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RESEARCH ARTICLE

Oxygen limitation fails to explain upper chronic thermal limits and the temperature size rule in mayflies

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ABSTRACT

An inability to adequately meet tissue oxygen demands has been proposed as an important factor setting upper thermal limits in ectothermic invertebrates (especially aquatic species) as well as explaining the observed decline in adult size with increased rearing temperature during the immature stages (a phenomenon known as the temperature size rule, or TSR). We tested this by rearing three aquatic insects (the mayflies *Neocloeon triangulifer* and two species of the *Cloeon dipterum* complex) through their entire larval life under a range of temperature and oxygen concentrations. Hyperoxia did not extend upper thermal limits, nor did it prevent the loss of size or fertility experienced near upper chronic thermal limits. At moderate temperatures, the TSR pattern was observed under conditions of hyperoxia, normoxia and hypoxia, suggesting little or no influence of oxygen on this trend. However, for a given rearing temperature, adults were smaller and less fecund under hypoxia as a result of a lowering of growth rates. These mayflies greatly increased the size of their gills in response to lower dissolved oxygen concentrations but not under oxygen-saturated conditions over a temperature range yielding the classic TSR response. Using ommatidium diameter as a proxy for cell size, we found the classic TSR pattern observed under moderate temperature conditions was due primarily to a change in the number of cells rather than cell size. We conclude overall that a failure to meet tissue oxygen demands is not a viable hypothesis for explaining either the chronic thermal limit or TSR pattern in these species.

KEY WORDS: Chronic hypoxia, Hyperoxia, TSR, OCLTT, Gill allometry

INTRODUCTION

Temperature is critical to all biological processes and environmental temperature is especially important to ectotherms, whose body temperature is more or less at its mercy. Understanding what limits upper thermal tolerance for an organism, both in the short term (acute or critical) and longer term (chronic or whole-life) may help explain the loss of performance observed at warm but sub-lethal values (Verberk et al., 2016a). In recent years it has been proposed that the temperature-dependent performance of animals is shaped by the capacity for oxygen delivery in relation to oxygen demand, a hypothesis referred to as oxygen- and capacity-limitation of thermal tolerance (OCLTT; Pörtner, 2010). This mismatch between metabolic oxygen demand and an animal's ability to supply it adequately as temperature rises has been implicated as a proximate cause of upper lethal limits (Verberk and Calosi, 2012). Moreover, oxygen limitation has been proposed (Hoefnagel and Verberk, 2015) as an explanation for the widely observed trend in ectotherms known as the temperature size rule (TSR; Atkinson, 1994), whereby individuals grow and develop slowly but reach a large adult size under cooler conditions, while under warm conditions they grow and develop quickly but reach a smaller adult size (see also Verberk et al., 2020, for a recent review).

Verberk et al. (2016b) reviewed the evidence supporting the role of oxygen in setting upper thermal limits in crustaceans and insects and found strongest support for OCLTT in species relying on underwater gas exchange. Oxygen uptake is expected to be more challenging for aquatic than terrestrial species because the oxygen concentration in water is only about 3% that in air and the oxygen diffusion rate in water is 3×10^5 times lower than in air (Verberk et al., 2011), making its extraction from water much more difficult. Add to that the higher density and viscosity of water and we find that as much as a third of resting metabolism in some fish may be devoted to ventilation (Forster et al., 2012) compared with ≤2% in humans (Peters, 1969).

Upper thermal limits are most often characterized by the critical thermal maximum (CT_{max}), the temperature at which animals subjected to thermal ramping over a relatively short period stop moving or die. Oxygen availability has been shown to affect CT_{max}, with hypoxia often lowering it and hyperoxia sometimes raising it (e.g. Verberk and Calosi, 2012; Verberk and Bilton, 2015; Verberk et al., 2018; Whitney, 1939). Although CT_{max} values may have little direct applicability to real-world thermal limits (Chou et al., 2018; Sweeney et al., 2018), at least one study has revealed a concordance between thermal tolerance as revealed by CT_{max} in the laboratory and the occurrence of two mayfly species in streams of varying oxygen and temperature conditions (Verberk et al., 2016a).

In a recent review of the evidence regarding oxygen and the TSR, focusing mainly on fish, Audzijonyte et al. (2018) concluded: 'Despite decades of research, we remain uncertain whether the TSR is an adaptive response to temperature-related physiological (enzyme activity) or ecological changes (food, predation and other mortality), or a response to constraints operating at a cellular level (oxygen supply and associated costs)'. For arthropods, a quantitative comparison of temperature–size responses and latitude–size clines by Horne et al. (2015) found their direction and magnitude to co-vary among 12 arthropod orders. Body size in aquatic species generally diminished with both warming and decreasing latitude, whereas terrestrial species had much more reduced or even opposite responses. The authors concluded such patterns support the prediction that oxygen limitation is a major controlling factor in water, but not in air. Furthermore, voltinism explained much of the variation in temperature–size and latitude–size patterns in terrestrial but not aquatic species. While body size decreased with warming and with decreasing latitude in multivoltine terrestrial arthropods, size increased on average in univoltine species over those conditions, consistent with predictions from size versus season-length trade-offs.

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At the cellular level there are data to suggest that, under colder conditions, larger adult size results from larger cell size (with cell number remaining relatively constant) and that this may be a result of higher oxygen concentrations (e.g. Atkinson et al., 2006; Forster et al., 2012; Horne et al., 2015). Thermal plasticity in cell size may have adaptive value for ectotherms because there are different optimal cell membrane to cell volume ratios at different temperatures (Kierat et al., 2017). At high temperatures, the demand for oxygen is high and the relatively large membrane surface of small cells might allow higher rates of oxygen transport into the cell. Conversely, at low temperatures, the metabolic costs of maintaining those membranes are expected to become more important, favouring larger cells (Szarski, 1983).

Here, we investigated the role of oxygen in both setting upper chronic (whole-life) thermal limits and explaining the TSR in mayfly species for which thermal reaction norms are now well established (Chou et al., 2018; Funk et al., 2019; Kim et al., 2017; Sweeney et al., 2018; and herein). We attempted to answer the following questions through a series of laboratory experiments involving aquatic larvae of one or more of three mayfly species. (1) Can hyperoxia increase the upper chronic thermal limits of mayflies? (2) Does the pattern of TSR change under conditions of hypoxia, normoxia and hyperoxia? (3) Do morphological characters known to be affected by oxygen stress (gill size, leg length, cell size) correlate well with upper thermal limit and the TSR? To address the first question, we tested whether artificially increased oxygen availability (hyperoxia) can enable larvae of one species to survive (i.e. 'rescue' them from possible internal hypoxia) at a temperature previously determined to be chronically lethal under environmental normoxia (i.e. 30°C). We also tested whether hyperoxia can restore fertility in a second species at the highest non-lethal temperature (i.e. 32°C), known from previous studies to result in infertility. For the second and third questions, we examined the response of larval growth and development rates, adult size and structural allometry over a range of oxygen concentrations and over a range of non-stressful temperatures known to elicit a classic TSR pattern.

MATERIALS AND METHODS

Test species

The three mayfly (Ephemeroptera) species involved in this study are members of the family Baetidae. *Neocloeon triangulifer* (McDunnough 1931) is native to eastern North America and has been used extensively for laboratory testing of environmental and ecological hypotheses because of its relative ease of culture (Sweeney et al., 1993; Weaver et al., 2015), clonally parthenogenetic mode of reproduction (Funk et al., 2006), sensitivity to environmental challenges (Struewing et al., 2015), and established cDNA sequence for molecular work (Kim et al., 2017). Specimens used for this study were from clone WCC-2 that is maintained at the Stroud Water Research Center, Avondale, PA, USA (original source: White Clay Creek, PA, USA, 39.86072°N, 75.78390°W). Thermal reaction norms for this clone of *N. triangulifer* have been documented (Chou et al., 2018; Kim et al., 2017; Sweeney and Vannote, 1984) and are supplemented herein. This species has relatively narrow thermal requirements, with a thermal zone of acclimation spanning only 8°C, and is generally found along the edges and in backwaters of streams that are well oxygenated.

Cloeon dipterum (Linnaeus 1761) is a Palearctic species complex consisting of at least four species in mainland Europe (Rutschmann et al., 2017). Two of these species have recently become established in North America (Funk et al., 2019) and were used in the present study: *Cloeon dipterum*-IS1 and *Cloeon dipterum*-CT1 (both as designated by Rutschmann et al., 2017) (source for the former: pond adjacent to White Clay Creek, PA, USA, 39.86535°N, 75.81782°W; for the latter: pond adjacent to Bartlett Brook, VT, USA, 43.68619°N, 72.53530°W). Thermal reaction norms have been documented for these populations of *C. dipterum*-IS1 (Sweeney et al., 2018) and *C. dipterum*-CT1 (Funk et al., 2019). Both *C. dipterum* species have relatively wide thermal requirements, with a thermal zone of acclimation spanning 16°C, and are often found in still and/or temporary waters where oxygen concentrations may be low.

