Why adult mayflies of *Cloeon dipterum* (Ephemeroptera: Baetidae) become smaller as temperature warms

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Abstract: We reared *Cloeon dipterum* from egg hatch to adult at 10 constant temperatures (12.1–33.5°C) to test 3 hypotheses (thermal equilibrium hypothesis, temperature size rule [TSR], and O_2- and capacity-limited thermal tolerance [OCLTT]) that account for variation in life-history traits across thermal gradients. Male and female adult size declined ~67 and 78% and larval development time declined ~88% with warming; chronic survivorship (thermal limit for population growth) was highest from 16.2 to 23.9°C (mean = 85%) and declined to 0 at 33.5°C; thresholds for 0 growth and development were 10.0 and 10.7°C, respectively; peak rate of population increase (r) occurred at 27.8°C; rates of growth and development were maximal at 30°C; fecundity was greatest at 12.1°C; and between 14.3 and 30°C, growth and development rates increased linearly and the number of degree days (>10.7°C) to complete development was nearly constant (mean = 271). Acute survivorship during short-term thermal ramping was 0 at 40°C. Warming temperature caused development rate to increase proportionately faster than growth rate; male and female adult size to decrease as per TSR, with adult females ~5× larger at 12.1 than 31.7°C; adult size to decrease proportionately more for females than males; and fecundity to decrease proportionately more than adult female size. TSR was related to differences in the responses of growth and development rates at temperatures above thresholds rather than to thresholds for growth or development per se. Respirometry suggested that OCLTT is more applicable to acute than chronic thermal limits. *Cloeon dipterum* appears to have a thermal 'acclimation zone' between 14.3 and 30°C where development and growth rates change linearly and degree-day requirements to complete metamorphosis are constant. The optimum temperature is ~27.8°C where r is maximum. We propose 5 hypotheses to explain these patterns.
Key words: temperature, growth, development, fecundity, respiration, aquatic insect, aerobic scope

About a half-century ago, freshwater scientists became alarmed by the local extinction of macroinvertebrates in streams and rivers below power plants and dams, which were subject to artificial warming and cooling (Coutant 1962, 1972, Pearson et al. 1968, Hilsenhoff 1971, Spence and Hynes 1971a, b, Lehmkuhl 1972, 1974). This phenomenon was puzzling because the degree of warming or cooling often appeared to be within the range of temperatures to which the species were exposed on a seasonal basis or throughout their known geographic range, which suggested that the warming and cooling ought to be nonlethal. These concerns triggered a new ecological subdiscipline, thermal ecology, to study the structure and function of aquatic ecosystems influenced by temperature (see Gibbons and Sharitz 1974).

In response, Sweeney and Vannote (1978, p. 445) proposed 2 thermal equilibrium hypotheses (TEHs) to explain the extinction patterns for aquatic insects in thermally modified streams and rivers and the potential role played by temperature in limiting the natural geographic distribution of species along latitudinal gradients. Specifically, they proposed that: 1) maximum adult size reflects an equilibrium between several developmental processes that appear to be temperature dependent (the rate and duration of larval growth, the specific time in larval development when adult structures begin maturing, and the rate of this mat-

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uration process); and 2) a species' distribution locally within drainage systems and over large geographic areas is limited, in part, by lowered fecundity as adult size gradually diminishes in streams of increasingly cold or warm temperature cycles. The basis for these hypotheses was intuitive—that the growth rate and the time spent growing determine size and fecundity. However, both hypotheses were implicit in that the response of larval growth rates and development rates to temperatures that are cooler or warmer than some optimum would alter the growth–development relationship such that growth was consistently compromised more than development, which produced a reduction in size and fecundity. Therefore, Sweeney and Vannote (1978) proposed that the small adult size and reduced fecundity (hence low levels of population recruitment and growth) were the basis for the extinctions of aquatic populations caused by the warming and cooling of stream and river thermal regimes, either artificially from dams or power plants, or naturally from thermal gradients associated with stream order, elevation, or latitude. Their proposal suggested that a "species distribution both locally within drainage systems and over large geographic areas was limited, in part, by lowered fecundity as adult size gradually diminishes in streams of increasingly cold or warm temperature cycles" (Sweeney and Vannote 1978, p. 445). Vannote and Sweeney (1980) then proposed a conceptual ecophysiological model for the energetics and developmental dynamics involved in regulating fitness (as a function of adult size and fecundity) and in the geographic distribution of aquatic insects. The model was designed to provide a new perspective for establishing guidelines for stream and river temperature management—namely, that temperature regimes, both warmer and cooler than some optimum, could lead to extinction of aquatic insects by diminishing fitness at both the individual (size and fecundity) and population levels (effective population size). These novel hypotheses were based on limited laboratory studies of a few species reared over a limited temperature range, buttressed by anecdotal field observations of a few natural species in eastern North America.

Subsequent investigators raised significant questions about aspects of TEH, but they also supported some of the underlying assumptions. For example, Atkinson (1994, 1996) used data on a large number of terrestrial and aquatic ectotherms to propose a temperature size rule (TSR), whereby individuals grow more slowly at cold temperatures but attain a larger adult size (recently reviewed by Kingsolver and Huey 2008, who refer to this phenomenon as "hotter is smaller" [p. 257], Forster and Hirst 2012, Forster et al. 2012, Klok and Harrison 2013). Thus, the TSR suggested that maximum adult size did not occur at some point between the warmest and coolest temperature (as suggested by Sweeney and Vannote 1978) but instead always occurred at the colder end of the species' range of temperature tolerance. Cabanita and Atkinson's (2006) experimental data and analysis also called into question the decline in adult size at lower temperatures as the TEH had suggested. Atkinson's (1994, 1996) TSR was based largely on studies in which the range of temperatures included only temperatures that were nonstressful to growth because, as he acknowledged in the paper, the logical expectation was that extreme temperatures, colder or warmer than the nonstressful range for a species, would produce declining body sizes.

On the other hand, Kingsolver and Huey's (2008, p. 253) review provided support for TEH that increasing size equates to increasing fitness (sensu larger adult body size contributes positively to mating success, fecundity, and overall survival, or "bigger is better" in their words). However, we caution, that the bigger-is-better idea may not hold for insect species that have >1 life cycle/y (Zeuss et al. 2017). For these species, a tradeoff might exist between maximizing individual body mass (hence fitness), which depends on longer development times, vs having shorter development times but more generations of offspring completing development during the year. Thus, temperatures that maximize individual fitness may differ from those that maximize population growth rates, which require balancing fecundity, development time, and survivorship.

Understanding the physiological basis for TEH or TSR is critical to interpreting the life-history patterns observed in nature and to predicting life-history phenomena in response to changing climate. This necessity makes it tempting to consider the TSR a special case of Bergmann's (1848) Rule (i.e., populations and species of larger size are found in colder environments). However, this viewpoint may not be very instructional because although TSR and thermal equilibrium-type responses appear to be outcomes of phenotypic plasticity, latitudinal gradients of size (such as a Bergmann cline) may reflect a genetic gradient (Horne et al. 2015). Moreover, no simple, general explanation for a Bergmann cline has been forthcoming, and one may not exist (see Angilletta and Dunham 2003 for discussion). A better approach might be to seek underlying physiological mechanisms in individual species. For example, Van der Have and de Jong (1996) proposed a biophysical model showing that the temperature coefficient for growth must be lower than the temperature coefficient for development for the size of ectothermic animals to decrease as temperatures increase. In a review of growth and development data for marine copepods, Forster et al. (2011b) found empirical evidence that growth and development rates were uncoupled with increasing temperature, and Forster and Hirst (2012) subsequently showed that these differences were ontogenetic for the brine shrimp *Artemia franciscana*.

Understanding the mechanisms involved in the responses of larval growth and development to temperature changes will lead to a better understanding of TEH and TSR. Recent advances in our understanding of the underlying physiology associated with the completion of develop-

ment (metamorphosis) and its timing in terrestrial insects (Hatem et al. 2015, Nijhout 2015) have provided significant new insights into the underlying mechanisms controlling variation in adult size. These authors have proposed 2 different mechanisms for controlling "the specific time in larval development that adult structures begin maturing and the rate of that maturation process" (sensu TEH of Sweeney and Vannote 1978).

First, studies of *Manduca sexta* (L.) (reviewed by Hatem et al. 2015, Nijhout 2015) suggest that a minimum critical mass must be exceeded for the secretion of juvenile hormone (JH) to stop and the catabolic enzyme, JH-esterase, to be up-regulated, which then removes JH from the system and releases the production of ecdysone. This combination leads to metamorphosis. The mechanism for sensing when the critical mass is achieved appears to involve O_2 limitation within the insect's tracheal respiration system. Thus, an individual advances toward metamorphosis when it senses that its respiration system can no longer provide sufficient O_2 to sustain advanced body growth.

