, the outgassing rate appeared to be faster than the temperature increase rate, leading to decreasing oxygen saturation. The lower dissolved oxygen at the end of the 0.10°C protocol—despite being at the same temperature as the other protocols—further suggested that the longer time spent at each temperature allowed more oxygen to outgas than it did when using the faster increase rates.

Previous studies with fish and terrestrial insects have also demonstrated differences in CT_{max} relative to temperature increase rate, although the specific results are somewhat contradictory with each other and with our study. Ribeiro et al. (2012) found that the ant *Atta sexdens* L. generally exhibited higher CT_{max} as temperature increase rates increased from 0.16 to 2.00°C per minute, although the authors did not test for differences between their specific rates. Elliot and Elliot (1995) found that speeding up the temperature increase rate from 0.0002°C per minute to 0.02°C per minute resulted in increasingly higher CT_{max} in two species of trout. Further increasing to 0.25°C per minute promoted no further CT_{max} increase. Becker and Genoway (1979) and Mora and Maya (2006) found similar results except the former study demonstrated an increased CT_{max} to a 1°C per minute rate with the sunfish *Lepomis macrochirus*, Rafinesque, and the salmon *Oncorhynchus kisutch*, Walbaum, whereas the latter actually found decreasing CT_{max} at 1°C per minute with the blenny *Acanthemblemaria hancocki*, Myers and Reid. The authors of the latter study suggested that differences in the species used and slight differences in experimental design between studies may account for the differing results.

One source of potential error in our experiment was a succession of power outages that caused the temperature in the Living Stream to increase to 18.3–18.5°C prior to 6 trials. Due to the effect of acclimation temperature on CT_{max} , it is possible that specimens *de facto* acclimated to this higher temperature may have had an artificially high CT_{max} . The lack of correlation between pre-trial Living Stream temperature and CT_{max} , however, suggests that the warmer temperatures did not have an important effect on our overall experiment. Studies on acclimation temperature usually acclimate specimens for 4–7 d before trials (Moulton et al. 1993, Galbraith et al. 2012), whereas our specimens were exposed to the warmer temperatures for ~2–3 h. Moreover, half of the affected trials were of the 0.10°C protocol, which had the lowest CT_{max} . Thus, if CT_{max} was artificially higher in the trials affected by the higher Living Stream temperature, then our observed differences between the 0.10°C protocol and the other protocols may actually have been underestimated.

Overall, our results suggest that temperature increase rate can affect CT_{max}, even within our limited testing range. More specifically, our results suggest that fundamental differences in temperature and dissolved oxygen profiles, as well as in CT_{max}, may occur between the 0.10°C and 0.33°C per minute temperature increase rates. Our results also indicate that organisms tested at increased rates between 0.33 and 0.70°C per minute, which encompass the majority of aquatic insect CT_{max} trials in the literature (Dallas and Rivers-Moore 2012), are comparable with each other. Further research will be needed to elucidate the specific interactions between temperature and dissolved oxygen in creating thermal stress in aquatic organisms. More basically, the differing results between studies on this topic strongly suggest the need for further basic research as well as a standardized experimental design.

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