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RESEARCH ARTICLE

Physiological responses to short-term thermal stress in mayfly (*Neocloeon triangulifer*) larvae in relation to upper thermal limits

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ABSTRACT

Understanding species' thermal limits and their physiological determinants is critical in light of climate change and other human activities that warm freshwater ecosystems. Here, we ask whether oxygen limitation determines the chronic upper thermal limits in larvae of the mayfly *Neocloeon triangulifer*, an emerging model for ecological and physiological studies. Our experiments are based on a robust understanding of the upper acute (~40°C) and chronic thermal limits of this species (>28°C, ≤30°C) derived from full life cycle rearing experiments across temperatures. We tested two related predictions derived from the hypothesis that oxygen limitation sets the chronic upper thermal limits: (1) aerobic scope declines in mayfly larvae as they approach and exceed temperatures that are chronically lethal to larvae; and (2) genes indicative of hypoxia challenge are also responsive in larvae exposed to ecologically relevant thermal limits. Neither prediction held true. We estimated aerobic scope by subtracting measurements of standard oxygen consumption rates from measurements of maximum oxygen consumption rates, the latter of which was obtained by treating with the metabolic uncoupling agent carbonyl cyanide-4-(trifluoromethoxy) pheylhydrazone (FCCP). Aerobic scope was similar in larvae held below and above chronic thermal limits. Genes indicative of oxygen limitation (LDH, EGL-9) were only upregulated under hypoxia or during exposure to temperatures beyond the chronic (and more ecologically relevant) thermal limits of this species (LDH). Our results suggest that the chronic thermal limits of this species are likely not driven by oxygen limitation, but rather are determined by other factors, e.g. bioenergetics costs. We caution against the use of short-term thermal ramping approaches to estimate critical thermal limits (CT_{max}) in aquatic insects because those temperatures are typically higher than those that occur in nature.

KEY WORDS: Temperature, Aerobic scope, Hypoxia, Aquatic insect, Gene expression

INTRODUCTION

Freshwater ecosystems support a disproportionate percentage of the earth's biodiversity (Dijkstra et al., 2014) and are among the most threatened by human activities and global climate change (Carpenter et al., 1992). Aquatic insects often dominate freshwater ecosystems in terms of biodiversity and ecological processes such as organic material processing and energy flow (Merritt et al., 2008; Wallace and Webster, 1996). The broad range of environmental sensitivity among insect species and their ecological importance has led to the widespread use of insects in ecological monitoring programs worldwide (Hawkins et al., 2000; Hawkins, 2006; Resh and Jackson, 1993). Although it is well established that temperature plays a major role in the geographic distribution of species, timing of species life history characteristics and structure of aquatic insect communities (Hynes, 1970; Sweeney and Vannote, 1978, 1984; Sweeney et al., 1990; Vannote and Sweeny, 1980; Ward and Stanford, 1982), both the thermal limits of individual species and the fundamental physiological mechanisms that determine those thermal limits remain poorly understood in this important but understudied faunal group.

We are concerned that studies used to infer the thermal limits of different aquatic insects often focus on short-term acute thermal challenges (critical temperature or CT_{max} type experiments) or thermal ramping rates (see Sorensen et al., 2013) that are not typically experienced by organisms in nature. Despite excellent work showing the importance of temporal aspects of thermal change and exposure durations (Angilletta, 2009; Schulte et al., 2011; Dillon and Frazier, 2013), short-term thermal challenges remain the most common experimental approach used to study thermal physiology. It remains unclear how acute CT_{max} and the physiological processes that occur under these conditions relate to the thermal limits of aquatic insects in the natural environment (i.e. long-term thermal limits for successful metamorphosis and reproduction). In the field of toxicology, it is well understood that the mode of toxicity of a given chemical in a given species may be very different depending on exposure intensity, duration and frequency. It is highly likely that the physiological mechanisms underpinning chronic and acute thermal limits are also different (Clark et al., 2013a).

The oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis has emerged as a compelling framework for understanding and studying the thermal limits of ectotherms (e.g. Pörtner, 2001, 2002, 2010; Pörtner et al., 2006). The core concept is that the 'mismatch between the demand for oxygen and the capacity of oxygen supply to tissues is the first mechanism to restrict whole-animal tolerance to thermal extremes' (Pörtner, 2010). To date, this concept has been supported as applicable to acute thermal tolerance in aquatic insects. For example, Verberk and Bilton (2011) showed that CT_{max} estimates could be increased with oxygen supplementation or decreased by oxygen depletion in the stonefly *Dinocras cephalotes*. This finding was also observed (Verberk and Bilton, 2013) in four additional species, and further work (Verberk and Bilton, 2015) showed that in the air-breathing aquatic insect *Ilyocoris cimicoides*, hypoxic water did not influence heat tolerance, whereas hypoxic water reduced heat tolerance in a plastron (dissolved oxygen)-breathing *Aphelocheirus aestivalis*. To our knowledge, no studies in aquatic

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insects have previously explored whether oxygen limitation occurs at more ecologically relevant chronic thermal limits.