Second, studies of *Drosophila* (Hatem et al. 2015) also suggest a minimum critical mass that is sensed, either through O_2 restriction or, more likely, through a nutrient-sensitive pathway (i.e., diet leading to increased ecdysone levels). However, in contrast to *Manduca*, JH does not regulate ecdysone production and is absent in the last instar of *Drosophila*. In addition, these reviews indicate that, for both *Manduca* and *Drosophila*, the insulin/target of rapamycin (TOR) pathway, which is a conserved regulator of cell and organism growth in metazoans, plays a key role during the final larval growth phase, although its effect is suppressed by the presence of JH (JH). Thus, Hatem et al. (2015, p. 10) suggested that, overall, the "trade-offs between body size and developmental speed are mediated by levels of JH," such that "JH serves to delay metamorphosis until a large enough size is attained, whereas in the absence of JH, attaining sufficient nutrition promotes the onset of metamorphosis." Regardless of which of the 2 mechanisms is correct, Ghosh et al. (2013) suggested that thermal plasticity in the critical minimum size could mediate the responses described by TSR and TEH.

The degree to which the above insights from terrestrial holometabolous insects can be applied to hemimetabolous aquatic insects, such as mayflies (Insecta:Ephemeroptera), which are the focus of our study, remains to be seen. However, mayflies are very primitive insects, whose ancestral traits stem back to the first flying insects, and their primitive traits are likely to represent the precursor of related traits in more advanced insect groups like *Manduca* and *Drosophila*. Regardless, the work of Hatem et al. (2015) and Nijhout (2015) sheds new light on a recent proposal that O_2, either in addition to or in combination with temperature, is another key factor affecting the geographic distribution of ectothermic animals and patterns, such as proposed by TEH and TSR (Atkinson et al. 2006). The central argument regarding O_2 is that 'aerobic scope' declines at temperatures beyond the 'thermal optimum' of a species and vanishes at extremely warm temperatures, when anaerobic (mitochondrial) metabolism predominates because of the insufficient capacity of an organism's circulation/ventilation system and the declining availability of O_2. This proposition was originally termed the oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis, which was developed in a series of papers by van Dijk et al. (1999), Pörtner (2001, 2002, 2010), and Pörtner et al. (2006) and was expounded further by Atkinson et al. (2006). Thus, in aquatic organisms, O_2 may become limiting at warm temperatures because of a mismatch between high demand (as a result of high metabolism) and low supply (because of low solubility) (see Verberk et al. 2011, Hoefnagel and Verberk 2015). Clearly, environmental O_2 concentrations must be factored into the discussion of TEH and TSR, given the effect of tissue O_2 concentrations on critical mass in determining the timing and size at metamorphosis (Hatem et al. 2015, Nijhout 2015).

Further progress in applying these concepts to insects requires that we better understand the thermal reaction norms of individual study species, which will enable us to link physiological processes explicitly to well-defined life-history outcomes. For aquatic insect species, such thermal reaction norms have been elusive because of the difficulty of rearing individuals of a given species in the laboratory across its entire range of thermal tolerance for its entire larval growth and development period (i.e., egg hatch to adult) and quantifying key life-history characteristics, such as overall growth, survivorship, and adult fecundity. We have resolved that challenge and provide those data for a mayfly species, *Cloeon dipterum* L., which is part of a multivoltine species complex distributed throughout Europe, Eurasia, and eastern North America (Sowa 1975, Bae and Park 1997, Randolph et al. 2002).

We propose that *C. dipterum*, and mayflies in general, are ideally suited for this type of analysis because the adults do not feed. Energy for growth and reproduction is accumulated only during the larval stage, so larval rearing conditions (temperature, O_2, food) completely determine adult size and fecundity. We used laboratory data on *C. dipterum* to provide insights into the role of temperature regarding the following 4 important issues (and associated questions) related to the thermal ecology of aquatic insects:

1. **Adult size and fecundity (TEH vs TSR).** *Question 1*: does maximum individual size and fecundity occur near the middle of the thermal range of a species (sensu TEH of Sweeney and Vannote 1978) or at temperatures closer to the lower thermal limit (sensu TSR of Atkinson 1994, 1996)?
2. **Adult size and the developmental and bioenergetic aspects of metamorphosis.** *Question 2*: does variation in adult size reflect, in part, an energetic disequilibrium between the partitioning of assimilated

energy between growth and metabolism as it affects the rate and duration of larval growth (sensu Vannote and Sweeney 1980)? *Question 3*: do the current hypotheses for the physiological basis of insect metamorphosis (Hatem et al. 2015, Nijhout 2015) reconcile with Vannote and Sweeney's (1980) hypothesis that maximum adult size reflects an equilibrium between several developmental processes that appear primarily temperature dependent (viz., the rate and duration of larval growth, the specific time in larval development that adult structures begin maturing, and the rate of this maturation process)?

3. **O_2 availability, larval size, and the timing of metamorphosis.** *Question 4*: does declining O_2 availability as temperature increases create a mismatch between O_2 supply and demand in individual larvae, such that the O_2 limitation per se sets the physiological limits for reproduction and survival of a given species (sensu the OCLTT hypothesis). *Question 5*: does O_2 limitation set the limits for reproduction and survival of a given species by reducing the minimum critical larval mass/size at which individuals in a population become committed to metamorphosis (sensu physiological models of Hatem et al. 2015 and Nijhout 2015)?
4. **Thermal optimum vs thermal acclimation zone.** *Question 6*: for aquatic insect species, what is the relationship between the thermal acclimation zone, where rates of growth and development increase or decrease linearly and degree-day requirements to achieve metamorphosis are constant, and the thermal optimum where the intrinsic rate of population increase (r) is maximized?

METHODS

Study animals

Mayflies used in our study were collected from a small (0.144 ha), shallow (≤1 m), fishless pond situated at lat 39.865118°N, long −75.817749°W adjacent to the western fork of the East Branch of White Clay Creek, Chester County, Pennsylvania, USA. These specimens fit Sowa's (1975) concept of *Cloeon cognatum* Stephens 1835, where *C. cognatum* is generally treated as a junior synonym of *C. dipterum*. Under this broad concept, genetic analysis of *C. dipterum* has recently shown that it includes ≥4 species in Europe, 2 of which are now also present in eastern North America (Rutschmann et al. 2017). Specimens used in our study were referred to as species IS1 by Rutschmann et al. (2017). Two representatives from the population we used were sequenced for the mitochondrial gene cytochrome *c* oxidase subunit I (COI; GenBank® accession numbers HM399117 and HM399118). The sequences were nearly an identical match of the sequence for specimens from 2 locations in southern Europe (Italy and Bulgaria). Nomenclatural changes have not yet been formalized, so we follow current convention and refer to our test mayflies as *C. dipterum*.

Laboratory culturing of mayflies

We maintained mayflies for these experiments in laboratory culture for up to 8 generations. Mayflies are unique among insects in that larvae metamorphose into a winged subimago stage, which then molts (without feeding and usually within 24 h) into the imago or true adult stage. Hereafter, we refer to the winged mayflies produced in our experiments simply as adults. We induce mating between male and female adults in the laboratory by using a technique similar to that described by Huff and McCafferty (1974) for *Stenonema femoratum*. After mating, females are held at room temperature (20–24°C) prior to oviposition. *Cloeon dipterum* is ovoviviparous with fertilized eggs that develop inside the adult female and are released into the water when embryos are fully formed. At room temperature, females oviposit 15 to 19 d after mating. Eggs hatch in <1 min of oviposition.

Experimental rearing of mayflies for whole life-cycle tests

We reared mayfly larvae from 1st instar (<1 h old) to the adult in 1.9-L glass vessels starting with 40 or 50 individuals/vessel. We immersed jars in a water bath held at constant temperature (see below). An air stone in each jar ensured O_2 saturation at a given temperature throughout the experiment. Food consisted of a 1- to 3-mm-thick coating of periphyton (predominantly diatoms) grown on 23- × 6.4- × 0.16-cm acrylic plates. The diatom coating was produced by streaming White Clay Creek (WCC) water continuously over the plates for ~4 wk in a greenhouse. Food was provided ad libitum. We placed plates colonized with algae in the rearing jars, and larvae grazed the algae directly from the plates as needed. Providing the algal food in this manner allowed easy assessment of the status of food in each vessel, and we exchanged new algal plates as necessary to ensure an abundance of high-quality food throughout the experiment. A screened cage was fitted tightly over the top of each rearing vessel to capture newly emerged adults. Subimagos were collected and counted daily and then either reared to the imago stage (<24 h) in cages or placed directly in a drying oven (50°C) for a minimum of 5 d, then weighed individually on a microbalance to the nearest 0.01 mg.

Thermal treatments were achieved with the aid of a custom-designed system involving 5 independent recirculating water baths, with temperature controlled to within ±0.05°C of the set-point temperature in each bath. We ran constant temperature experiments at 10 temperatures ranging from 12.1 to 33.5°C (Table S1). Four to 8 replicates (rearing vessels), each begun with 40 or 50 1st-instar larvae were involved at each temperature. Simulated daylight was pro-

vided by 2.43-m long fluorescent grow lights. All experiments involved a 15 : 9-h light : dark cycle.