Laboratory rearing methods

Whole-life rearing methods were described previously (Sweeney et al., 2018). In summary, larvae were reared in White Clay Creek water in 1.8 l glass vessels submersed in a water bath that maintained the desired temperature to ±0.1°C. Food was provided *ad libitum* as periphyton (predominantly diatoms) grown on acrylic plates. Flow conditions in the vessels were similar to those in preferred habitats of both *N. triangulifer* and *C. dipterum*, i.e. still water, with localized flow driven by the action of bubblers. Both species are able to beat their gills as necessary and were observed to engage in this behaviour, especially under low oxygen concentration. Manipulation of oxygen concentration was achieved by bubbling air or a premixed combination of oxygen and nitrogen (or in some cases pure nitrogen) through an air stone (diffuser) in each vessel at a rate of about 30 ml min^{-1}. Oxygen concentration was monitored at the beginning of each experiment and on average every 4 days thereafter using an optical dissolved oxygen probe (RDO, Orion 087003, Thermo Fisher Scientific, Waltham, MA, USA). Each oxygen test treatment consisted of 4 replicate vessels, each starting with 50 first instar larvae (<1 day old). Larvae were reared under constant photoperiod (15 h:9 h, light:dark) for the duration of larval development. Emerging subimagos were dried at 50°C and weighed individually. Survivorship (to adult), adult dry mass, development time and instantaneous growth rate were determined using the methods described earlier (Sweeney et al., 2018).

Question 1: can hyperoxia increase the upper chronic thermal limits of mayflies? We performed two experiments (experiments 1 and 2 of Table 1) to explore this question. First, we tested whether artificially elevated oxygen concentrations can enhance survival of *N. triangulifer* at 30°C (the lowest temperature known to result in complete mortality of this species under normoxia). Newly hatched larvae were reared to metamorphosis (subimago) at three treatment levels: 45% oxygen [yielding an O_2 partial pressure (P_{O_2}) of ∼42 kPa]; 20% oxygen (yielding a P_{O_2} of ∼18 kPa); and normal atmospheric aeration (also yielding a P_{O_2} of ∼19 kPa). Second, we performed similar tests on *C. dipterum*-CT1 but at 32°C, which is a temperature previously determined to be survivable but at which females cannot produce viable offspring (i.e. zero fertility as per Funk et al., 2019; Sweeney et al., 2018). Again, there were three treatments: 45% oxygen (yielding a P_{O_2} of ∼40 kPa); 20% oxygen (yielding a P_{O_2} of ∼18 kPa); normal aeration (yielding a P_{O_2} of ∼18 kPa; see Table 1, experiments 1 and 2, for details).

Question 2: does the pattern of TSR differ under conditions of hypoxia, normoxia and hyperoxia? To explore this question, we reared *N. triangulifer* and *C. dipterum*-IS1 from newly hatched larvae to metamorphosis (subimago) at a range of oxygen concentrations, achieved by aeration with gases ranging from 45% O_2 to pure N_2, at 20 and 25°C (see Table 1, experiments 3 and 4, for details). Initially, for the *C. dipterum*-IS1 experiments at 20°C, we had two normoxic treatments (20% O_2 and atmospheric aeration). Although the lack of CO_2 in the 20% oxygen treatment resulted in a

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Table 1. Experimental temperature and oxygen treatments for chronic (whole-life) tests with *Neocloeon triangulifer* and *Cloeon dipterum*-IS1

Experiment	Species	Temperature (°C)	O_2 treatment	O_2 saturation (%)	O_2 concentration (mg l^{-1})	P_{O_2} (kPa)
1	*N. triangulifer*	30	45% O_2	205	15.4	41.6
		30	Air	91	6.9	18.6
		30	20% O_2	87	6.6	17.7
2	*C. dipterum*-CT1	32	45% O_2	197	14.4	39.9
		32	Air	89	6.5	18.0
		32	20% O_2	88	6.4	17.8
3	*C. dipterum*-IS1	20	45% O_2	218	19.5	44.7
		20	Air	101	9.1	20.7
		20	20% O_2	95	8.3	19.0
		20	3% O_2	25	2.2	5.1
		20	N_2	12	1.1	2.5
3	*C. dipterum*-IS1	25	45% O_2	195	16.0	39.9
		25	Air*	92	7.6	18.1
		25	6.5% O_2	33	2.7	6.2
		25	3% O_2	19	1.5	3.6
		25	N_2	6	0.5	1.2
4	*N. triangulifer*	20	Air*	98	8.8	20.3
		20	6.5% O_2	41	3.7	8.3
		20	3% O_2	29	2.5	5.8
4	*N. triangulifer*	25	Air*	94	7.8	19.3
		25	6.5% O_2	37	3.1	7.7
		25	3% O_2	24	2.0	4.9

Each treatment consisted of four replicate vessels with 50 larvae per vessel.
*Only air was used to approximate normal (20 kPa) O_2 partial pressure because in experiments 1–3 no difference was detected between that and a 20% O_2 mix.

somewhat higher pH relative to the air treatment (by about 0.2–0.4), as detailed later, we found no significant differences in survivorship, adult size or growth and development rate between those normoxic treatments and so, for the 25°C experiments with *C. dipterum*-IS1, as well as all subsequent experiments with *N. triangulifer*, we only used normal aeration for the normoxia treatment. Preliminary tests with *N. triangulifer* resulted in 100% mortality at oxygen concentrations $\leq$1 mg l^{-1} (i.e. aeration with pure nitrogen). We therefore modified the three oxygen treatment levels for experiments with *N. triangulifer* as follows: normoxia via atmospheric aeration and two levels of hypoxia using 6.5% O_2 and 3% O_2 (see Table 1). Note that hyperoxic concentrations were tested for *C. dipterum*-IS1 but not *N. triangulifer*.

In all the above experiments, larval development was allowed to proceed until metamorphosis, when subimagos (hereafter referred to as adults) were collected, dried and weighed as above. Survivorship, adult dry mass, median development time and instantaneous growth rate were analysed using ANOVA (with Tukey's *post hoc* tests) in SAS (SAS Institute, Cary, NC, USA). We modelled the response variables as a function of oxygen, temperature and their interaction to explicitly test whether thermal reaction norms are modified by oxygen. However, the analysis was confounded by temperature and oxygen covariance (i.e. a strong discrepancy between Type III and Type I sums of squares). So, to determine whether thermal reaction norms were modified by oxygen, we first ran simple linear regressions for each of the measures versus temperature and then ran a second regression using the residuals from the first versus log-transformed (measured) P_{O_2}. This allowed us to consider the impact of oxygen after removing variability explained by temperature.

Size analysis of larval gills, legs (femur) length and ommatidia (compound eye) cells

Question 3: do morphological characters known to be affected by oxygen stress (gill size, leg length, cell size) correlate well with the upper thermal limit and the TSR? During the course of our rearing experiments 3 and 4, it became apparent that mayflies reared under hypoxic conditions developed enlarged gills relative to those reared under hyperoxia or normoxia. In order to quantify this difference (as well as provide material for the measurement of larval mesothoracic femur length and ommatidia size), we collected all larval exuviae that could be individually associated with an adult at the time of emergence for both *N. triangulifer* and *C. dipterum*-IS1. This provided us with a random subset of individuals from each oxygen/temperature treatment. In order to increase the range of body size and temperature for our normoxic baseline, we collected additional exuviae from individuals reared in vessels kept at 15 and 23°C for *N. triangulifer*, and 14, 15, 30 and 32°C for *C. dipterum*-IS1, all with oxygen maintained near saturation using atmospheric aeration. And finally, exuviae were collected from one vessel of *C. dipterum*-IS1 that was maintained at 32°C and aerated with a 3% oxygen mix, which resulted in a 23% saturation (1.7 mg l^{-1}, 4.7 kPa).

Thus, for *C. dipterum*-IS1, measurements were taken from 85 individuals from treatments within the thermal zone of acclimation (i.e. 14–30°C; Sweeney et al., 2018), 34 from hypoxic treatments and 51 from normoxic or hyperoxic treatments. Another 8 individuals were measured from 32°C treatments, 5 from normoxic and 3 from hypoxic. For *N. triangulifer*, measurements were taken from 77 randomly chosen individuals: 45 from hypoxic treatments (aeration with 6.5% or 3% oxygen at both 20 and 25°C) and 32 from normoxic treatments (air only, at 15, 20, 23 and 25°C).

For each individual sampled, a temporary slide mount (in water) of each abdominal gill was photographed using a Canon T1i (Canon, Inc., Tokyo, Japan) camera body on a Nikon Labophot-2 (Nikon Corporation, Tokyo, Japan) trinocular microscope with a 4× objective and a 2.5× projection eyepiece. Gill surface area was determined from digital images using Affinity Photo software (v.1.6.7; Serif Europe Ltd, Nottingham, UK) to enumerate pixels within a selection. Pixel counts were converted to area using a factor determined from images of a stage micrometer photographed with the same set up. As gill lamellae are essentially 2-dimensional, area was multiplied by 2 and summed over all 14 gills to arrive at total gill surface area.