In the present study, we ask if the mismatch between oxygen supply and demand is apparent at the chronic upper thermal limits of the mayfly *Neocloeon triangulifer* (McDunnough 1931) (Ephemeroptera: Baetidae). This parthenogenetic species (see Funk et al., 2006) has emerged as a useful laboratory model for ecological (Sweeney and Vannote, 1984) and physiological/toxicological studies (Sweeney et al., 1993; Conley et al., 2009, 2011, 2013, 2014; Kim et al., 2012; Kunz et al., 2013; Soucek and Dickinson, 2015; Xie et al., 2010; Xie and Buchwalter, 2011; Johnson et al., 2015). We established chronic upper thermal limits by rearing newly hatched eggs to adulthood across several temperatures. We used both respirometry and gene expression (RT-qPCR) to seek evidence for oxygen limitation in larvae subjected to temperatures bracketing the chronic thermal limits. Specifically, we used the metabolic uncoupling agent carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) to stimulate maximum oxygen consumption rates for the estimation of aerobic scope. We also identified genes responsive to environmental hypoxia and thermal stress and examined their relative expression patterns in larvae exposed to different thermal and oxygen conditions.

MATERIALS AND METHODS

Assessing chronic thermal limits

To establish chronic thermal limits on mayfly survival rate, hatchlings (<12 h old) of *N. triangulifer* [WCC-2 clone, isolated from White Clay Creek (WCC), Chester County, PA, USA] (Sweeney et al., 1993; Funk et al., 2006) were reared from first instar larvae to the subimago stage in 1.9-liter glass jars immersed in water baths held at constant temperature (±0.05°C) at the Stroud Water Research Center (SWRC) (Avondale, PA, USA). Rearing was completed at nine temperatures ranging from 14 to 30°C in 2°C increments. Five to 11 replicates (glass jars), each containing 50 larvae at the start, were involved in each temperature treatment. An air stone in each jar insured conditions of oxygen saturation throughout the experiment. Simulated daylight was provided by 8-foot (2.44-m) fluorescent 'grow lights' and all experiments involved a 15 h:9 h light:dark cycle. Food was provided *ad libitum* and consisted of a 1–3 mm thick coating of periphyton (i.e. a natural algal mix consisting of predominantly diatoms) attached to 23×6.4×0.16 cm acrylic plates placed in each glass rearing jar. The colonization of algae on the plates was achieved by streaming raw stream water (from WCC) continuously over the acrylic plates for approximately 4 weeks in a greenhouse. Plates were replaced as necessary throughout the experiments to assure an abundance of high-quality food. A screened cage was fitted tightly over each rearing jar to capture/contain emerging subimagoes and adults. Emerging subimagoes/adults were collected and enumerated daily.

Assessing acute thermal limits

To assess acute thermal limits, 23-day-old larvae (approximately 80% through larval development) that had been reared at 20°C were individually held in 30 ml beakers in a water bath at SWRC. A diatom slurry was prepared from the algal plates described above and was equally provisioned across the 30 exposure beakers. Temperature in the water bath was increased at a rate of 0.75°C h^{-1}, and cumulative mortality was recorded until all larvae died.

Mayfly husbandry for physiological experiments

The WCC-2 clone of *N. triangulifer* is also maintained at North Carolina State University (Raleigh, NC, USA) on the laboratory benchtop at room temperature (21–22°C) using food (natural WCC periphyton) provided by SWRC as described above. Reconstituted artificial soft water (American Society for Testing and Materials) was produced from ACS grade salts and deionized water as follows: 48 mg l^{-1} $NaHCO_3$, 30 mg l^{-1} $CaSO_4{\cdot}H_2O$, 30 mg l^{-1} $MgSO_4$ and 2 mg l^{-1} KCl; pH 7.6±0.4 for rearing. In all physiological experiments, control larvae were held in a temperature-controlled water bath at 22±1°C.

Respirometry experiments

To examine the effect of temperature on oxygen consumption rates, we used a fiber-optic-based, intermittent flow respirometry system (Loligo Systems, Tjele, Demnark) as described previously (Camp et al., 2014). We examined oxygen consumption in larvae subjected to a thermal ramp of 1°C h^{-1}, beginning at their rearing temperature of 22.0±1.0°C. This rate of diel thermal change represents the higher end of typically observed rates in temperate stream ecosystems (Vannote and Sweeny, 1980). This experiment used a single larva per test chamber. Chamber volumes were 1.28±0.1 ml and each chamber was continually stirred by a mini magnetic stir bar. A small piece of stainless steel mesh separated the larvae and stir bar. When oxygen consumption rates changed markedly at 38°C, chambers were checked to assess survival. Chambers were re-checked at 40°C to confirm mortality had occurred in all chambers.