Experiments to determine acute lethal thermal tolerance (Ct_{max})

We tested 2 size classes of *C. dipterum* larvae: large (i.e., nearly full grown by mass) and small (~¼-grown by mass), with both sets of larvae reared at 20.2°C. On day 1, we placed 30 larvae of each size class individually in separate 30-mL beakers with 15 mL WCC water and kept them at 22°C from 1100 to 1630 h, when temperature was increased at a rate of 0.75°C/h. Observations were begun (at ~0740 h on day 2) when experimental temperatures reached 33.7°C and were repeated hourly until all larvae had died.

Respirometry experiments at constant temperatures

We conducted respirometry on larvae at North Carolina State University (NCSU). We used artificial soft water (ASW; 48 mg/L $NaHCO_3$, 30 mg/L $CaSO_4{\cdot}H_2O$, 30 mg/L $MgSO_4$, and 2 mg/L KCl; ASTM International, West Conshohocken, Pennsylvania) as the culture and experimental water in all experiments. We conducted initial experiments with larvae reared from the eggs of a single *C. dipterum* adult. The eggs were initially deposited into a Petri dish containing ASW at 22°C. Eggs hatched upon wetting and were immediately divided into 3 groups, with each group kept at a different constant temperature (17, 22, or 27°C) in incubators with 12 : 12-h light : dark cycles. We performed subsequent experiments at 19.5 and 24.5°C. Rearing chambers were 2-L mason jars containing 1.8L ASW, and larvae were provided with periphyton plates (as described above) produced in Pennsylvania (Stroud Water Research Center) and shipped overnight to NCSU. Jars were aerated and covered with parafilm to prevent evaporative loss. We maintained larvae until they reached an adequate size (≥ 0.34 mg dry mass [DM]) for the measurement of O_2 consumption as individual larvae.

We conducted all respirometry experiments with a 4-channel, fiber-optic-based, intermittent-flow respirometry system (Loligo Systems, Tjele, Denmark) equipped with AutoResp® software. Each vertical chamber (1.28 ± 0.1 mL) was fitted with a glass spacer ring and stainless steel mesh to separate larvae from a magnetic stir bar. The stir system ensured that the chambers were well mixed for dissolved O_2 measurements. A peristaltic pump refreshed chamber solutions in programmed 15-min intervals, with each respirometry cycle consisting of a flush phase (300 s), a wait phase (350 s), and a measurement phase (250 s). We submerged chambers in an aerated, temperature-controlled water bath (~4 L), which was monitored continuously for dissolved O_2 and temperature. Temperature was controlled by a programmable heater/chiller (12108–30; Cole Palmer, Vernon Hills, Illinois). A stainless steel heat-exchange coil was immersed in the well of the temperature controller, and a water pump continuously circulated water (5 L/min) from the respirometry water bath through the heat-exchange coil. An inline 5-W ultraviolet filter was installed to inhibit microbial activity during all respirometry experiments.

Thermolimit respirometry

In a separate experiment, we chose 4 larvae representing individuals ~⅓ to ½ grown at random from a group being reared at 22°C at North Carolina State University. They were placed in the respirometer at 22°C and subjected to a temperature increase of 1°C/h until they died.

Data analysis: life-cycle testing

For all full-life-cycle tests, we treated individual rearing vessels as replicates. We calculated survivorship as the percentage of 1st-instar larvae surviving to the adult stage, and we arcsine-transformed these data for statistical analysis. We calculated development time as the number of days from the start of the experiment (egg hatch) to the median day of adult emergence from a given rearing vessel and development rate as the reciprocal of median development time. Instantaneous growth rate (IGR) was the average growth in mass/d from 1st-instar to adult and was calculated as:

$$\mathrm{IGR} = \frac{ln\frac{W_f}{W_i}}{t}, \qquad \text{(Eq. 1)}$$

where W_f is final adult DM (mg; gravid DM in the case of females), $W_i = 0.0009$ mg (hatchling DM), and t is development time (d). Temperature thresholds (and their confidence intervals [CIs]) for growth and development were extrapolated from the linear portion of the instantaneous growth- and development-rate curves (i.e., 14.3–30°C) based on regression with replication (Zar 1999). Adult DM was averaged for each rearing vessel and increased by 4% to account for lost mass associated with molting from the subimago stage to the true adult. Estimation of fecundity of *C. dipterum* adults was challenging because the species is ovoviviparous. However, like other mayflies, *C. dipterum* adults do not feed, and thus, females emerge from the aquatic larval stage with sufficient energy reserves to enable the maturation of eggs. Therefore, we estimated fecundity for each female by mating the individual, holding her 15 to 19 d until oviposition, and then counting the number of hatchlings.

Eventually, we were able to predict fecundity empirically from female DM as follows. First, 98 individual females, representing the entire range of body sizes observed in our study, were mated and held until oviposition. We counted the number of hatchlings (fecundities ranged from 0 to 2427) and measured the wing length of each female. The relationship between wing length (y) and fecundity (x) was: $y = 416.55x - 2320$ ($R^2 = 0.88$; Fig. 1A). Second, we measured

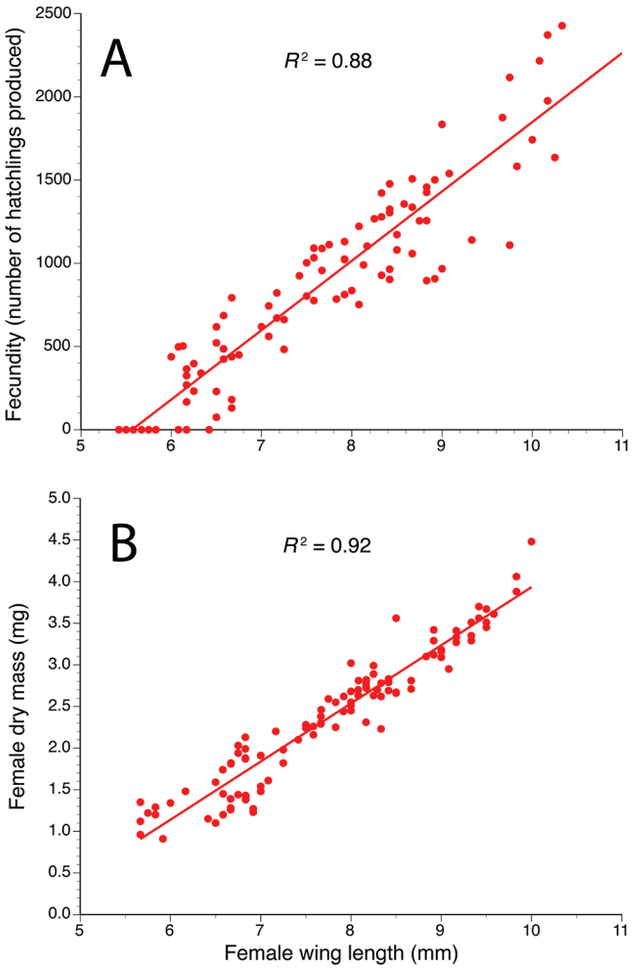

Figure 1. The relationship between female wing length of *Cloeon dipterum* and fecundity (A) and female dry mass (B).

wing length of 105 individual females and then oven-dried (50°C) and weighed the females. The relationship between DM (y) and wing length (x) was $y = 1.312x + 4.652$ ($R^2 = 0.92$; Fig. 1B). Third, we combined these 3 regressions into a single formula describing fecundity (y) as a function of DM in mg (x): $y = 416.55(1.312x + 4.652) - 2320$.

r (per capita rate of population increase)

Oviposition in mayflies is a single event, so no need exists to construct female life- and age-specific fecundity tables and then calculate r by solving differential equations with an iterative approach (Birch 1948). Instead, we calculated r directly for each vessel as:

$$r = \frac{(lnN_t - lnN_0)}{t}, \qquad \text{(Eq. 2)}$$

where N_t is the number of females emerged × their mean fecundity/2 (assuming 50% will be female) × 0.94 (mean survivorship to oviposition as estimated with measurements on 359 mated females in the laboratory), N_0 is ½ the number of larvae starting in that vessel (assuming 50% will be female), t is the median development time from hatching to emergence of the adult for females in that vessel, with 1 d added for the time to molt from subimago to true adult and subsequently mate and 15 d added for gestation of embryos (assuming a constant air temperature of 24° during incubation). Thus, for *C. dipterum*, r is the per capita rate of population increase in a closed population. r incorporates constant age-specific schedules of survival and reproduction and assumes a stable age distribution. Because r is scaled to time, it is strongly affected by generation time. Given our focus on the effect of water temperature, we assumed a constant air temperature of 24°C for the adult stage and used the same survivorship and gestation time across all treatments. Moreover, we made no correction for mortality or fertility in the egg stage because our fecundity figures were based on counts of live, newborn 1st-instar larvae.

Data analysis: respirometry

The AutoResp® software produced an O_2 consumption rate from each measurement cycle and a corresponding correlation coefficient based on the slope of the line describing individual mass-specific respiration rate as a function of individual larval mass. Most respirometry cycles had R^2 values >0.9. We did not consider those that did not meet this threshold for analysis. We omitted data from the first several respirometry cycles at a given constant temperature to avoid considering data that may have been biased by initial handling stress. We made all statistical comparisons of respirometry experiments with t-tests in GraphPad Prizm (version 6.0; GraphPad Prizm, San Diego, California).