We measured the length of the mesothoracic femur for both *C. dipterum*-IS1 and *N. triangulifer* as this measurement has been

shown to be a good proxy for body size in the mayfly *C. dipterum* (Šupina et al., 2016). Each femur was photographed using the equipment described above for gills, and length was determined by comparison with a photograph of a stage micrometer. The right and left femora were averaged for analysis.

The effects of oxygen concentration, dry body mass and temperature on gill surface area and mesothoracic femur length were analysed using ANCOVA (with Scheffe *post hoc* tests). We then used least squares regression analysis of $\log_{10}$-transformed data to compare scaling of gill surface area and mesothoracic femoral length with adult dry mass between normoxic and hypoxic treatments. We used the method described by Glazier and Paul (2017) for determining the significance of differences in slope.

The size of ommatidia has been suggested as a good proxy for cell size by several authors (e.g. Blanckenhorn and Llaurens, 2005; Kierat et al., 2017). Thus, compound eyes from the same set of *C. dipterum*-IS1 exuviae used to measure gill size and femur length above were photographed and a minimum of 100 ommatidia from the central portion of each eye were delineated in Affinity Photo and the number of pixels quantified (in the case of males, only ommatidia from the ventral portion of the eye were included). From these data, mean ommatidial diameter was calculated and values from the left and right eyes were averaged. Data were analysed using ANOVA (with Scheffe *post hoc* tests). Scaling with body mass was analysed as described for gill surface area above. All analyses of gills, legs and ommatidia were performed in Data Desk® v.7.0.2 (Data Description, Inc., Ithaca, NY, USA).

RESULTS

Question 1: can hyperoxia increase the upper thermal limits of mayflies?

In experiment 1, where *N. triangulifer* larvae were reared at 30°C under either normoxia or hyperoxia, young larvae were observed in all vessels up until about day 23 but none of the larvae in any treatment survived to adulthood. Note that 30°C is the lowest temperature known to result in complete mortality under normoxia (Kim et al., 2017). Thus, in this experiment, more than doubling the oxygen concentration from 20% to 45% did not enhance survivorship of *N. triangulifer*.

In experiment 2, *C. dipterum*-CT1 were reared at 32°C under either normoxia or hyperoxia. In this case, 32°C is known to be the warmest temperature at which this species can survive to metamorphosis (although females produced at this temperature are very small and infertile; Funk et al., 2019). In this experiment, there were no significant differences among O_2 treatments in survivorship or development time, but both male and female adults were 20–31% smaller in the 45% O_2 treatment than in either 20% O_2 or normal aeration (Table 2). Females in all three treatments were less than 1 mg dry mass and unable to produce progeny. Thus, increasing oxygen concentration did not alleviate the negative effects of high temperature on adult size and fertility. Rather, elevated oxygen at 32°C appeared to have a negative effect on adult body size.

Question 2: does the pattern of TSR change under conditions of hypoxia, normoxia and hyperoxia for mayflies?

Earlier experiments with *C. dipterum*-IS1, *C. dipterum*-CT1 and *N. triangulifer* showed conclusively that all three follow the TSR under normoxic conditions (Funk et al., 2019; Kim et al., 2017; Kolpas et al., 2020; Sweeney et al., 2018). For *C. dipterum*-IS1 there was a dramatic decline in size (greater than 3-fold in females) with warming temperature (from 12 to 32°C), and a concomitant increase in growth and development rate over the same temperature range (Fig. 1, top row). The thermal acclimation zone as defined by Sweeney et al. (2018) is the range (in this case, between 14.3 and 30°C) where physiological and developmental adaptations enable larvae to complete development and metamorphose after exposure to a constant number of total degree days above a defined threshold. Within this range, the rate of development and growth responds linearly to temperature. The temperature above which a significant decrement in organismal performance occurs (30°C) is referred to as the upper pejus, T_p (Frederich and Pörtner, 2000), and is most easily visualized at the point where the growth rate decreases (Fig. 1, top row, right).

Species in the *C. dipterum* complex are known to be tolerant of low oxygen concentrations relative to other mayfly species (Nagell, 1977). Thus, in order to examine the effect of oxygen availability on the TSR, we reared *C. dipterum*-IS1 through their entire larval life at a wide range of oxygen concentrations at both 20 and 25°C (Table 1). ANOVA revealed no difference in survivorship, adult dry mass, development or growth rate between air and 20% O_2 treatments at 20°C, so in all subsequent tests, normoxia was represented only by normal aeration.

Regressions using all the data collected in experiment 3 revealed no temperature effect on survivorship or adult dry mass in either sex, but highly significant effects on growth and development in both sexes (P<0.0001). Subsequent regression of temperature residuals and log-transformed P_{O_2} revealed a highly significant oxygen effect on survivorship, dry mass, development and growth.

Under normoxic conditions (~20 kPa) in experiment 3, the decrease in adult body mass between 20 and 25°C was not significant, but the increase in growth and development rate was (Fig. 1, middle and bottom rows), consistent with the trends evident in the top row of Fig. 1. A similar pattern was evident under hyperoxia (45% O_2): no significant reduction in size but a significant increase in growth and development rate. Pairwise statistical comparisons between 20 and 25°C in the hypoxia treatments must be viewed with caution for *C. dipterum*-IS1 because measured oxygen concentrations in the 3% O_2 and pure nitrogen treatments were each somewhat lower in the 25°C tests (see Table 1). With that caveat, comparison of 20 versus 25°C data showed a significant decrease in mass in the 3% O_2 and nitrogen treatments as well as an increase in both growth and development rates.

Although performance appeared to be slightly diminished by hyperoxia (similar to what we observed at 32°C, at these more

Table 2. Experiment 2: chronic (whole-life) outcomes of hyperoxic and normoxic treatments for *C. dipterum*-CT1 reared at 32°C

Aeration treatment	P_{O_2} (kPa)	Mean survivorship (%)	Mean median development time (days)	Male mean dry mass (mg)	Female mean dry mass (mg)
45% O_2	39.9	48.5±4.4	17.8±0.5	0.43±0.02*	0.59±0.02*
20% O_2	17.8	46.5±6.1	18.5±0.3	0.53±0.03	0.78±0.06
Air	18.0	55.0±8.4	17.1±0.7	0.54±0.02	0.85±0.05

Data are means±s.e.m.
*Male and female dry mass were significantly less in the 45% O_2 treatment relative to the 20% O_2 and air treatments.

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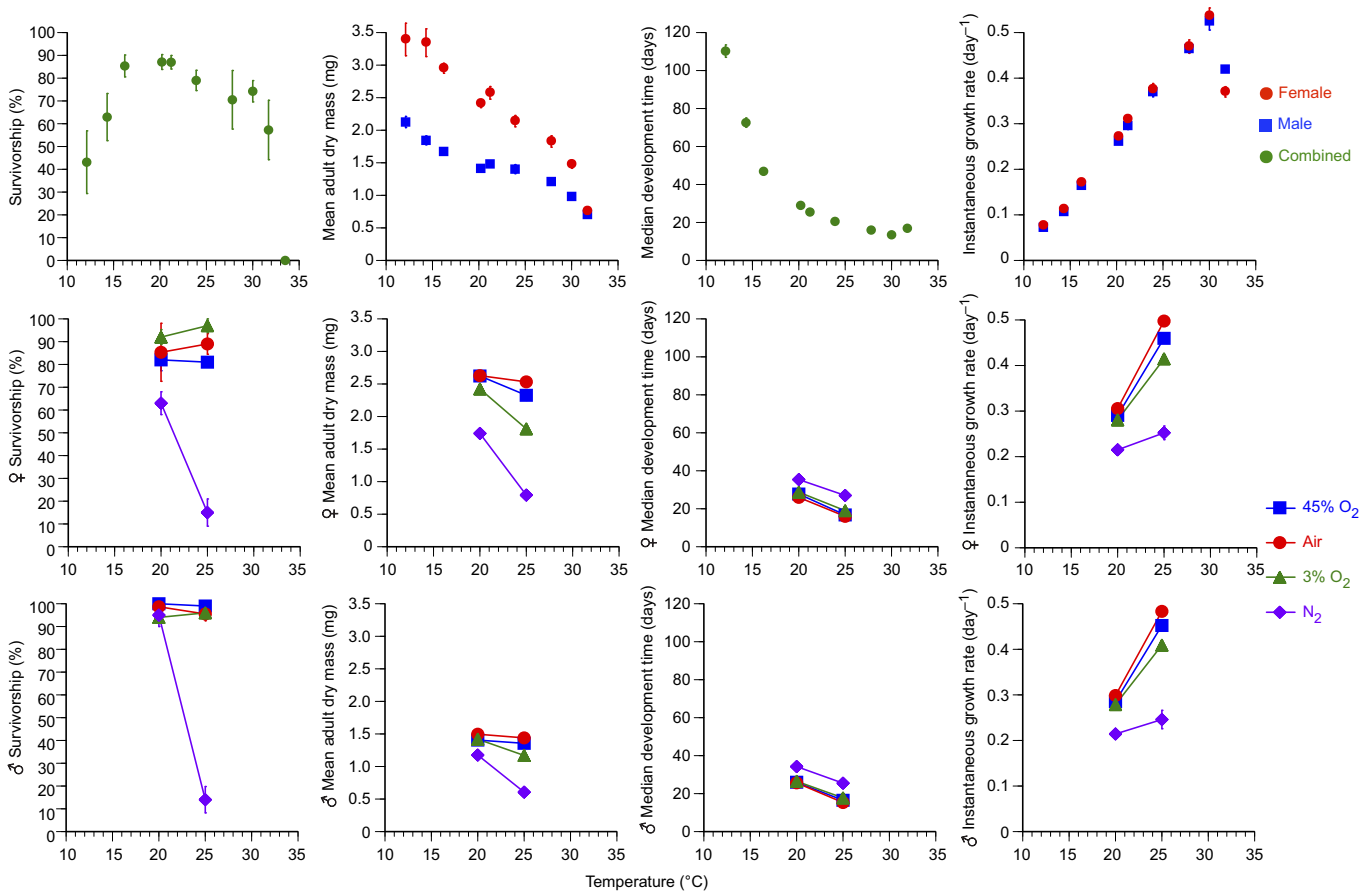