To estimate aerobic scope at different temperatures, we used the metabolic uncoupling agent FCCP to stimulate maximum oxygen consumption rates. This agent disrupts the mitochondrial proton gradient and decouples oxygen consumption from the generation of ATP (Luz et al., 2015a,b). In our experiments, we held individual larvae in rearing water and monitored oxygen consumption for four to eight respirometry cycles (200 s each for flush, hold and measure phases). The mean rates of oxygen consumption for this portion of the experiment were taken as the standard oxygen consumption rate. Then, source water for the treated larvae was switched to an aerated solution containing 30 µmol l^{-1} FCCP, 2% DMSO in rearing water using the same respirometry cycles (200 s each for flush, hold and measure phases) as were used in standard oxygen consumption measurements. Exposure of larvae to this concentration stimulates a rapid and dramatic increase in oxygen consumption rates, and larvae ultimately die from exposure. We take the maximum observed oxygen consumption rate as an estimate of maximum oxygen consumption rate for each larva and we take the difference between each larva's maximum oxygen consumption rate and standard oxygen consumption rate as an estimate of aerobic scope. We acknowledge that our estimates of maximum oxygen consumption rate may have a slight low bias as FCCP stimulates rapid increases in oxygen consumption rates until the larvae die (and oxygen consumption rates drop quickly). The temporal resolution of our respirometry (600 s between measurements) could potentially miss the absolute oxygen consumption peak. This bias would also slightly under-estimate aerobic scope. Separate studies demonstrated that DMSO did not affect standard oxygen consumption rates and the exposure solutions did not affect the performance of the sensor spots (data not shown). FCCP experiments were conducted with larvae reared at 22°C and were performed at 22°C (n=3), 26°C (n=7) and 30°C (n=5). Larvae were held at experimental temperatures for a minimum of 1–2 h prior to introducing them to the respirometry chambers.

Gene expression and lactate experiments

To understand whether thermal stress influenced the expression of genes indicative of physiological oxygen limitation, we first

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identified hypoxia responsive genes. We exposed *N. triangulifer* larvae to a series of short-term hypoxia and gradual temperature change experiments (see below) and quantified the expression of certain genes (Table 1) and gene products (lactate) in the larvae as our principal response variable. Lactate was measured (n=2) using the Lactate Assay Kit (MBL® International Corporation, Woburn, MA, USA) reading absorbance at 570 nm in a FLUOstar OMEGA® (BMG Labtech Inc., Cary, NC, USA).

Target/reference gene selection and primer design

Primers (Table 1) were designed based on a *de novo* assembly of compiled *N. triangulifer* cDNA sequence data (both 454 and Illumina platforms) resulting in ~23,000 contigs with associated bioinformatics. Based on these sequences, we selected potential genes of interest to further develop qPCR probes. For this study, the heat shock protein genes *Hsp40* and *Hsp90* were used as indicators of thermal stress whereas lactate dehydrogenase (*LDH*) and the oxygen-sensing gene *EGL-9* were used as indicators of oxygen limitation. Both *TATA-box binding protein* and *18S rRNA* were evaluated and *18S rRNA* was selected as a reference gene. Each gene of interest was inserted into a pCR2.1®-TOPO® TA vector (Life Technologies, Carlsbad, CA, USA) expression vector and produced sequences that were independently confirmed. All primers were designed with IDTSciTools (http://www.idtdna.com/SciTools/SciTools.aspx) or Primer3 v. 0.4 (http://frodo.wi.mit.edu/primer3/) and were synthesized by Life Technologies. Amplicons generated ranged from 179 to 272 base pairs.

RT-qPCR

Total RNA was isolated from *N. triangulifer* following the SV Total RNA Isolation System protocol (Promega, Madison, WI, USA). First-strand cDNA was synthesized from the same amount of each total RNA by ImProm-II™ reverse transcriptase using random primers (Promega) and all thermocycling was performed using a Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA, USA). Quantitative real-time PCR was performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA) using default parameters. Amplification mixtures consisted of 12.5 µl of SYBR Green Master Mix (Applied Biosystems), 10 µmol l^{-1} primers, 20 ng template cDNA and nuclease-free water in a total volume of 25 µl. qRT-PCR conditions were 2 min at 94°C, followed by 40 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. After the PCR reactions, the melting curve for each PCR product was determined following the manufacturer's protocols. Relative expression of each amplicon was calculated by the corrected $\Delta\Delta C_t$ method (Pfaffl, 2001), with all expression normalized to 18S rRNA levels in initial control samples. Relative levels of 18S rRNA were confirmed to be approximately equal across all treatments.

Heat and hypoxia gene expression

To validate the potential genes that will respond to hypoxia or heat stress, an initial critical oxygen tension (P_{crit}) experiment determined that in mature *N. triangulifer* larvae, oxygen supply becomes limiting at 18% saturation at 22°C. This result was used to design the following hypoxia experiments to identify heat- and/or hypoxia-responsive genes. Larvae in hypoxia treatments were first exposed to 40% oxygen saturation and then saturation was gradually lowered to either 15% or 5% saturation over 60 min. Hypoxia treatments were maintained by simultaneously bubbling nitrogen and air at different rates to maintain the desired oxygen saturation levels. A mesh filter was used to prevent hypoxia-treated larvae from accessing the air–water boundary layer and maintain them in the bottom half of the beaker where dissolved measurements were taken. All treatments had a 6 h exposure duration (inclusive of hypoxia pre-treatment time) as follows: controls (22°C, 100% oxygen saturation), hypoxia 1 (22°C, 15% oxygen saturation), hypoxia 2 (22°C, 15% oxygen saturation followed by 10 min of exposure to 5% oxygen saturation), heat challenge (32°C, 100% oxygen saturation) and heat+hypoxia challenge (32°C, 15% saturation). Sampled larvae from the hypoxia 1 treatment did not include individuals that had lost the ability to swim or maintain hold of the substrate, whereas sampled larvae from the hypoxia 2 treatment included impaired individuals. For all treatments, 10 groups of three pooled larvae were randomly collected and flash-frozen in liquid nitrogen for later RNA extraction and RT-qPCR.