RESULTS

Whole-life-cycle response to constant temperatures

Survivorship of *C. dipterum* from 1st-instar to adult was consistently high between 16.2 and 23.9°C and declined (and was more variable) at both warmer and cooler temperatures (Fig. 2A). At the coolest temperature (12.1°C), the sex ratio of survivors was 1.62 : 1 (male : female), which differed significantly from the expected 1 : 1 ratio (binomial test) and from results of experiments at all other temperatures. We observed complete mortality at 33.5°C.

Median development time declined exponentially with temperature (Fig. 2B), from 110 days at 12.1°C to 13.5 d at 30°C, and then rose slightly to 16.9 d at the highest sublethal temperature (31.7°C). Variability in development time was low in all treatments. The differences in development time as a function of temperature were significant (analysis of variance [ANOVA], $p < 0.05$), and all pairwise differences between temperature treatments were significant (except between 27.8 and 31.7°C; Tukey's test, $p < 0.05$).

Adult DM decreased with increased temperature by a factor of ~4.4 for females and ~3.1 for males (Fig. 2C). Fe-

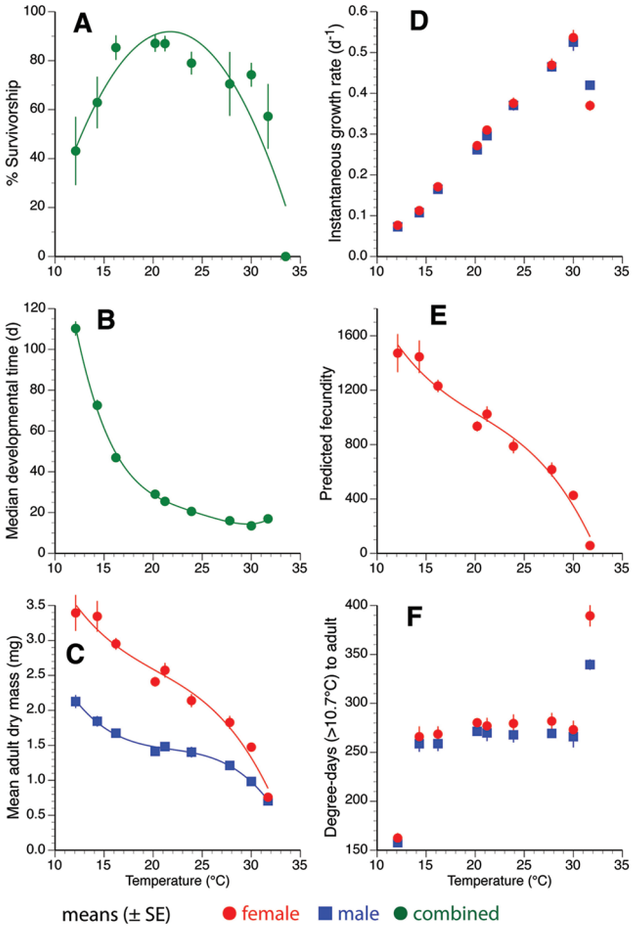

Figure 2. The relationships among water temperature and mean (±SE) survivorship (2nd-degree polynomial) (A), median development time (sexes combined; 4th-degree polynomial) (B), mean adult dry mass (3rd-degree polynomial) (C), instantaneous growth rate (D), fecundity (3rd-degree polynomial) (E), and degree days to reach adulthood (F).

males had a greater % change in mass/°C than males (4.4 vs 3.6%, respectively), even when we eliminated data points near the extreme (e.g., 31.7°C) from the analysis. This % change differed significantly between males and females based on a multiple regression relating adult DM to temperature, an indicator variable separating males (value = 0) from females (value = 1), and including the temperature × indicator variable interaction. The interaction-term coefficient was significantly different from 0 ($p < 0.007$), confirming the sex-dependent relationship between adult DM and temperature where females proportionately lost mass faster than males as temperature increased (Appendix S1, Fig. S1).

IGR increased linearly with temperature up to 30°C, but declined precipitously in the 31.7°C treatment (Fig. 2D). Fecundity was proportional to female DM (Fig. 2E), and predicted means ranged from 1438 at 12.1°C to 30 at 31.7°C, but no adult female from the 31.7°C treatment actually produced hatchlings.

Thermal thresholds for development and degree-day requirements

We used regressions relating IGR and development rate to temperature to calculate a developmental threshold (i.e., 0 development) of 10.7°C for both males and females (females: CI = 8.8–12.6°C, males: 8.6–12.9°C) and a growth threshold (i.e., 0 growth) of 9.9°C (CI = 7.0–12.7°C) for females (Fig. 3A) and 10.1°C (CI = 7.2–13.1°C) for males (Fig. 3B). Based on the developmental threshold, we estimated that *C. dipterum* required nearly the same number of degree days (mean: ~266; range: 259–271) in all treatments between 14.3 and 27.8°C to complete development (Fig. 2F). However, fewer degree days were required at 12.1°C (males: 158, females: 162), and substantially more were required at 31.7°C (males: 340, females: 389).

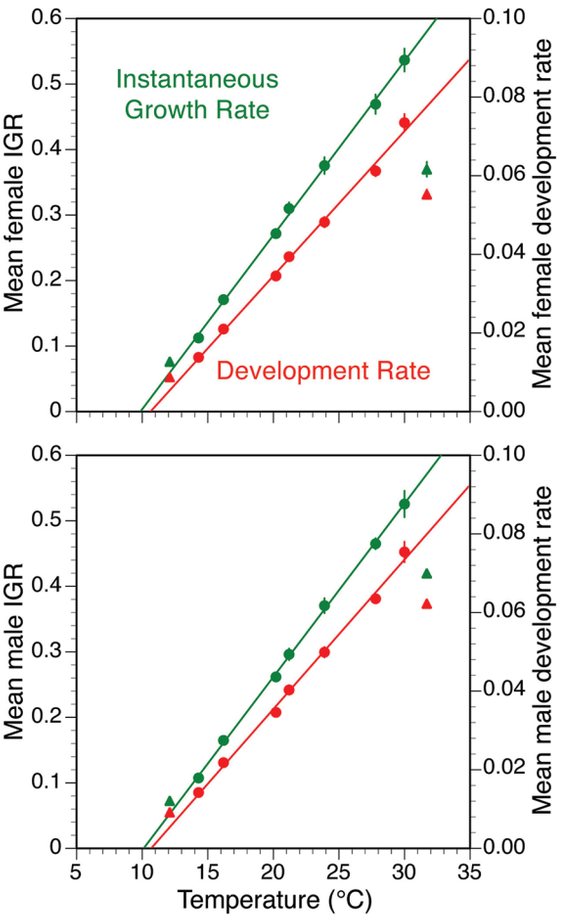

Figure 3. Mean (±SE) instantaneous growth rate (IGR) and development rates for females (A) and males (B) as a function of temperature, with trend lines for the linear portion of the curves (i.e., 14–30°C). Repeated measures regression was used to extrapolate thresholds (and their confidence intervals) for growth and development.

r(per capita rate of population increase)

r increased with temperature to a maximum at ~27.8 to 30.0°C and then declined significantly at 31.7°C (Fig. 4).

Acute lethal thermal tolerance (CT_{max})

Cumulative mortality during acute exposure to high temperatures depended on larval size (Fig. 5). Small larvae (i.e., ¼ grown) began dying at lower temperatures (34–36°C) than large larvae (full grown; >36°C). However, the temperatures associated with 50 and 100% mortality levels were about the same for small and large larvae (~39 and 40.2°C, respectively). Thus, CT_{max} appears to be ~40°C. However, we did not test statistically for differences between small and large larvae with respect to their sensitivity to heat (e.g., by fitting survival functions).

Respirometry

On an individual basis, O_2 consumption rates as a function of body size increased in a linear fashion across all temperatures (Fig. 6A). When respiration rates were expressed and analyzed on a mass-specific basis, the slopes of lines relating O_2 consumption/unit tissue mass to larval size were all negative or flat (with 1 exception), and no significant differences were observed among the slopes based on analysis of covariance on log(*x*)-transformed data (data not shown). However, for an animal of a specific size, mass-specific O_2 consumption rates increased as a function of temperature (Fig. 6B). Further study (viz., thermolimit respirometry experiments) showed that the increase in O_2 consumption (metabolic demand) per unit tissue with temperature for different size individuals was well described by an exponential relationship between tissue O_2 consumption and temperature but better (higher overall r^2) described by 2 distinct linear relationships (one for temperatures ~22–~32°C, one for temperatures ~32–~38°C (Fig. 6C) with the break point (~32°C) representing the highest temperature conducive to successful metamorphosis.