Fig. 1. Whole-life response of *Cloeon dipterum*-IS1 to a broad range of temperatures under different oxygen levels. Survivorship, mean adult dry mass, median development time and instantaneous growth rate under oxygen saturation (top row; data from Sweeney et al., 2018) and four oxygen treatments from experiment 3 ranging from hyperoxia (aeration using 45% O_2) to hypoxia (3% O_2 and pure N_2) at 20 and 25°C for females (middle row) and males (bottom row). Vertical bars show s.e.m.

moderate temperature treatments of 20 and 25°C), differences were not statistically significant. However, extreme hypoxia reduced adult size and larval growth and development rates (Fig. 1).

Hypoxia did not result in significant mortality until oxygen levels fell below 1 mg l^{-1} (2 kPa) at 25°C. The smallest females produced under hypoxia (at 25°C with P_{O_2} of 1.2 kPa) were able to produce viable progeny, unlike similar-sized females reared at 32°C under normoxia or hyperoxia.

Neocloeon triangulifer also exhibits a classic TSR, but with a narrower thermal zone of acclimation (16–24°C) and a pejus of about 25°C (Fig. 2). Regressions of the 20 versus 25°C data from experiment 4 showed that temperature affected adult size, development and growth rate, but not survivorship. Oxygen concentration altered survivorship, adult size and growth rate, but not development time.

Pairwise comparisons (ANOVA) between 20°C and 25°C (by oxygen treatment) all showed the classic TSR pattern: mayflies reared at 25°C had greater growth and development rates but adults were smaller than at 20°C. Pairwise comparisons (by temperature) indicated no difference in survivorship, growth, development or adult size between normoxia and the milder hypoxia treatment (air versus 6.5% O_2). However, mortality was greater and growth rate and adult size were reduced at oxygen concentrations below 3 mg l^{-1} (kPa <6) at both 20 and 25°C (Fig. 2). Hypoxia did not affect development rate.

Question 3: do morphological characters known to be affected by oxygen stress (gill size, leg length, cell size) correlate well with upper thermal limit and the TSR in mayflies?

While running experiment 3, we noticed that mayfly larvae in hypoxic treatments appeared to develop larger gills than those individuals reared under normoxia or hyperoxia. A comparison of two intact larval exuviae from the final larval moult of two similar-sized male *C. dipterum*-IS1 (Fig. 3) shows that gills (visible on abdominal segments 1–7) are substantially larger for the individual reared under hypoxia (left specimen) relative to normoxic conditions at the same temperature (right specimen).

In this species, gills have two lamellae on abdominal segments 1–6 and one on segment 7. Measurement revealed that the individual reared under hypoxia (4.2 kPa) had more than double the total gill surface area compared with the individual reared under normoxia (19.3 kPa), despite its smaller body mass (Fig. 4). A similar pattern was evident in *N. triangulifer*, for which all gills consist of a single lamella (Fig. 5).

Gill size in *C. dipterum*-IS1

Gill measurements made on 69 individuals reared from a range of temperatures (14–30°C) and oxygen conditions (hyperoxic to hypoxic) revealed no significant differences among normoxic (air or 20% oxygen) and hyperoxic (45% oxygen) treatments (Scheffe *post hoc* tests). Similarly, no significant differences were found

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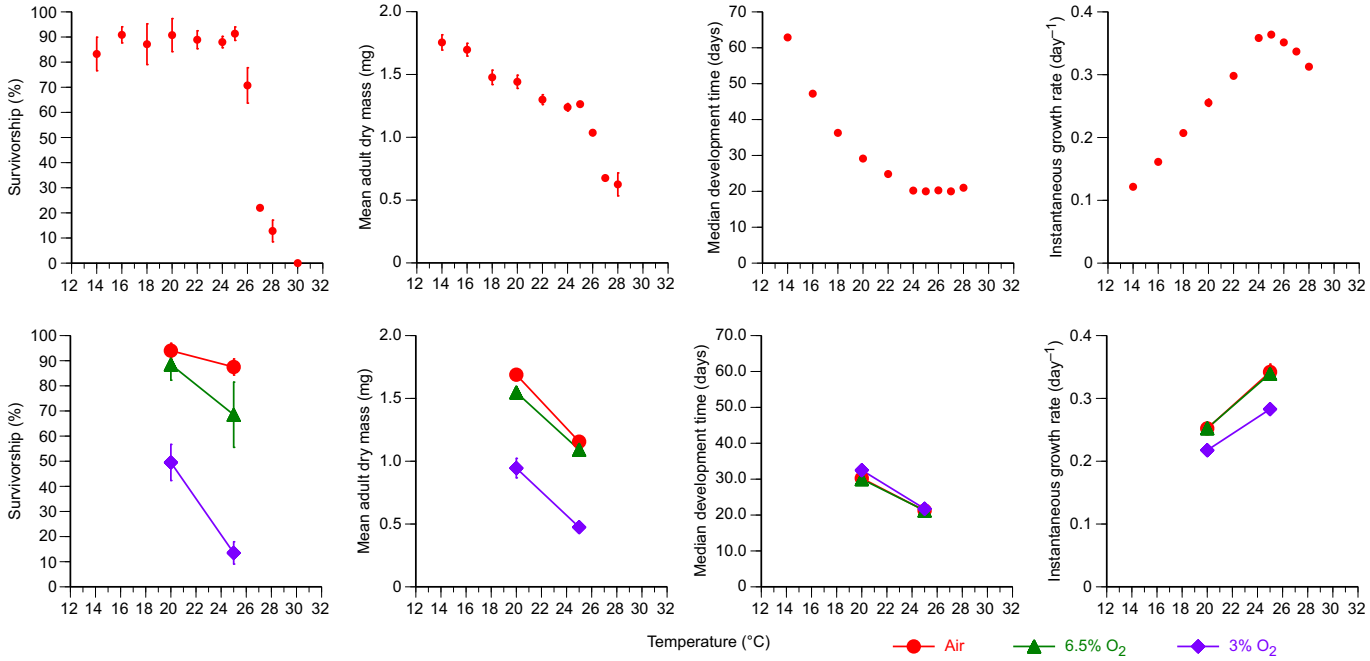

Fig. 2. Whole-life response of the parthenogenetic mayfly *Neocloeon triangulifer* to a broad range of temperatures under different oxygen levels. Survivorship, mean adult dry mass, median development time and instantaneous growth rate under conditions of oxygen-saturation (top row, from Kim et al., 2017; Kolpas et al., 2020) and three oxygen treatments from experiment 4 ranging from normoxia (normal aeration) to hypoxia (6.5% and 3% O_2) at 20 and 25°C. Vertical bars show s.e.m.

among the three hypoxic treatments (6.5% oxygen, 3% oxygen and pure nitrogen). However, all comparisons between hypoxic versus normoxic or hyperoxic treatments revealed highly significant differences (P≤0.0003). We therefore combined the three hypoxic treatments for comparison with the combination of two normoxic and one hyperoxic treatment and performed an ANCOVA, which confirmed highly significant (P≤0.0001) effects of oxygen treatment and adult dry mass on gill size, and their significant interaction suggests a difference in scaling under hypoxia (red versus blue symbols in Fig. 6A). Regression of total gill area as a function of adult dry mass (both $\log_{10}$ transformed) for all individuals from the normoxic plus hyperoxic group (at 14–30°C) showed that gill size was very predictable when oxygen was near or above saturation (y=0.589x+1.26, R^2=0.79, P≤0.0001). The 95% confidence interval (CI) for the slope was 0.493–0.685. A similar regression on the hypoxic group yielded y=0.382x+1.64 (R^2=0.59, P≤0.0001), with a CI of 0.251–0.513. Using the criteria described in Glazier and Paul (2017) (i.e. if the slope of each is outside of the other's 95% confidence limits they are considered significantly different), the indication from the ANCOVA that the scaling coefficients for normoxic versus hypoxic treatments were different was confirmed by the regression analysis.