Thermal ramp experiments

To study whether hypoxia responsive genes respond to gradual thermal stress, larvae were subjected to a gradual rise in temperature or 'thermal ramp' at a rate of 1°C h^{-1} from 22 to 34°C. Controls consisted of 30 larvae collected at the beginning of the experiment (just prior to increasing temperature) and larvae held at 22°C for the duration of the experiment (i.e. a 'time' control). At 4°C intervals, 30 larvae were removed from the experiment and flash-frozen in liquid nitrogen. This allowed us to compare the mRNA expression levels of individual genes at 22, 26, 30 and 34°C. Ten groups of three pooled larvae each comprised the samples for each temperature in the RT-qPCR analysis.

Thermal ramp and hold experiments

To determine how genes respond to prolonged exposure to thermal stress, larvae were exposed to a gradual increase in temperature or 'ramped' from a starting temperature of 22°C as described above. However, in this experiment, when the temperature reached 30°C, two jars were removed from the water bath and transferred to a 30°C incubator. When the water bath temperature reached 34°C, two additional jars were transferred to a 34°C incubator. Larvae were then sampled from the 30 and 34°C incubators after 14 and 24 h of

Table 1. List of primer sequences for qPCR gene expression studies

Gene name	Accession number	Primer sequence
CT18SrRNA	HM132045	Forward; 5′-AACGATACGGGACTCATCCGAGG-3′ Reverse; 5′-GGCTGTCCGATACACAGATCCAAC-3′
CTLDH	JX675218	Forward; 5′-ACACAAGCGTTCCTGTTTGGTCTG-3′ Reverse; 5′-TTTCTGAGAATGGTCTGCACCAGG-3′
CTEGL9	JF697592	Forward; 5′-CTGACCAGGAACGACCTGAAGAC-3′ Reverse; 5′-TGTTCGGATTGTCCACGTGCTTC-3′
CTHSP90	JF682769	Forward; 5′-TGAAAGATCCGCCAGCAGATGACT-3′ Reverse; 5′-ACCTGGTAAACAACCTGGGAACGA-3′
CTHSP40	JF697593	Forward; 5′-AAAGCCGGCACCAAGATCACTTTC-3′ Reverse; 5′-TCTCGCCCGTCAAGTTGATTGAGA-3′

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holding time at each temperature. Larvae held at 22°C were used as controls and larvae held at 22°C, 15% oxygen saturation were used for hypoxia treatments. To support this experiment, we measured the concentration of lactate in whole larvae and used it as an indicator of anaerobic metabolism from all treatment groups (excluding 30°C held for 24 h). Briefly, ∼45 larvae were used for each of two replicates for each treatment. Larvae were homogenized and deproteinated through successively smaller filtration spin columns in preparation for lactate measurements (as above).

Data analysis

Data analysis was performed using GraphPad Prism (v6, GraphPad Software, La Jolla, CA, USA). For survivorship and gene expression studies, one-way ANOVA was performed and Tukey's multiple comparisons test was used to analyze the differences between temperatures/hypoxia groups. Results were significant when adjusted $P \leq 0.05$. Error bars represent ±s.e.m. throughout. For respirometry studies, Q_{10} estimates were obtained from the slopes of $\log_{10}$-transformed data and a nonparametric (Mann–Whitney) test was performed to estimate aerobic scope between different temperature groups.

RESULTS

Chronic and acute thermal responses

Survivorship ranged between 83.2 and 90.9% for larvae reared from newly hatched, first instar larvae to the subimago stage at constant temperatures between 14 and 24°C (Fig. 1A). However, at 26°C, survivorship declined to 78.3% and then declined precipitously to 12.8% at 28°C. One-way ANOVA and Tukey's multiple comparisons test was performed across all rearing temperatures. The survivorship of *N. triangulifer* between 26 and 28°C was significantly different ($P<0.0001$). Larvae failed to metamorphose to the subimago stage at 30°C (but did survive for a few weeks before dying). When 30 larvae were challenged with an acute thermal ramping rate of 0.75°C h^{-1}, no larvae survived past 40°C (Fig. 1B).

Respirometry experiments

Mass-specific respiration rates ($\dot{M}_{O_2}$) of larvae exposed to a 1°C h^{-1} ramping rate rose continuously from 21.7 to ∼37°C (Fig. 2). Between 37 and 38°C, respiration rates became irregular (declined) in all larvae and chambers were checked for survival. All larvae were alive at 38.2°C and dead at 39.8°C following a second peak of consumption in three of four larvae. Data from 21.7 to 36.03°C were linearized by log transforming the oxygen consumption data (R^2=0.91–0.97, $P<0.0001$) and metabolic Q_{10} estimates derived from the slopes of the linearized data ranged from 1.70 to 1.96. Peak oxygen consumption was 1.7- to 2.4-fold higher than consumption rates at 30°C, a temperature that exceeds the long-term thermal limits of this species (Fig. 1).