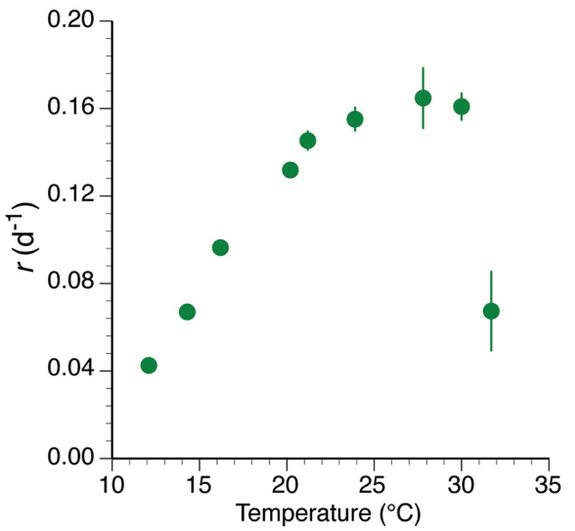

Figure 4. Per capita rate of population increase (*r*) for female *Cloeon dipterum* across a range of rearing temperatures.

DISCUSSION

We attempted to minimize confounding variables (photoperiod, food, and crowding effects) in the design of our study to unravel the effects of chronic (egg hatch to adult) exposure of larvae to a wide range of water temperatures to a degree not possible in previous studies (Sweeney and Vannote 1978, 1981, Vannote and Sweeney 1980). Our approach was not perfect, but it was an improvement over the past because O_2 levels were controlled by keeping them at saturation (but not manipulated), photoperiod was kept constant, food was high quality and nonlimiting, and animal densities in rearing containers were kept at levels conducive to producing high survivorship and larval growth. Exposure to temperature for the entire larval period, with a well-defined beginning (egg hatch) and end (adult emergence), provided much better consistency from one experiment to the next. These data provide new insights into, and perspective on, the importance of temperature to aquatic insect ecology. Below, we group the discussion of these insights in the context of the 4 issues and associated questions set forth at the beginning of the paper, discuss each in detail, and summarize our overall conclusions.

Issue 1: adult size and fecundity (TEH vs TSR)

In our experiments, *C. dipterum* followed the TSR (Atkinson 1994, 1996); i.e., adult body size of males and females was greatest at the coolest temperatures and declined with increasing temperature (Fig. 2C). These data confirm Cabanita and Atkinson's (2006) suggestion based on more limited data that *C. dipterum* conforms to TSR. The pattern we observed was especially evident in females, whose DM and fecundity ranged over a factor of 4.5 from the coldest to warmest treatments (Fig. 7). *Cloeon dipterum* did not decrease in size near the lower thermal limit (sensu TEH; Sweeney and Vannote 1978). At the coolest temperature (12.1°C), males were only 63% of the size of females, whereas at the warmest temperature (31.7°C), males and females were both substantially smaller and more equal in size (males 93.4% of females' mass). Females were unable to produce offspring at the warmest temperature that produced adults (31.7°C). These data differ from results of earlier mayfly studies (Sweeney and Vannote 1978) in which adult size in some mayfly species was reduced at temperatures at or near both the lower and higher thermal limits. One possibility for this discrepancy with previous experiments is that Sweeney and Vannote (1978) began their experiments with partially grown field-collected larvae rather than with newly hatched larvae. Consequently, the field conditions experienced by the larvae at or before collection may have in-

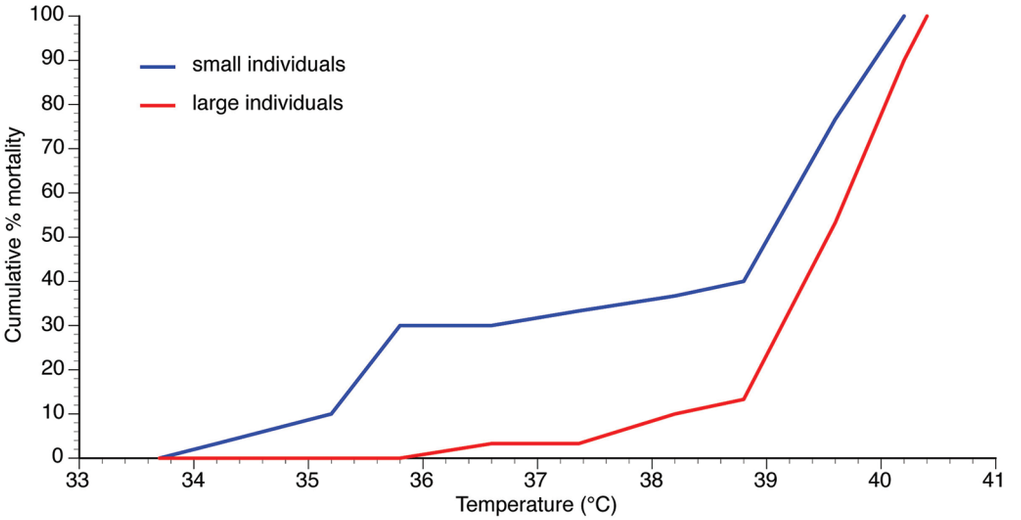

Figure 5. Cumulative % mortality for 30 small and 30 large *Cloeon dipterum* larvae acclimated to 22°C and exposed to thermal ramping at a rate of 0.75°C/h until death.

fluenced their developmental trajectories (see below). Another possible confounding difference is that Sweeney and Vannote (1978) did not control for changing photoperiod (whereas photoperiod was kept constant in our study). Cabanita and Atkinson (2006) showed for *C. dipterum* that changing photoperiod length can alter thermal responses related to TSR.

TSR in *C. dipterum* might be explained by different temperature thresholds for growth and development. As Walters and Hassall (2006) have suggested, this mechanism would require a significantly lower temperature threshold for growth (TT_G) than for development (TT_D). Thus, their model predicts that adult size (mass) would decrease with temperature in ectotherms where $TT_G < TT_D$, increase with temperature where $TT_G > TT_D$, and remain unchanged with a change in temperature where $TT_G = TT_D$. In their test species, TT_G was distinctly $>TT_D$ (by ~5°C) and the species did not adhere to TSR. For *C. dipterum*, we estimate TT_G at 9.9°C (CI: 7.0–12.7°C) for females and 10.1°C (CI: 7.2–13.1°C) for males. Our estimate of TT_D (males: 10.7°C, CI = 8.8–12.6°C; females: 10.7°C, CI = 8.6–12.9°C) is ~0.7°C higher than TT_G but the difference between TT_G and TT_D was not statistically significant and was substantially less than the difference Walters and Hassall (2006) reported (i.e., ~5°C). Therefore, although TT_G seems to be about equal to TT_D for *C. dipterum*, the adult size data for *C. dipterum* clearly follow the TSR. We have confidence in our estimates of TT_D and TT_G. The number of degree days required to complete development for *C. dipterum* was relatively constant at ~266 in the 14.3 to 30°C range (Fig. 2E), suggesting that our estimate of TT_D is good. Moreover, our attempts to grow this species at temperatures ≤10°C have been unsuccessful, also suggesting a TT_G of ~10°C is good. Thus, our results do not adhere to the model published by Walters and Hassall (2006). Furthermore, 3 other mayfly species (*Neocloeon triangulifer*, *Procloeon rivulare*, and another species in the *C. dipterum* complex, CT1 [Rutschmann et al. 2017], all multivoltine species in the family Baetidae) likewise exhibit a TT_D slightly, but not significantly, greater than TT_G, and all follow the TSR (Stroud Center, unpublished data). Thus, the Walters and Hassall (2006) model is appealing, both intuitively and empirically, but it seems insufficient to provide an explanation for TSR in species, such as *C. dipterum*, where TT_G appears to be equal to TT_D.

The apparent lack of applicability of Walter and Hassall's (2006) model to our system could be because their model states that TSR is observed when the ratio between the rates of development and growth change with temperature, such that development and growth have significantly different temperature thresholds. However, these ratios also can change with temperature without significant differences in thresholds. This situation has been explored both theoretically and empirically across a number of metazoan species (see Forster et al. 2011a, b), and these analyses suggest that TSR can, in fact, occur if growth and development have nonlinear reaction norms to temperature, *and* growth is less sensitive to temperature change than is development.

Our results are aligned with the hypothesis by Forster et al. (2011a, b) that the underlying basis for TSR in *C. dipterum* is not related to TT_G or TT_D per se but rather to differences in *the response of growth and development rates at temperatures warmer than TTG and TTD*, such that the rate of larval development increases faster per °C than the rate of larval growth at warmer temperatures. For example, adult females of C. *dipterum* reared at 30°C were, on average, 56.6% smaller (3.39 vs 1.47 mg) than those reared at 12.1°C because the growth rate increased 705% from 12.1 to 30°C while the development rate in-