Additional measurements of gill size on 5 individual *C. dipterum*-IS1 reared at 32°C under normoxia (from the 'air' treatment in experiment 2, Table 2) were made to test whether high temperature might elicit a gill enlargement response even under oxygen concentrations near saturation. Those individuals are represented by the open blue symbols in Fig. 6. All 5 individuals fell slightly above the regression line that had been derived from individuals reared at temperatures within the zone of acclimation (i.e. 14–30°C). The 95% CI (based on the t-distribution) for the mean of the residuals for those 5 individuals was 0.027–0.152. Because these do not include zero, we conclude that gills were indeed slightly enlarged at 32°C.

For comparison, larvae reared at the same temperature (32°C) but under hypoxic conditions (3% oxygen; see Materials and Methods for conditions) produced distinctly enlarged gills (open red symbols in Fig. 6) relative to normoxia/hyperoxia treatments and consistent with the overall trend in hypoxia.

Gill size in *N. triangulifer*

Gill measurements of *N. triangulifer* made on 45 larval exuviae from hypoxic treatments and 32 from normoxic treatments showed the effects of both oxygen and dry mass to be highly significant (P≤0.001), but unlike for *C. dipterum*-IS1, not their interaction. *Post hoc* tests indicated there were significant differences between normoxia (air) and each of the hypoxic treatments, but not between the two hypoxic treatments (i.e. 6.5% and 3% O_2). The pattern of gill size for the normoxia treatments (blue symbols in Fig. 7A) was similar to that seen in *C. dipterum*-IS1. However, although gill enlargement occurred in both hypoxic treatments for *N. triangulifer*, gills for larvae in the 6.5% O_2 treatment did not differ in size between the 20 and 25°C treatments even though adult dry mass was significantly greater at 20°C (see red symbols in Fig. 7A). ANCOVA revealed no significant difference in gill size between the two hypoxic treatments (nor their interaction with adult dry mass) and their regression was not significant. For regression of the normoxic treatments, we excluded the 25°C treatment because that temperature falls right at the pejus (see Fig. 2) and ANCOVA indicated gill size at 25°C differed from that for the other temperature treatments under normoxia (P=0.009). The restricted normoxic regression was significant (y=0.531x+0.964, R^2=0.70, P≤0.0001, n=25). The 95% CI for the slope was 0.381–0.682. Based on that regression, individuals from the 25°C air treatment had slightly enlarged gills (95% CI for the mean of the residuals was 0.015–0.063).

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Fig. 3. Exuviae from the final larval moult of two similar-sized male *C. dipterum*-IS1. The images illustrate the apparent difference in abdominal gill size when larvae were reared at 25°C in hypoxic conditions (left) versus normoxia (right).

Leg length for *C. dipterum*-IS1 and *N. triangulifer*

Because cuticular gas exchange in mayflies is not limited to tracheal gills (Eriksen and Moeur, 1990), other appendages with high surface to volume ratios might also be expected to respond to oxygen availability. We chose mesothoracic femur length as an indicator because this measure has been shown to be a good proxy for body size under normoxic conditions (Funk et al., 2019; Šupina et al., 2016) and it varied across our treatments by a factor of 2.7 in *C. dipterum*-IS1 and 1.3 in *N. triangulifer*.

Although there appeared to be some lengthening of the mesothoracic femur in *C. dipterum*-IS1 larvae reared under hypoxia (Fig. 6B), ANCOVA revealed a highly significant effect of dry mass ($P \leq 0.0001$) but not oxygen treatment ($P=0.052$) and their interaction was highly significant ($P=0.0004$); thus, the effect of oxygen treatment could not be distinguished from that of body mass. For larvae reared under normoxia or hyperoxia (at 14–30°C), regression of $\log_{10}$-transformed data (where y is the length of the mesothoracic femur and x is dry body mass) yielded $y=0.364x+0.012$ ($R^2=0.96$, $P \leq 0.0001$, $n=43$; 95% CI for the slope was 0.341–0.387). A slope of 0.333 is the expected allometry when comparing length with mass. Regression of the combined hypoxic treatments (at 14–30°C) yielded $y=0.233x+0.102$ ($R^2=0.87$, $P \leq 0.0001$, $n=31$; 95% CI for the slope was 0.199–0.267). Thus, the scaling coefficient was significantly lower for the hypoxic treatments.

For *N. triangulifer*, the relationship between femur length and body mass appeared to differ distinctly among oxygen treatments (Fig. 7B). ANCOVA indicated the effects of oxygen treatment and adult dry mass were highly significant ($P \leq 0.0001$), but not their interaction ($P=0.123$), suggesting their response curves had similar slopes. *Post hoc* tests indicated differences were significant between all three oxygen treatments, with lower oxygen concentrations resulting in longer femora. Thus, regressions on $\log_{10}$-transformed data yielded the following (where y is the length of the mesothoracic femur and x is dry body mass). For normoxia (excluding the 25°C treatment), $y=0.405x-0.006$ ($R^2=0.95$, $P \leq 0.0001$, $n=25$; 95% CI for the slope was 0.365–0.445. For 6.5% O_2, $y=0.251x+0.059$ ($R^2=0.76$, $P \leq 0.0001$, $n=26$; 95% CI for the slope was 0.166–0.286). For 3% O_2, $y=0.105x+0.096$ ($R^2=0.25$, $P=0.0275$, $n=19$; 95% CI for the slope was 0.013–0.197). Thus, the scaling coefficient of each O_2 treatment was significantly different from the others. Note, the values measured for the two hypoxic treatments were significantly lower, and for the normoxic treatment, significantly higher than the expected 0.333.

Ommatidium diameter for *C. dipterum*-IS1

We measured mean ommatidium diameter in final instar *C. dipterum*-IS1 larvae over a broad range of body sizes, from

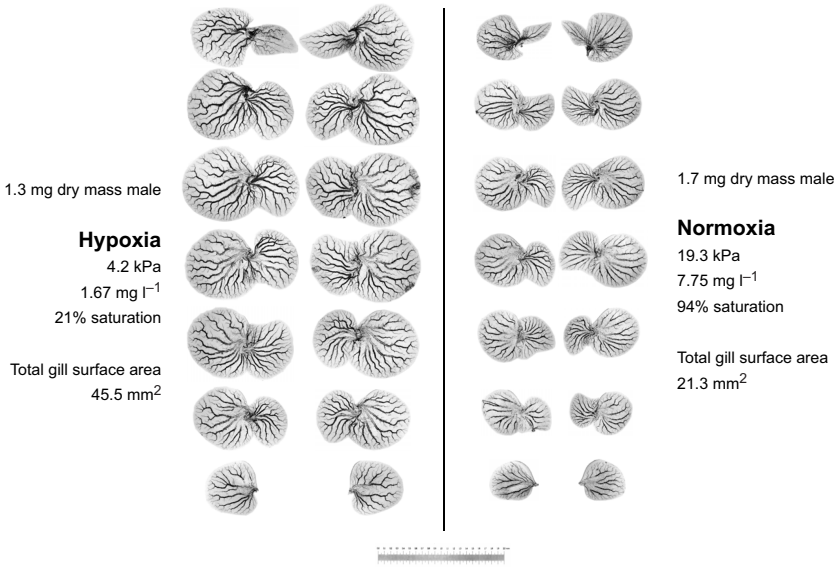

Fig. 4. Gills from two *C. dipterum*-IS1 reared under hypoxic and normoxic conditions at 25°C. Images are of the two *C. dipterum*-IS1 shown in Fig. 3, with gills arranged in order (those from segment A1 are at the top). Although the individual on the left, reared under hypoxia, was slightly smaller in terms of dry mass, its gills had more than twice the surface area of those from the individual reared under normoxia. Scale bar: 2 mm.