An example of the effect of FCCP treatment on an individual mayfly larva is shown in parallel to an untreated larva in Fig. 3A and estimates of aerobic scope in all FCCP-treated larvae are shown in Fig. 3B. We focused our efforts on the 26°C- and 30°C-treated larvae because these temperatures bracket our estimated chronic thermal limit (between 28 and 30°C; see Fig. 1A) in this species. A nonparametric (Mann–Whitney) test was performed and estimates of aerobic scope were not statistically different between 26°C (1921±253 μg O_2 g^{-1} h^{-1}) and 30°C (1853±200 μg O_2 g^{-1} h^{-1}). On average, maximum observed metabolic rates were 2.22±0.45- and 1.98±0.37-fold higher than mean standard metabolic rates at 26°C and 30°C, respectively.

Gene expression experiments

Hypoxia experiments

For all experiments, genes expressed from the control group were normalized to 1 and Tukey's multiple comparisons test was used to analyze the differences of gene expression between treatments. Larvae with 6 h hypoxia treatment had significantly elevated mRNA expression levels for the oxygen-sensing gene prolyl hydroxylase, also known as Egg Laying Defective Nine (*EGL-9*) (∼9.2-fold higher than controls; $P<0.0001$), and *LDH* (∼6.9-fold higher than controls; $P=0.0002$; Fig. 4). These two genes provided our strongest indication of oxygen limitation at the whole organism level. Slight increases in heat shock protein (*HSP-90* and *HSP-40*) mRNA expression were also observed in response to hypoxia treatment, but were only statistically significant for *HSP-40* in the extreme hypoxia (hypoxia 2) treatment group ($P=0.03$) (Fig. 4). In contrast, increasing temperature from 22 to 32°C under conditions of normoxia stimulated significant *HSP-90* mRNA expression (∼4.9-fold higher than controls; $P<0.0001$), whereas *HSP-40* expression was only slightly elevated and not statistically significant. Thus, *HSP-90* appears to be the most thermally responsive gene, with its expression further increasing (by ∼12.5-fold; $P<0.0001$) under a treatment combination of

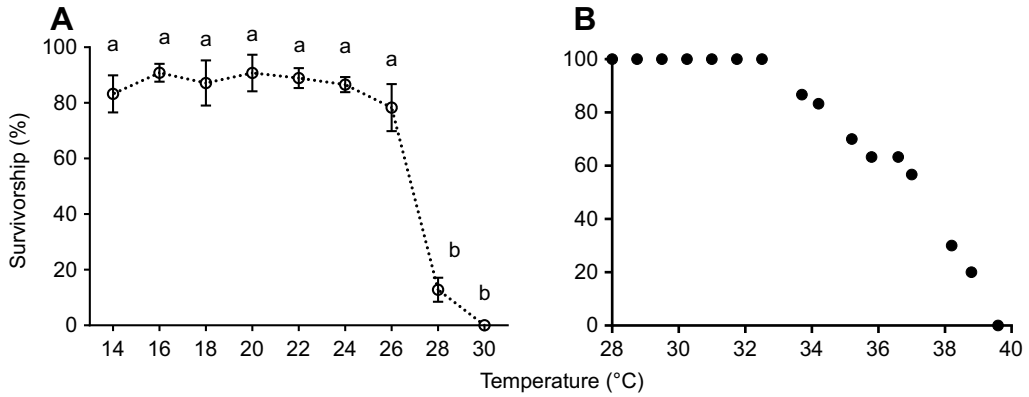

Fig. 1. Comparison of life cycle and short-term thermal limits in *Neocloeon triangulifer*. (A) Survivorship as a function of temperature in *Neocloeon triangulifer* life cycle rearing experiments. Each point represents the mean survival from rearing jars [14°C (n=8), 16°C (n=7), 18°C (n=7), 20°C (n=8), 22°C (n=11), 24°C (n=10), 26°C (n=7), 28°C (n=5) and 30°C (n=11); 50 larvae per jar]; error bars represent standard deviations from the means. Different lowercase letters indicate significant differences in survivorship between temperatures (one-way ANOVA, Tukey's multiple comparisons test; $P<0.05$). (B) Acute survival of 30 *N. triangulifer* larvae subjected to a temperature ramping rate of 0.75°C h^{-1}.

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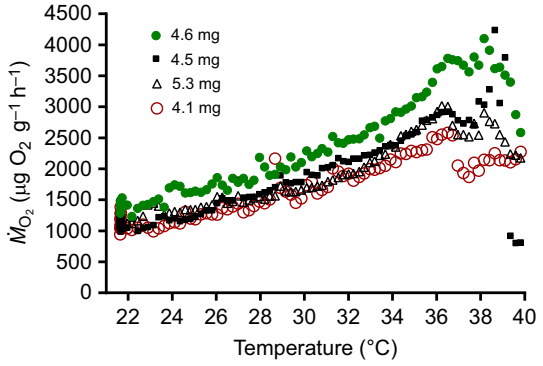

Fig. 2. Thermolimit respirometry of *N. triangulifer* larvae subjected to a thermal ramp of 1°C h^{-1} (*n*=1 larva per chamber). Larvae were confirmed to be live at 38°C and dead at 40°C. $\dot{M}_{O_2}$, metabolic rate.

higher temperature and hypoxia. It should be noted that the hypoxia-responsive gene *EGL-9* was not stimulated by heat alone, and its expression in response to the combination of heat and hypoxia was considerably smaller than for hypoxia alone. *LDH* (but not *EGL-9*) expression was mildly but not significantly stimulated by heat alone.