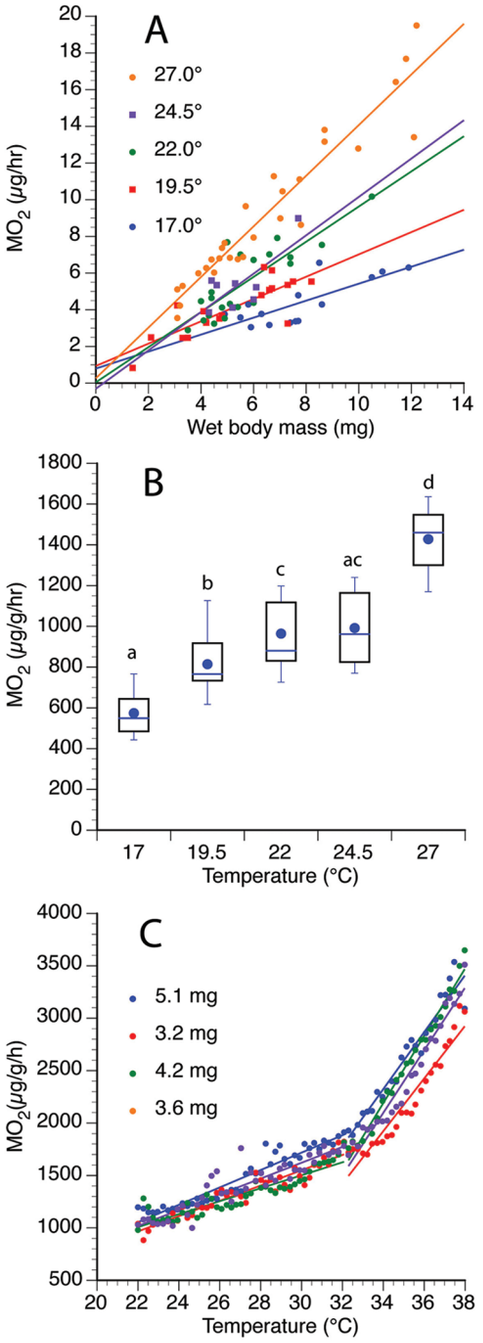

Figure 6. A.—*Cloeon dipterum* O_2 consumption rate/h as a function of larval wet mass (linear regression; all $p < 0.05$ and regressions all significantly different ($p < 0.01$) except 22 vs 24.5°C. B.—Box-and-whisker plot for wet-mass-specific O_2 consumption. Dots are means, lines in boxes are medians, box ends are quartiles, and whiskers are 10th and 90th percentiles. Boxes with the same lowercase letters are not significantly different (Tukey's multiple range test). C.—Mass-specific O_2 consumption rates measured continuously as temperatures were increased for larvae in 4 size classes. Lines show 2 significant ($p < 0.05$) linear relationships for each size class. One regression was calculated for temperatures between 22 and ~32°C and the other for temperatures between ~32° and ~38°C.

creased even more (925%). This result suggests that the propensity for larvae to get bigger increases with increased temperature above TT_G or TT_D but at a progressively slower rate than does the propensity to develop and metamorphose to the adult over the same temperature range. This decoupling of growth and development seems key to the final size of *C. dipterum* and its gradual decline with increased temperature.

Issue 2: adult size and the developmental and bioenergetic aspects of metamorphosis

As noted earlier, Vannote and Sweeney (1980) hypothesized that variation in adult size in aquatic insects reflected, in part, an energetic disequilibrium in the partitioning of assimilated energy between growth and metabolism as it affects the rate and duration of larval growth. If true, this disequilibrium also could be a factor in the decoupling of growth from development described above. Our study provides considerable perspective and insight into this possible decoupling (see below), but it lacks sufficient data to provide a definitive test for this hypothesis. We measured rate and duration of larval growth carefully, but variation in these parameters does not necessarily reflect only differences in energy partitioning. Other factors, such as temperature-induced changes in feeding and ventilation rates or activity patterns, which we did not control or address, make attributing variation in adult size solely to changes in rate and duration of larval growth difficult. For example, we did not measure ventilation rate in our study, but Verberk and Atkinson (2013) showed that energy expended in ventilation can be substantial and is both size- and temperature-dependent. Ventilation rates help explain size differences in heat tolerance and, perhaps more important for this discussion, contribute to a different energetic balance between males and females. Thus, larger body size of females might be advantageous for ventilation (larger gill structures for ventilating) while simultaneously freeing them from the larger metabolism normally associated with a larger body size because much of a female's mass is reproductive tissue which, although not trivial to maintain (i.e., increases metabolic costs ~8% in invertebrates), has substantially lower metabolic costs than other tissues (Griffen 2017). Last, our attempt to control for photoperiod in our experiments by keeping day length constant also fell a bit short (Table S1) because, as Cabanita and Atkinson (2006) pointed out, the same day length can be perceived differently at different temperatures by mayflies.

Regardless of these shortcomings, our results provide new insights into developmental and bioenergetic aspects of mayfly metamorphosis. For example, Vannote and Sweeney (1980) hypothesized that maximum adult size reflects equilibrium between the rate and duration of larval growth and both the specific time in larval development when adult structures begin maturing and the rate of this maturation process.

Figure 7. Adult female *Cloeon dipterum*. Specimen on left is from overwintering generation and weighed 4.2 mg (dry) and produced 2427 hatchlings. Specimen on right was reared under laboratory conditions (diel cycle with mean of 27.8°C and diel amplitude of 7.7°C) and weighed 1.4 mg and produced 235 hatchlings.

We found different magnitudes of responses for development time, larval growth rate, and adult size of female *C. dipterum* to warming (Table S1, Fig. 2B, C, D). As temperature increases over the range 16.2 to 31.7°C, 4 notable responses occur: 1) larval development rate increases proportionately faster than larval growth rate, 2) both male and female adult size is inversely related to temperature (as per the TSR), 3) females decrease in size proportionately more than males, and 4) the decrease in fecundity is proportionately greater than the decline in adult female size.

These findings enable us to expand on the original TEH by proposing 3 additional hypotheses to describe how accelerated development or slower growth of specific adult tissues might affect the size and fecundity of *C. dipterum*. Fundamental to all of these new hypotheses are data showing that the relative amount of time in the *C. dipterum* life cycle available for getting bigger was declining faster in response to warming than was the ability to grow (e.g., the development rate increased 171% from 16.2 to 30°C, whereas the growth rate increased only 106%). Hypothesis 1 (H1): *With increased warming, the decline in adult size and fecundity is caused largely by the substantially faster increase in developmental than in growth rate*. H2 and H3 address the mechanisms leading to this differential response of development and growth to warming temperature—specifically, that the observed acceleration of development is caused by either H2: *The "critical weight"* (sensu Hatem et al. 2015, Nijhout 2015) *occurring at a smaller larval size (mass) as ambient temperature warms*, or H3: *Warming temperatures affect the expression of genes related to reducing the body levels of juvenile hormone* (JH; causing adult tissues to develop earlier than usual) *while increasing the levels of ecdysone* (causing molting and growth to continue and increase).

For H2, acceleration in the onset of development and metamorphosis and shortening of the larval growth period without an equal and concomitant acceleration of growth would lead to the decline in size of both males and females as observed. We did observe an increase in growth, but it was not equal in magnitude to the observed change in rate of development. Regardless, the key point of H2 is that as temperatures increase, metamorphosis is initiated earlier in development and at a smaller size, but once initiated, it proceeds as usual. For H3, no minimum larval size is associated with initiation of the metamorphosis process, only for the concentrations of JH to decline faster than ecdysone in response to warming. This response could be caused by producing less JH or degrading more of it.

H2 and H3 are speculative, and they are not mutually exclusive. However, even if they, or some combination of them, explain why increasing temperatures shorten the life cycle and result in smaller males and females for *C. dipterum*, neither hypothesis explains the following observations. In response to an increase in temperature from 16.2 to 31.7°C, why did adult size decline at a significantly greater rate for females than for males and why did fecundity decline faster and to a greater extent than adult female size? These observations may be unique and may have come to

light in our study because of the unusually precise data associated with the life-history characteristics in these experiments. To this point, we note that authors of a wider meta-analysis of sex-specific temperature–size responses in arthropod species were unable to show significant differences between females and males (Hirst et al. 2015). However, Cabanita and Atkinson (2006) did show a stronger temperature–size response for females than for males for a species closely related to *C. dipterum*. Given these 2 independent observations and relatively little variation in our adult mass vs temperature regressions (ln[x]-transformed regressions: R^2 = 0.96 and 0.95 for females and males, respectively), we have confidence that our observed rate of loss of adult mass is significantly greater for females than males.

One way to understand these sexual differences is in the context of bioenergetics and the partitioning of energy between growth and reproduction. Four known aspects of mayfly biology are relevant to this discussion: 1) net growth efficiencies have been reported to be as much as 2× higher for females than males (Sweeney 1978); 2) by the time larvae are ~½ grown in field populations, female larvae are already considerably larger than males despite hatching from eggs at or about the same time (Sweeney and Vannote 1981); 3) females seem to consistently constitute a disproportionate percentage of large individuals of a given species (i.e., in the upper ½ of the mass structure for a population) at a point halfway through its life cycle in a natural stream (Sweeney and Vannote 1981); and 4) mass-specific respiration appears to increase with temperature, and the increase seems disproportionately greater for smaller larvae (reviewed by Sweeney 1978). From these 4 fundamentals, we think it reasonable to conclude from our experiments that 2 propositions are true. First, females must have dominated the upper ½ of the size (mass) structure of our laboratory populations by the mid-point in any given experiment at a given temperature. Second, both males and females were exposed to the same temperatures (and, thus, high metabolic rates at the start of all experiments because of small size following egg hatch), but males remained smaller *longer* than females. Therefore, as rearing temperature increased from 16.2 to 31.7°C across the various experiments, the relative increase in metabolic cost/individual must have been substantially greater for males than for females, thereby contributing further to the males' lower net growth efficiency. Given that these 2 propositions are likely to be true, we would have expected a priori to find a greater decline in adult size for males than for females. Instead, we observed the opposite: that increased temperature caused female adults to lose size faster than males and to lose fecundity even faster than size.