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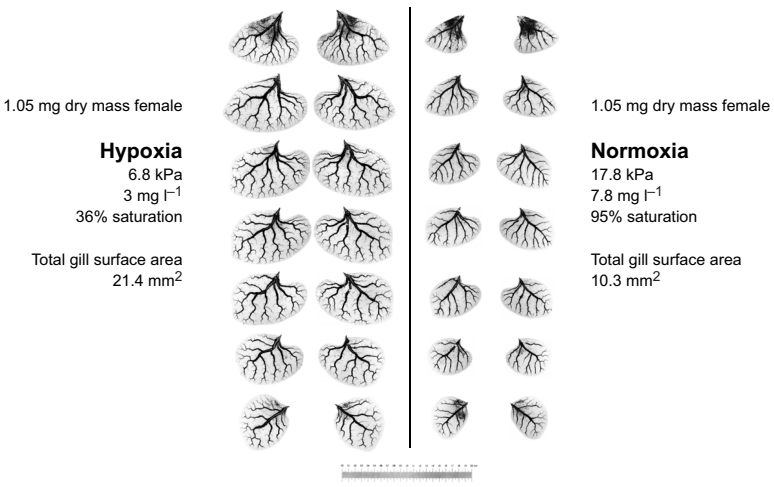

Fig. 5. Gills from two *N. triangulifer* reared under hypoxic and normoxic conditions at 25°C. The two were identical with respect to adult dry mass, but the individual reared in hypoxia had gills with more than twice the surface area of those of the individual reared in normoxia. Scale bar: 2 mm.

0.37 to 4.99 mg dry mass (a factor of nearly 13.5). Overall, ommatidium diameter increased with body size (Fig. 8A). In order to evaluate the effects of O_2, we began with an ANCOVA with $\log_{10}$ ommatidium diameter as the response variable and the factors oxygen treatment, $\log_{10}$ adult dry mass and their interaction. The results indicated there was a very significant dry mass effect on ommatidium diameter ($P \leq 0.0001$), but no significant O_2 effect, and their interaction was not significant. We then combined the two normoxic (20% O_2 and atmospheric aeration) and the hyperoxic (45% O_2) treatments into one category, and the three hypoxic (6%, 3% and pure N) treatments into another and performed a linear regression of $\log_{10}$-transformed ommatidium diameter as a function of $\log_{10}$ body mass. Regressions were significant for both groups. For the normoxic+hyperoxic group: $y=0.084x+1.199$ ($R^2=0.68$, $P \leq 0.0001$, $n=51$; CI for slope was 0.068–0.101). For the hypoxic group: $y=0.112x+1.196$ ($R^2=0.77$, $P \leq 0.0001$, $n=31$; CI for slope was 0.089–0.136). Because each slope estimate falls outside the CI of the other, we consider them to be significantly different. Here, we assume that if the diameter of ommatidia is a reliable proxy for cell size, the slope of the regression will indicate the relative contribution of cell size to changes in organ or body size (as long as they are measured in the same dimension) (Stevenson et al.,

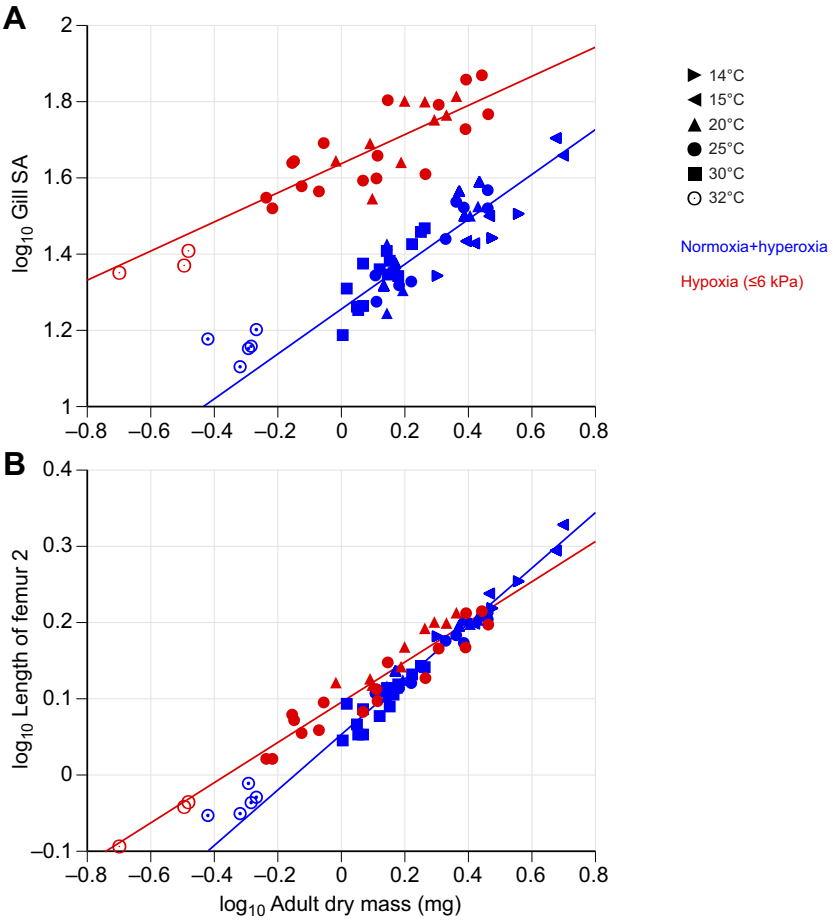

Fig. 6. Gill surface area and femur length of *C. dipterum*-IS1 reared under different oxygen levels at various temperatures. (A) Total gill surface area (SA; mm^2) in final instar larvae as a function of adult dry mass (mg) for *C. dipterum*-IS1 (males and females) reared from hatchlings under normoxic or hyperoxic (blue) and hypoxic (red) conditions at different temperatures. (B) Mesothoracic femur length (mm) as a function of adult dry mass (mg) for the same individuals. See Results for regressions. Trend lines are based only on individuals reared at temperatures within the zone of acclimation (14–30°C; Sweeney et al., 2018) (i.e. excluding the 32°C treatments).

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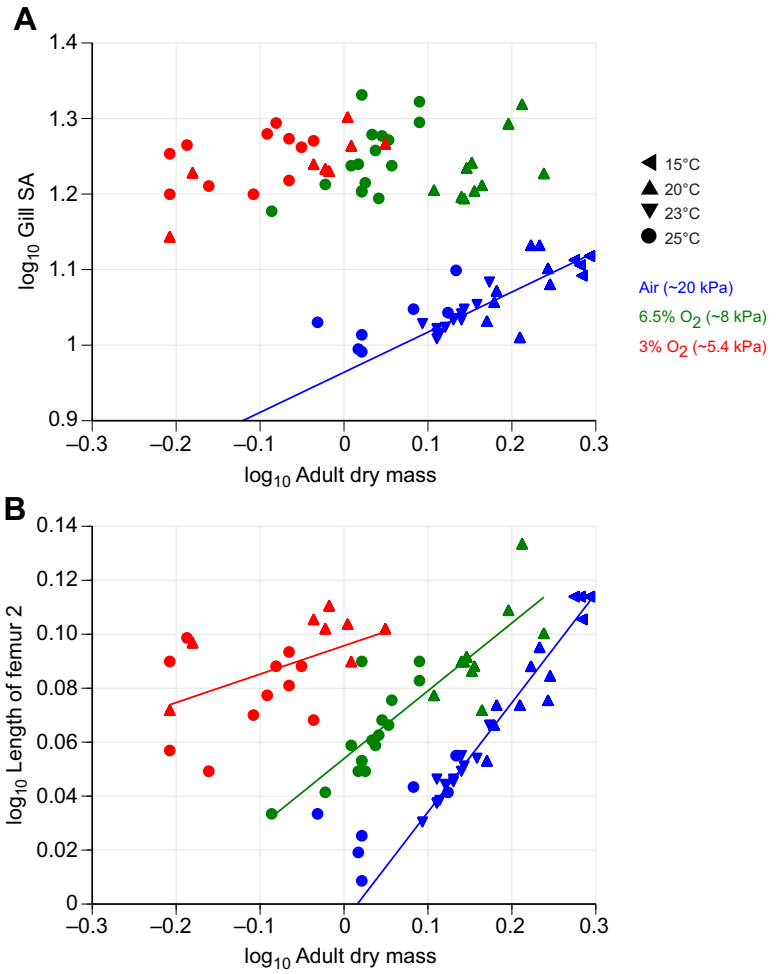

Fig. 7. Gill SA and femur length of *N. triangulifer* reared under different oxygen levels at various temperatures. (A) Total gill SA (mm^2) in final larval instar as a function of adult dry mass (mg) for *N. triangulifer* reared from hatchlings under normoxic (blue) and hypoxic (green and red) conditions at different temperatures. Regression for normoxia (air) does not include 25°C data (see Results). (B) Length of the mesothoracic femur (mm) in the final larval instar as a function of adult dry mass (mg). See Results for regressions.

1995). Thus, because our comparison was between cell diameter and body mass, a slope of 1/3 (rather than 1) would indicate 100% of the change in mass is due to cell size. Our regressions show that 25.3% of the variation in body mass is due to cell size for the normoxic group and 33.7% for the hypoxic group.