Thermal ramp experiments

HSP-90 was the most thermally responsive gene and consistently increased with increasing temperature to significant levels (above controls) when temperatures rose to 30 and 34°C (P<0.0001; Fig. 5A).

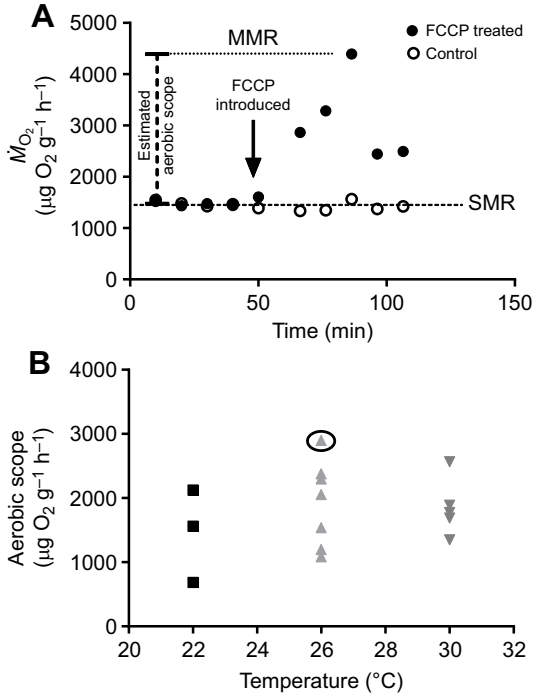

Fig. 3. Use of the metabolic inhibitor FCCP to stimulate maximum oxygen consumption rates for aerobic scope estimates. (A) An example of respiratory patterns in a larva treated with carbonyl cyanide-4-(trifluoromethoxy) pheylhydrazone (FCCP) relative to a control larva. Aerobic scope is taken as the difference between the maximum observed metabolic rate (MMR) and the standard metabolic rate (SMR). (B) Aerobic scope estimates in individual larvae as a function of temperature: 22°C (*n*=3), 26°C (*n*=7) and 30°C (*n*=5). One-way ANOVA was performed; there was no statistical difference between different temperature groups. The example shown in A is circled in B.

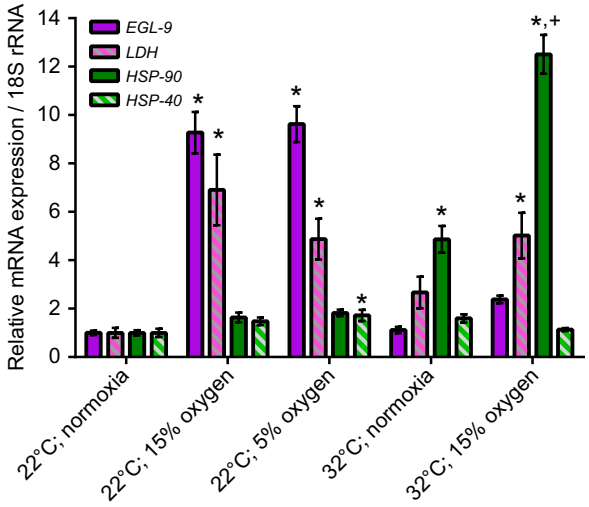

Fig. 4. Relative mRNA expression of an oxygen-sensing gene (*EGL-9*), lactate dehydrogenase (*LDH*) and two heat shock proteins (*HSP-40*, *HSP-90*) in *N. triangulifer* larvae subjected to hypoxia and/or heat treatment. Larvae were pooled in three for one biological replicate (*n*=10). All data are normalized to control (22°C) 18S rRNA. One-way ANOVA was performed and Tukey's multiple comparisons test was used to analyze the differences of gene expression between treatments. Error bars represent standard deviations from the mean. *Significant difference (P<0.05) compared with control (22°C; normoxia). $^{+}$Significant difference (P<0.05) compared with heat control (32°C; normoxia).

A similar response pattern was observed for *HSP-40* expression, but this gene was not as responsive either temporally or in magnitude relative to *HSP-90*. Neither *EGL-9* nor *LDH* responded significantly to temperature change at or below the ecologically relevant temperature of 30°C, but *LDH* expression increased significantly at 34°C (~2.2-fold higher than controls; P<0.0001).

Thermal ramp and hold experiments

Levels of gene expression for *EGL-9*, *LDH*, *HSP-90* and *HSP-40* in larvae ramped from 22 to 30°C and held at 30°C for 14 or 24 h were not strikingly different but were elevated (<2-fold) from control levels (i.e. larvae kept at 22°C and normoxia; Fig. 5B). Elevated levels were statistically significant for the temperature-sensitive genes *HSP-90* (P=0.02 at 14 h exposure to 30°C; P=0.0007 at 24 h exposure to 30°C) and *HSP-40* (P=0.008 at 14 h exposure to 30°C; P=0.007 at 24 h exposure to 30°C) but not for the hypoxia genes (except *LDH* at 14 h exposure to 30°C; P=0.03). In contrast, larvae ramped from 22 to 34°C and held for 14 h at 34°C showed very strong gene responses, particularly significant for the *HSP*s (P<0.0001) and *LDH* (P<0.0001) but not for EGL-9. In contrast, EGL-9 was strongly responsive to hypoxia (P<0.0001), while the *LDH* response to hypoxia (P<0.0001) and the 34°C treatment was comparable. Larvae did not survive being held for 24 h at 34°C. It is also noteworthy that the observed increased levels in *LDH* mRNA gene expression were associated with measures of whole-animal lactate levels in larval tissue by colorimetric assay [though small sample sizes (*n*=2) limit this analysis]. Lactate levels in larvae from the 30 and 34°C treatments held for 14 h were 1.2- and 1.9-fold higher but not statistically significant than controls, respectively. Lactate levels were significantly higher (3.2-fold higher than controls; P=0.03) in hypoxia-treated larvae.