This disparity between observed and expected outcomes suggests that, from an energy partitioning standpoint, getting bigger may be more challenging for females than for males, especially as temperatures warm. Males need energy primarily to grow physically bigger because the production of sperm (which has low mass and energy content) is relatively benign to the male's overall energy budget. In contrast, females need much more energy per individual per unit time than males because they have to increase in size substantially more than males *and* to accumulate reproductive tissue (lipids, eggs) that has a substantially higher mass and energy content than for males. Larvae need to metamorphose to the adult stage with all their sperm and eggs largely formed because mayflies do not feed as adults.

The bioenergetics of this system is complicated, especially for females, because the mass associated with female reproductive tissue (lipids and nondeveloping eggs) represents ~50% of the total energy value of a mature female (Sweeney and Vannote 1981). However, fatty tissue (i.e., stored energy for reproduction) in invertebrates typically has a lower metabolic rate than other tissues and only represents ~8% of an individual's total metabolic costs (Griffen 2017). Thus, as female mayflies get larger, the actual respiration costs that we measure must be dominated proportionately by the O_2 demand of their nonreproductive tissue. The energy requirements of females are necessarily substantially greater than those of males, given that females are larger than males, must produce and maintain more nonreproductive tissue, and must increase their physical structure more per day leading up to metamorphosis. The female disadvantage is exacerbated by the additional need to accumulate and store a much greater mass of high-energy reproductive tissue (eggs) than males whose sperm biomass is small.

In our experiments, as temperature rose, the time available for males and females to add both non-reproductive and reproductive tissue declined (because development rates increased faster than growth rates). We propose that females simply cannot accumulate energy at a rate sufficient to maintain the tissue they have, accumulate new tissue (get bigger), and store energy for reproduction as efficiently as males, which are smaller and have lower overall energetic costs associated with their reproductive tissue. We suggest that this "reproductive starvation" provides at least a partial explanation for the observed pattern for *C. dipterum* associated with TSR: size and fecundity decline proportionately faster for females than for males.

Why fecundity declines faster than female size also may have a bioenergetics explanation but probably is complicated by factors beyond the scope of our study. To understand from an ecological/evolutionary perspective why increasing temperatures cause adult females to decline more extensively in both mass and physical size than males (but at a lower rate than their decline in fecundity) will require a better understanding of the differences between the sexes in the bioenergetics of producing reproductive tissue. Sperm have little mass and are energetically cheap to make, so the production of reproductive tissue is a relatively small part of an individual male's overall energy budget. In contrast, the production of reproductive tissue is energetically expensive

(mayfly eggs contain mainly lipids and protein and have a high caloric content; Harvey et al. 1980) for females because they must: 1) produce a nontrivial number of high-caloric eggs and 2) successfully metamorphose into a body capable of transporting those eggs out of the aquatic habitat into the air, swarm as an adult above the water to find a mate, copulate in flight to assure fertilization, and return to the water for oviposition. All of this comes at significant cost to a female's overall energy budget. If mayfly populations emerge as adults and reproduce synchronously on both a diel and seasonal basis and if this strategy is adaptive (Sweeney and Vannote 1982), then ecophysiological adaptations determine how much of a female's ingested and processed energy is partitioned between growth and fecundity as she approaches metamorphosis. Our data and the discussion above suggest that when temperatures warm, the metabolic costs of tissue maintenance increase, the time to accumulate energy before metamorphosis decreases, and overall stress on a female's energy budget is greater than on that of a male. Unless food resources and the ability to ingest and process them are unlimited, each female must balance getting physically bigger with becoming more fecund.

Our experiments showed that at the highest nonlethal temperatures of the study (30 and 31.7°C), fecundity declined to 426 and 57, respectively, or ~29 and 4% of the maximum level observed for the total range of temperature in our study. However, in response to the same thermal conditions (30 and 31.7°C), female size declined to 1.47 and 0.76 mg, respectively, or ~43.4 and 22.4%, respectively, of the maximum adult size for our experiments. So, females appear to sacrifice fecundity for body size at or near the maximum thermal limit of *C. dipterum*. This conclusion suggests that under limiting or stressful levels of resources (or other conditions), leaving the stressful environment sooner with fewer eggs leads to more successful reproduction than delaying metamorphosis to produce a larger, more fecund adult. These insights may be new for mayflies, but they are not new science because shunting of energy from increased size or fecundity under increasingly stressful conditions was proposed long ago as an effective evolutionary strategy for amphibian metamorphosis (Wilbur and Collins 1973).

To summarize this part of the discussion, we propose the following hypotheses for *C. dipterum* and possibly for mayfly species in general. H4: *Female adults get proportionately smaller as temperatures warm because females cannot ingest and process sufficient energy to support and grow their body structure while accumulating high-energy reproductive tissue (lipids, eggs), whereas the reproductive tissue of males is light in both mass and calories, which means that proportionately more processed energy is available for structural tissue growth*. H5: *Female fecundity decreases faster than female size in response to increased temperature because reduced egg production allows more energy to be directed to the structural growth needed for successful metamorphosis, flight, mating, and oviposition*.

Issue 3: O_2 availability, larval size, and the decision to metamorphose

The current understanding in insects is that JH must be present in a given larva to delay metamorphosis and allow time for energy to be stored for reproduction but must be absent for a given larva to actually metamorphose (Hatem et al. 2015, Nijhout 2015). The observed decline in adult fecundity with increased temperature for *C. dipterum* suggests that larvae metamorphose before they can accumulate sufficient energy reserve for egg production. In other words, JH declines to 0 progressively earlier in larval development at higher temperatures. However, whether the premature decline in JH levels reflects the cessation of JH production by gene downloading or the consumption (degradation) of JH by catabolic enzymes, such as esterases, via gene uploading is unclear from our data. Additional experimentation is clearly needed on this front.

Regardless of the exact mechanism, our data suggest that the decision to begin metamorphosis is initiated at a much smaller larval size at higher (30–31.7°C) than at lower temperatures (12.1–14.3°C). Our measurements do not allow us to suggest an exact minimum critical mass for *C. dipterum* or to resolve the current disagreement about what happens after critical minimum size is achieved and the decision to metamorphose is made (e.g., *Manduca* vs *Drosophila* physiological models as per Hatem et al. 2015 and Nijhout 2015, respectively). However, the models agree that once critical minimum size is attained, O_2 limitation in the insect's tracheal system probably is involved in initiating the path to metamorphosis.

The OCLTT hypothesis involves O_2 as a key factor for understanding the thermal limits of ectotherms, but it does not identify O_2 limitation as an important factor in initiating metamorphosis. Rather, the OCLTT suggests that warming temperatures create a mismatch between O_2 supply from the water and O_2 demand of the organism, which sets physiological limits for reproduction and survival. Evidence supporting this paradigm to explain CT_{max} does exist (Verberk and Bilton 2013, Verberk et al. 2013), but the thermolimit respirometry experiments we conducted on *C. dipterum* larvae (measurements of respiration rates of larvae as they were being warmed 1°C/h until death) revealed that O_2-consumption rates were nearly 2× as high near CT_{max} (i.e., 38–40°C) as they were at considerably lower temperatures (i.e., 31.7°C) where adult emergence occurred but females produced no offspring (i.e., $R_o = 0$ in *C. dipterum* life-cycle studies; Fig. 4). This pattern also was observed in the mayfly *Neocloeon triangulifer*, where aerobic scope was similar at tolerated and chronically lethal temperatures (Kim et al. 2017). This result suggests

that larvae still have substantial aerobic capacity at a temperature that results in 0 reproductive output.

Our experiments were not designed to test whether O_2 limitation is involved in reduced survival or growth in response to temperature. For example, we did not manipulate O_2 levels and did not include important ancillary data regarding metabolomics, the expression of genes related to oxygen stress, and other physiological parameters. However, we can report that measurements of metabolism in our study and recent results of aerobic-scope studies for other mayflies (Kim et al. 2017) suggest that mayfly larvae seem to have sufficient capacity to extract O_2 at CT_{max}. A recent test of the OCLTT (Verberk et al. 2016) showed that thermal responses exacerbated the sensitivity of test organisms to hypoxia in the field and the laboratory and often at O_2 levels well above those considered stressful. In the context of our study, what we do not know is whether mayflies and other aquatic invertebrates exhibiting TSR can shift their energy allocations at O_2 levels that are not limiting to prevent O_2 from becoming limiting, or whether O_2 may be limiting only during certain points in the organism's life (e.g., during molting, as pointed out recently by Camp et al. 2014). It is not surprising that thermal limits for growth and reproduction are lower than lethal limits (which require only that the animal survives) and that resulting physiological activity associated with the lower limits for growth and reproduction would raise O_2 demand. Suffice it to say that the ability to sustain aerobic scope for acute exposure to warm temperatures (i.e., near CT_{max}) is very different from chronic exposure for weeks or months to those same temperatures. We maintained O_2 saturation continuously at all temperatures, but our experiments were not designed to test whether O_2 limitation at the tissue level plays a role in sublethal effects, such as the TSR (as suggested by Verberk et al. 2011). Therefore, we cannot use the results of our study to test their OCLTT hypothesis directly, but we did find that O_2 availability in the rearing water declined ~32% throughout our experiments (from ~10.8 mg/L at 12.1°C to ~7.3 mg/L at 31.7°C). At this time, we cannot rule out that the 32% decline in O_2 availability could have caused the observed 66% decline in adult size over the same range of temperature.