The normoxic plus hyperoxic treatment group included measurements from individuals reared at a wide temperature range, from 14 to 32°C. The only significant differences were between 25 versus 30°C treatments (one-way ANOVA with Scheffe, P=0.02) and 32°C versus any other temperature ($P \leq 0.0001$; see Fig. 8). Recall that we know that 32°C is the highest survivable temperature for *C. dipterum*-IS1 and is above the T_p of 30°C (see Fig. 2), and that we know females reared at this temperature are infertile. When we included in our regression only those temperatures between 14 and 30°C where the species is fertile (Sweeney et al., 2018), we found the following relationship:

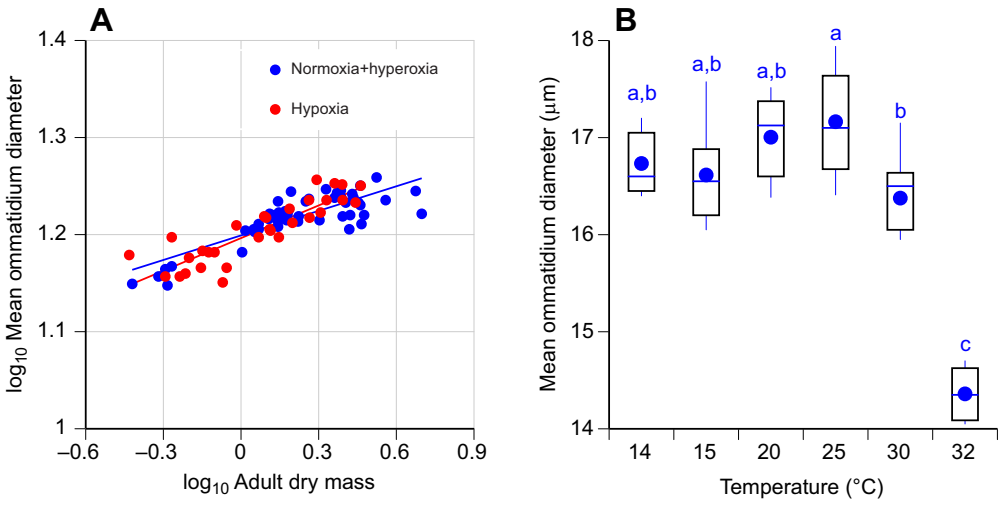

Fig. 8. Mean ommatidia diameter in *C. dipterum*-IS1. (A) Scatterplot of $\log_{10}$ mean ommatidia diameter (µm) as a function of $\log_{10}$ adult dry mass (mg) in *C. dipterum*-IS1. See Results for regressions and explanation of groupings. (B) Boxplot of mean ommatidia diameter under normoxia or hyperoxia [showing mean (circles) and median (horizontal line) values, upper and lower quartiles (box) and bottom and top decile (whiskers)]. Treatments sharing the same letter are not significantly different (ANOVA Scheffe *post hoc* tests).

y=0.051x+1.121 (R^2=0.35, P≤0.0001, n=46; CI for slope was 0.030–0.073). This indicates that only 15.5% of the change in mass was due to cell size inside the zone of thermal acclimation.

DISCUSSION

Question 1: can hyperoxia increase the upper chronic thermal limits of mayflies?

Rescue/extension of survivorship/fertility at the upper thermal limit

We found that artificially increased oxygen availability (hyperoxia) was not able to 'rescue' *N. triangulifer*, i.e. allow them to survive and reproduce at a temperature (30°C) that had earlier been determined to be chronically lethal under conditions of normoxia. These results appear to reinforce a recent study of the same mayfly species (Kim et al., 2017) that concluded chronic thermal limits do not appear to be caused by oxygen limitation based on available capacity to provide oxygen above maintenance requirements (i.e. aerobic scope) and the expression of genes indicative of hypoxia challenge.

Performance enhancement above pejus/near the upper thermal limit

Hyperoxia did not appear to benefit *C. dipterum*-CT1 at 32°C. Not only was there no increase in survivorship or development rate but also adults of both sexes were significantly smaller in the hyperoxic treatment and, like their siblings in normoxia, females were infertile. However, given that no individuals of *C. dipterum*-CT1 survived to adulthood when oxygen concentration was lowered to 4.7 kPa (3% O_2 treatment) at 32°C, it does appear that hypoxia can lower the chronic thermal maximum in this species.

We are not aware of any previous studies investigating whether hyperoxia can extend chronic upper thermal tolerance in an aquatic ectotherm. However, our results contrast with findings for terrestrial *Drosophila* (Frazier et al., 2001) where hyperoxia 'generally increased mass and growth rate at higher rearing temperatures. At lower rearing temperatures, however, hyperoxia had a very small effect on mass, did not affect growth rate, and lengthened time to eclosion'. Also note that the highest temperature where hyperoxia increased mass and growth in their study (31.5°C) was above T_p (which is consistent with our study), and this temperature resulted in low survivorship under both normoxia and hyperoxia.

Much more is known regarding the effects of hyperoxia on acute thermotolerance. During acute exposure of six species of terrestrial insects to temperatures above the upper T_p, mild hyperoxia (i.e. 35% O_2) did not improve heat tolerance and extreme hyperoxia (95% O_2) actually lowered heat tolerance in half of the species (McCue and De Los Santos, 2013). For aquatic gill-breathing insects, hyperoxia at both 36 and 60 kPa did raise CT_{max} in the stonefly *Dinocras cephalodes* (Verberk et al., 2013), but hyperoxia at 60 kPa did not for the damselfly *Calopteryx virgo* (Verberk and Calosi, 2012).

Question 2: does the pattern of TSR change under conditions of hypoxia, normoxia and hyperoxia?

Testing whether growth follows the TSR should be preceded by defining the temperature range that is 'physiologically comfortable' for the species under study, i.e. between minimal and optimal for population growth rates (Walczyńska et al., 2016). Testing species outside of this 'comfort range' may actually be an examination of the stress response. These limits (i.e. the thermal acclimation zone, *sensu* Sweeney et al., 2018) are clearly defined for *C. dipterum*-IS1 (*op. cit.*), *C. dipterum*-CT1 (Funk et al., 2019) and *N. triangulifer* (Kolpas et al., 2020). It is evident from the top row in Figs 1 and 2 of this study (and fig. SM1 of Funk et al., 2019) that *N. triangulifer*, *C. dipterum*-IS1 and *C. dipterum*-CT1 all exhibit a classic TSR pattern within their thermal zone of acclimation (12–24°C for *N. triangulifer*, 14–30°C for both *C. dipterum*-IS1 and *C. dipterum*-CT1). Indeed, adult body mass varies by about a factor of two for all these species across that range.

In our 20 and 25°C tests with *N. triangulifer*, we observed a classic TSR pattern under both normoxia and hypoxia. That is, as rearing temperature increased, growth and development rates also increased, and adult size decreased. A similar pattern was evident in *C. dipterum*-IS1, even though the decrease in dry mass from 20 to 25°C under normoxia and hyperoxia was not significant. This was likely because that temperature range represents a relatively narrow slice from the middle of the comfort zone (14–30°C) where size trends tend to flatten for both sexes (Fig. 1 herein and fig. SM1 of Funk et al., 2019). Sequential regression showed that oxygen level significantly altered thermal reaction norms for survivorship, adult size and growth rate in both species, as well as development rate in *C. dipterum*-IS1. In *N. triangulifer*, the lowest tolerable oxygen concentration (3% mix) lowered survivorship, and reduced size and growth rate (Fig. 2). A similar pattern was evident in *C. dipterum*-IS1, where the most severe hypoxia (resulting from aeration using pure nitrogen) both steepened the size reduction and reduced the increase in growth rate at the higher temperature (Fig. 1). Hyperoxia had no effect and patterns were consistent with the TSR under all oxygen treatments.

Several previous studies testing the interactive effects of oxygen and temperature on growth and adult size have shown that temperature–size responses depend on oxygen conditions. Our results contrast with some findings in each of these studies. For example, Hoefnagel and Verberk (2015) tested the freshwater isopod *Asellus aquaticus* and observed the TSR only under hypoxic conditions; under both normoxia and hyperoxia, the TSR was reversed. In our mayflies, hypoxia resulted in a significant size reduction, but the TSR was expressed under all conditions (hyperoxia, normoxia and hypoxia). Atkinson et al. (2006) found that while growth and development were always reduced by hypoxia in colonial protozoans, increased temperature increased these rates only under normoxic conditions. In our mayflies, higher temperature increased growth and development rate at all oxygen levels. Also, their protozoans did not reproduce under high temperature plus hypoxia. But unlike similar-sized females reared under normoxia at stressfully high temperature (32°C; Sweeney et al., 2018), the smallest female *C. dipterum*-IS1 produced in our experiment 3 (severe hypoxia at 25°C) were still able to produce progeny. And Frazier et al. (2001) found that hyperoxia increased mass and growth rate in *Drosophila*, at least at higher temperatures, but hyperoxia had no such effect on our mayflies (and was actually detrimental near the upper thermal limits).