DISCUSSION

Because insects are so important to the ecology of freshwater ecosystems and the focal group for biomonitoring programs

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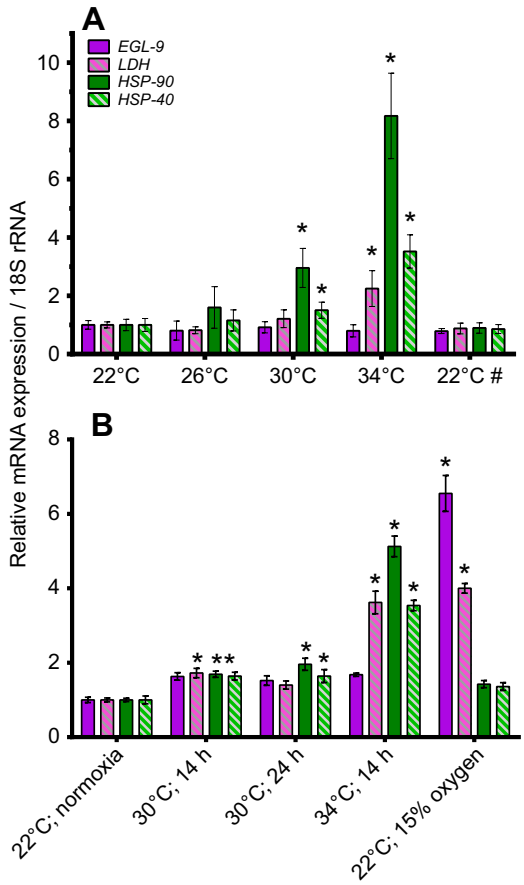

Fig. 5. Relative mRNA expression of an oxygen-sensing gene (*EGL-9*), lactate dehydrogenase (*LDH*) and two heat shock proteins (*HSP-40*, *HSP-90*) in *N. triangulifer* larvae subjected to a thermal ramping rate of 1°C h^{-1} at normoxia. Larvae were pooled in groups of three for each biological replicate (n=10). (A) Larvae were sampled at 4 h (4°C) intervals from 22°C. 22°C # larvae were held for 12 h and serve as a time control. (B) Larvae were held for 14 and 24 h at 30°C and 14 h at 34°C. All data are normalized to control (22°C) 18S rRNA. One-way ANOVA was performed and Tukey's multiple comparisons test was used to analyze the differences of gene expression between treatments. Error bars represent standard deviations from the mean. *Significant difference (P<0.05) compared with control.

worldwide, it is important that we develop a more robust understanding of the linkages between their physiology and performance. The mayfly *N. triangulifer* is emerging as a laboratory model species to study chemical pollutants (Conley et al., 2009; Kim et al., 2012; Xie et al., 2010; Struewing et al., 2014), salinity (Johnson et al., 2015; Soucek and Dickinson, 2015) and temperature (Sweeney and Vannote, 1984). Here we used life cycle rearing experiments to establish thermal reaction norms to provide context for physiological changes in *N. triangulifer* associated with increasing temperatures.

Our data are concordant with others (Kingsolver et al., 2011; Kingsolver and Woods, 2016) showing that insects can tolerate short-term thermal challenge at temperatures that far exceed their chronic thermal limits. Similarly, recent work has failed to demonstrate any correlation between CT_{max} measurements and the inferred thermal maxima of several aquatic insect species derived from ecological niche models (C. P. Hawkins, personal communication). The maximum temperature recorded from WCC, where *N. triangulifer* has maintained a viable population for many years, was 23.4°C (taken in 2012). However, our studies show that it can survive for a short time at temperatures approaching 40°C, which are exceedingly rare in temperate aquatic ecosystems. Nevertheless, in larval rearing experiments, it failed to complete metamorphosis to the adult at 30°C (Fig. 1). Thus, there is no single thermal limit, as exposure durations are critical to consider when discussing thermal limits of taxa (Vasseur et al., 2014; Schulte et al., 2011; Dillon et al., 2007; Dillon and Frazier, 2013).

Our study thermally challenged *N. triangulifer* larvae with temperatures ranging from those supporting good long-term performance to chronically lethal temperatures to better understand the physiological changes associated with single diel temperature change. Respirometry experiments performed on a thermal ramp showed expected exponential increases in oxygen consumption ceased at ∼37°C, but larvae were still live at 38°C. In both the short-term ramp experiment at 0.75°C h^{-1} and the respirometry ramp experiment at 1.0°C h^{-1}, 100% mortalilty was observed at ∼39–40°C. Regardless, the increase in oxygen consumption rates observed when temperatures were ramped from 30°C (past the chronic thermal limit) to temperatures near death (∼38°C) was substantial.