The relationship between larval size and O_2 level of larval tissue as it relates to metamorphosis needs more study. In particular, tests are needed that span the complete range from egg hatch to larval metamorphosis while O_2 levels in the aquatic environment are manipulated. If such experiments show that larvae initiate metamorphosis at a small size under hypoxia (while temperature is kept constant), then that would strongly suggest O_2 shortage as the stimulus. In contrast, as in our study, larvae metamorphosis at a smaller size may or may not be caused by O_2 shortage. This question becomes even more confounded because, under aquatic conditions of fully saturated O_2, some data indicate that O_2 supply need not decline and may even increase (Verberk et al. 2011) because, although the solubility of O_2 decreases with increased temperature, the effect is mitigated by the fact that O_2 diffusivity actually increases. Therefore, O_2 availability at the surface of mayfly gills tends to increase with temperature. However, even if the O_2 supply remains high, a mismatch can occur between demand and availability if the demand for O_2 changes dramatically because of thermally induced increases in metabolism. One step toward unraveling the question may be to include metabolomic parameters in the experimental design as an independent measure of what is happening inside these animals as developmental decisions are being made. For example, recent studies of the mayfly *N. triangulifer* strongly suggest that genes indicative of physiological hypoxia did not respond in larvae exposed to temperatures approaching their chronic thermal limit (i.e., 30°C for *N. triangulifer*; Kim et al. 2017). Similar metabolomic data gathered on animals exposed to various temperature and O_2 conditions during life-cycle testing would greatly clarify these issues.

In summary, our experiments suggest that the relative importance of factors affecting adult size emphasized by Vannote and Sweeney (1980) can be further clarified as follows: 1) the specific time in larval development that metamorphosis is stimulated and final adult structures are formed (i.e., the point when critical mass/size is achieved) is as important as the rate of larval growth or the rate of the maturation process associated with adult structures per se, and 2) the point when critical mass/size is achieved and the trigger to proceed to metamorphosis is made appears to be strongly temperature dependent and may be related, in some still-unknown way, to levels of O_2 associated with internal tissues.

Issue 4: Thermal optimum vs thermal acclimation zone

Our results suggest that *C. dipterum* has a well-defined thermal acclimation zone (Fig. 8) between 14.3 and 30°C. The zone is a range of temperatures where physiological and developmental adaptations enable larvae to complete development and metamorphose after exposure to a constant number of total degree days above some threshold. Individuals reared at temperatures warmer (31.7°C) and colder (12.1°C) than the acclimation zone required substantially more and fewer degree days, respectively, than those reared in the zone. Rates of development and instantaneous growth changed in linear but opposite directions throughout the acclimation zone, whereas survivorship peaked in the middle of the zone. Thus, identifying the temperature that is optimal for maximizing individual fitness for *C. dipterum* is not intuitive.

We calculated and presented r for *C. dipterum* as a function of temperature. We did not present R_0 because it is an inappropriate metric for gauging thermal optima

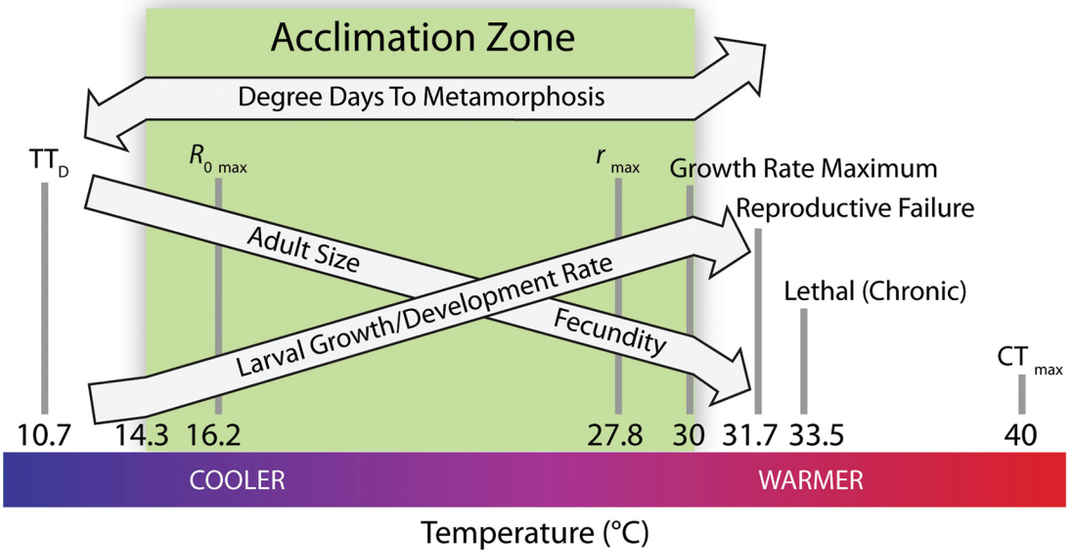

Figure 8. Conceptual model of the thermal acclimation zone for the mayfly *Cloeon dipterum* defined as the range of temperatures where the degree days needed to complete development are constant and rates of growth and development respond linearly to changes in temperature. Rising, falling, and horizontal arrows indicate increasing, decreasing, and unchanging rates of the parameters listed at experimental temperatures. TTD = calculated threshold temperature for larval development; CT_{max} = temperature at which larval mortality was 100%, R_{0max} = test temperature with the highest net reproductive rate, r_{max} = test temperature with the highest intrinsic rate of population growth. Reproductive failure = temperature at which females produced 0 hatchlings.

because generation time of *C. dipterum* is not constant at 1 y (univoltine). Given that *C. dipterum* is multivoltine, *r* is a more appropriate measure of fitness because it can be maximized by increasing the number of generations completed within a season (Zeuss et al. 2017; see also Frazier et al. 2006). For *C. dipterum, r* was relatively high between 20 and 30°C, reached a maximum at 27.8°C, and declined precipitously as temperatures increased >30°C (Fig. 4). This pattern has been observed before (Savage et al. 2004, Frazier et al. 2006), but we point out that *r* peaked near the temperature (30°C) that resulted in maximum rates of larval growth and development. Therefore, we propose that the optimum temperature for *C. dipterum* is close to 27.8°C (where *r* is maximized) at the upper end of its thermal acclimation zone and thermal range.

Conclusions

We have reached the following conclusions regarding the 4 important issues of *C. dipterum* thermal ecology addressed in our study. *Cloeon dipterum* follows Atkinson's (1994, 1996) TSR and not Sweeney and Vannote's (1978) TEH. TSR in *C. dipterum* does not appear to be caused by a difference in TT_G vs TT_D (sensu Walters and Hassall 2006). Rather, it seems to be related to the response of growth and development rates to temperature at temperatures above TT_G and TT_D because the propensity for larvae to get bigger increases with increased temperature above TT_G or TT_D but at a progressively slower rate than does the propensity to develop and metamorphose to the adult over the same temperature range.

For *C. dipterum*, warmer temperatures cause: 1) the larval development rate to increase proportionately faster than the larval growth rate, 2) male and female adult size to decrease, 3) adult females to decrease in size proportionately more than males, and 4) fecundity to decrease proportionately more than adult female size. We propose that some combination of 5 hypotheses (H1–5) can explain these patterns, and that the decline in adult size and fecundity is caused largely by substantially faster increases in larval developmental rate than growth rate as temperatures warm (H1). The observed accelerated development with warming occurs because either the "critical weight" (sensu Hatem et al. 2015, Nijhout 2015) occurs at a smaller larval size (mass) as ambient temperature warms (H2) or warming temperatures affect the expression of genes related to reducing the body levels of JH, which causes adult tissues to develop earlier than usual, while simultaneously increasing the levels of ecdysone, which cause molting and growth to continue and increase (H3). Moreover, female adults get proportionately smaller as temperatures warm because they cannot ingest and process sufficient energy to support and grow their body structure while accumulating high-energy reproductive tissue (lipids, eggs) (H4). In contrast,

the reproductive tissue of males is light in terms of mass and calories and, thus, proportionately more processed energy is available for structural tissue growth. Last, female fecundity decreases faster than female size in response to increased temperature because reduced egg production allows direction of more energy to the structural growth required for successful metamorphosis, flight, mating, and oviposition (H5).

Respirometry suggests that the OCLTT hypothesis is more applicable to acute thermal limits than to chronic thermal limits. Overall, it appears that *C. dipterum* has a thermal "acclimation zone" between 14.3 and 30°C, when development and growth rates increase or decrease linearly, degree-day requirements for completing metamorphosis are constant, and an "optimum temperature" close to 27.8°C, where r reaches a maximum.

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