The lack of benefit from hyperoxia in our tests (as well as the absence of gill size reduction) suggests that oxygen limitation is not responsible for the TSR in normoxia. Another possibility is that even though oxygen levels in our hyperoxic treatments (40 kPa) could be considered mild or moderate, they may have led to the formation of reactive oxygen species (Fridovich, 1977) that resulted in a chronic toxicity over their entire larval lifetime, thus neutralizing any potential benefits of hyperoxia at moderate temperatures. The formation of reactive oxygen species may also have been responsible for the lowered performance that we observed at stressfully high temperature (32°C). Insects must regulate internal P_{O_2} within a fairly narrow range to maintain aerobic metabolism while avoiding oxygen toxicity (Harrison et al., 2006). In at least some terrestrial insects, this may be achieved by systematic opening and closing of their spiracles (Hetz and Bradley, 2005). Although

the mayfly species in our experiments are able to actively beat their gills to increase ventilation when needed, they are not known to have functional spiracles at the intersection of gill and internal tracheae and this may limit their ability to regulate internal $\dot{P}_{O_2}$ under hyperoxic conditions, making them susceptible to oxygen toxicity.

Question 3: do morphological characters known to be affected by oxygen stress (gill size, leg length, cell size) correlate well with upper thermal limit and the TSR?

Like other insects, mayflies distribute respiratory gases by a combination of diffusion and convection within a branching tracheal system. In the terrestrial mayfly adult, these tracheae open up to segmental spiracles where they exchange gases with the atmosphere. The aquatic larvae, however, have a closed or apneustic tracheal system with tracheal gills attached to some abdominal segments where the adult spiracle will be. These gills, with their finely branched tracheae and large surface area, increase the capacity for cuticular gas diffusion. First instar mayflies have a very high surface area to volume ratio and are generally born without gills but as they grow and that ratio falls, gills appear, and gill area increases with body mass.

For the two species we tested (*N. triangulifer*, *C. dipterum*-IS1) larvae dramatically increased the size of their gills in response to low oxygen concentrations. A similar phenomenon has been observed once before in the mayfly *Stenacron interpunctatum*, where a population was found to have enlarged gills, apparently in response to depressed oxygen concentrations resulting from effluent from a paper pulp plant (Pescador and Rasmussen, 1995).

It appears that gill enlargement in our mayflies provides an easily recognized and measured morphological character indicative of a response to prevent/alleviate oxygen stress. For *C. dipterum*-IS1 reared under normoxic or hyperoxic conditions over a broad range of temperatures within their thermal acclimation zone (14–30°C; Sweeney et al., 2018), we observed a classic TSR pattern (Fig. 1, top row). Across this range of temperatures, gill size varied in a predictable manner with body mass (blue symbols in Fig. 6) and the slope of this relationship was not significantly different from 2/3, i.e. scaling was not disproportionate. Under hypoxia conditions at 20 and 25°C, larvae of *C. dipterum*-IS1 produced gills that were approximately double the size of those of comparably sized larvae reared in normoxia or hyperoxia. If the upper thermal limits for this species were the result of oxygen limitation, we would expect to have observed gill enlargement in larvae reared at temperatures near the upper chronic thermal limit of 32°C (i.e. at a temperature beyond the pejus where adult size is significantly diminished and adults are infertile) even when oxygen was near saturation. However, gills from these individuals (open blue symbols in Fig. 6) were only slightly enlarged, falling very near values predicted by the regression from individuals reared under non-stressful thermal conditions. In contrast, larvae reared at 32°C but at only 23% O_2 saturation (1.7 mg l^{-1}, 4.7 kPa) were observed to have greatly enlarged gills and a size consistent with the relationship of gill size and temperature in the reduced O_2 environment (open red symbols in Fig. 6). Thus, it seems that chronic thermal limits in this species are not the result of a failure to meet oxygen demands in a normoxic environment. Given mayflies' ability to greatly enlarge their gills in response to hypoxia within the thermal comfort zone (as evidenced by the 20 and 25°C data in Fig. 6) and assuming that this gill enlargement is a response to oxygen stress sensed internally, we propose that the absence of gill enlargement in individuals reared at the warm end of the comfort zone under environmentally normoxic conditions indicates the absence of oxygen stress. Thus, their smaller size at maturity appears to be the result of other factors and we conclude that the TSR for this species is not driven proximately by oxygen.

For *N. triangulifer*, we also showed that larvae greatly increased gill surface area in response to lowered oxygen concentrations. However, for this species, leg length also increased, indicating an increase in overall body surface area, not just gills. This is consistent with observations that even gilled aquatic ectotherms are known to meet part of their oxygen demand by other cutaneous oxygen uptake (Verberk and Atkinson, 2013). For example, in the mayfly *Siphlonurus occidentalis* (which has gills similar in structure and relative size to those of *C. dipterum*), cutaneous uptake accounted for 33% of the total oxygen consumed (Eriksen and Moeur, 1990), and some mayflies have been shown to survive the experimental removal of all their gills (Wingfield, 1939). We also know that the ratio of gill surface area to body mass in *N. triangulifer* (3.9 mm^2 per mg dry mass), a species whose gills each consist of only a single lamella, is considerably lower than that for *C. dipterum*-IS1 (7.4 mm^2 per mg dry mass), whose gills have double lamellae on abdominal segments A1–A6. The latter may explain why we observed lengthening of the leg in *N. triangulifer* but not *C. dipterum*-IS1 (although in *C. dipterum*-IS1, there was some indication of leg lengthening at the most extreme hypoxia). Regardless, it is interesting to note that under mild (6.5% O_2, ∼8 kPa) hypoxia treatment, the gills (and legs) of *N. triangulifer* were enlarged without any concomitant reduction in survivorship, adult size, growth or development under the conditions in our tests (Fig. 2). This suggests that gill enlargement is a relatively inexpensive response to oxygen stress and its absence in either *N. triangulifer* or *C. dipterum*-IS1 at warmer temperatures inside the thermal acclimation zone in a normoxic environment likely signals a lack of oxygen stress.

Although cell size (as indicated by ommatidium diameter) accounted for some portion of the changes in body mass in *C. dipterum*, the percentage was relatively low, especially under normoxia within the thermal acclimation zone (16%). The fact that cell size accounted for much more of the change in body mass under hypoxia (34%) supports the idea that smaller cell size may be adaptive under oxygen stress. And the significant drop in cell size for individuals reared at stressfully high temperature under normoxia (Fig. 8), as well as the slight enlargement of gills at this temperature, suggests oxygen does start to become a problem at the very highest survivable temperatures. However, the fact that lowered oxygen concentration in the environment can elicit a much larger response at that temperature suggests that oxygen availability does not set upper chronic thermal limits in these mayflies.

Summary and conclusion

In our thermal limit experiments, increased O_2 concentration (hyperoxia) did not rescue *N. triangulifer* larvae reared at the lowest temperature known to result in complete mortality under normoxia (30°C), reinforcing the conclusion from an earlier study (based on measurement of aerobic scope and gene expression) that oxygen limitation does not explain the chronic upper thermal limit for this species (Kim et al., 2017). Similarly, experimental hyperoxia did not mitigate the loss of fertility experienced by *C. dipterum*-CT1 reared at the highest survivable temperature under normoxia for that species (32°C), suggesting the upper chronic limit in that species is not set by oxygen limitation either.

In experiments at more moderate temperature (i.e. within the thermal acclimation zone), both *N. triangulifer* and *C. dipterum*-IS1

expressed the TSR under all oxygen concentrations – normoxia, hypoxia and, for *C. dipterum*-IS1, hyperoxia – although adults reared under hypoxia were smaller as a result of slower growth rates. Thus, at a given temperature, adult size across a gradient from normoxia to hypoxia showed a decreasing pattern similar to the TSR, but growth rate in *N. triangulifer*, and both growth and development rate in *C. dipterum*-IS1 slowed rather than increased. Structural indicators of oxygen stress under hypoxia (viz. increased gill size in both species, leg length in *N. triangulifer*, and decreased cell size in *C. dipterum*-IS1) were not evident in individuals expressing a classic TSR within the thermal zone of acclimation when dissolved oxygen was near saturation. Thus, we found no evidence to support oxygen limitation as a proximate cause of the TSR.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: D.H.F., B.W.S., J.K.J.; Methodology: D.H.F.; Validation: D.H.F.; Formal analysis: D.H.F.; Investigation: D.H.F., B.W.S., J.K.J.; Resources: B.W.S., J.K.J.; Data curation: D.H.F.; Writing - original draft: D.H.F.; Writing - review & editing: D.H.F., B.W.S., J.K.J.; Supervision: B.W.S., J.K.J.; Project administration: B.W.S., J.K.J.; Funding acquisition: B.W.S., J.K.J.

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