We used FCCP to stimulate estimates of maximum oxygen consumption rates, which enabled us to estimate aerobic scope in larvae challenged by a short-term exposure to a chronically lethal temperature (30°C). According to the OCLTT hypothesis, we might have expected to see a decrease in aerobic scope with warming temperatures in 22°C-reared larvae challenged at 30°C, especially considering their larger body size and tracheal architecture (Helm and Davidowitz, 2013) associated with their thermal history relative to larvae reared at a warmer temperature. However, we observed no significant change in aerobic scope, suggesting that this is not a critical factor underlying the chronic thermal limits of this species. Methods for measuring aerobic scope are not well developed for very small organisms such as mayfly larvae, and have generated controversy otherwise (Clark et al., 2013 a,b; Farrell, 2013). While our novel approach of using a chemical agent to elicit maximum oxygen consumption requires further validation in subsequent studies, it provides a promising technique for overcoming the technical challenges of estimating aerobic scope in water-breathing aquatic insects.

In our gene expression experiments (Figs 4 and 5), hypoxia treatment stimulated the expression of *LDH* and *EGL-9*. *EGL-9* is an oxygen-sensing prolyl hydroxylase that serves as an off-switch for hypoxia-inducible factor (*HIF1α*) signaling (Bishop et al., 2004; Epstein et al., 2001; Semenza, 2001; Shao et al., 2009; Shen and Powell-Coffman, 2003). *EGL-9* was largely unresponsive to thermal challenge in our gene expression experiments, indicating that exposure to high temperatures alone did not appear to activate hypoxia signaling or indicate systemic oxygen limitation. Similarly, the combination of increased temperatures and hypoxia treatment resulted in smaller increases in *LDH* and *EGL-9* expression than observed under hypoxia at cooler temperatures.

Although *LDH* and *EGL-9* expression were both stimulated by hypoxia treatment, they responded differentially to high temperature stress. *EGL-9* did not respond to high temperature stress, suggesting that *HIF1α* signaling was not stimulated by these temperatures. *LDH* expression did increase but only at temperatures exceeding ecological limits. This result could be interpreted in at least two ways. First, Li et al. (2013) demonstrated that hypoxia upregulates *LDH* gene expression in a HIF-independent manner in *Drosophila* larvae [late larval stage (L3)]. Therefore, the upregulation of *LDH* gene expression we observed in *N. triangulifer* at temperatures exceeding ecological thermal limits could be evidence of functional hypoxia. Second, we also note that we used whole mayfly larvae for gene

expression studies; therefore, the increased *LDH* expression may indicate that not all tissues received adequate oxygen supply and some anaerobic metabolism was occurring in the larvae. Third, thermal stress may have an effect on energy metabolism. While the rate of pyruvate entering the tricarboxylic acid (TCA) cycle is oxygen-dependent, the activity of LDH is oxygen-independent (Callier and Nijhout, 2014). We speculate that mayfly larvae undergo rapid aerobic glycolysis in response to short-term thermal challenge. In this scenario, the rate of pyruvate production exceeds the rate that it can be incorporated into the TCA cycle, and is transformed by LDH into lactate (Callier and Nijhout, 2014). Therefore, an observation of *LDH* gene upregulation does not necessarily indicate that larvae are oxygen deficient. One study in developing *Drosophila* larvae also showed the phenomenon of aerobic glycolysis, and associated it with rapid growth rates (Tennessen et al., 2011).

Taken collectively, our results suggest that although the OCLTT hypothesis may be appropriate for explaining responses to rapid short-term warming, it does not explain thermal limits in the mayfly *N. triangulifer* at temperatures that are chronically lethal, and perhaps more ecologically relevant. Respirometry experiments revealed no change in aerobic scope at temperatures that bracketed the chronic thermal limits of this species, and gene expression experiments showed marked differences between responses to heat and hypoxia and little evidence for widespread expression of hypoxia responsive genes at temperatures up to the ecologically relevant limit. Although the data presented here represent physiological responses to short-term thermal change, ongoing studies are evaluating gene expression and metabolomics changes in chronically reared mayflies. These processes are important to understand, particularly in relation to understanding responses to multiple stressors, which are predominant in natural ecosystems (Buchwalter et al., 2003; Piggott et al., 2012; Sokolova et al., 2006). However, our results do suggest that oxygen limitation may indeed play a significant role when *N. triangulifer* larvae are warmed beyond their ecologically relevant thermal limit up to their acutely tolerated temperatures. It is also possible that the respiratory challenge observed during the molt (Camp et al., 2014) may introduce transient oxygen limitation, which may become more important as larvae age. The field of toxicology has long realized that mechanisms associated with acute and chronic toxicity may be very different. Our data suggest that the same is likely true for temperature.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: B.S., D.F., D.B.; Methodology: H.C.; Investigation: K.K., H.C., D.F., D.B.; Writing - original draft: H.C., D.F., D.B.; Writing - review & editing: K.K., J.J., B.S.; Project administration: D.B.; Funding acquisition: D.B.

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