

## RESEARCH ARTICLE

# The effect of ambient oxygen on the thermal performance of a cockroach, *Nauphoeta cinerea*

Emily J. Lombardi\*, Candice L. Bywater and Craig R. White

## ABSTRACT

The oxygen and capacity-limited thermal tolerance (OCLTT) hypothesis proposes that the thermal tolerance of an animal is shaped by its capacity to deliver oxygen in relation to oxygen demand. Studies testing this hypothesis have largely focused on measuring short-term performance responses in animals under acute exposure to critical thermal maximums. The OCLTT hypothesis, however, emphasises the importance of sustained animal performance over acute tolerance. The present study tested the effect of chronic hypoxia and hyperoxia during development on moderate to long-term performance indicators at temperatures spanning the optimal temperature for growth in the speckled cockroach, *Nauphoeta cinerea*. In contrast to the predictions of the OCLTT hypothesis, development under hypoxia did not significantly reduce growth rate or running performance, and development under hyperoxia did not significantly increase growth rate or running performance. The effects of developmental temperature and oxygen on tracheal morphology and metabolic rate were also not consistent with OCLTT predictions, suggesting that oxygen delivery capacity is not the primary driver shaping thermal tolerance in this species. Collectively, these findings suggest that the OCLTT hypothesis does not explain moderate to long-term thermal performance in *N. cinerea*, which raises further questions about the generality of the hypothesis.

**KEY WORDS:** Hyperoxia, Hypoxia, Metabolic rate, OCLTT, Thermal performance, Growth rate

## INTRODUCTION

To predict the responses of species to environmental change, it is first important to understand the mechanisms responsible for limiting their physiological tolerances (Verberk and Bilton, 2011). The oxygen and capacity-limited thermal tolerance (OCLTT) hypothesis has emerged as one compelling framework for understanding and studying the thermal limits of animals (Fry, 1947; Pörtner, 2001; Pörtner et al., 2017). According to the OCLTT hypothesis, critical temperatures that affect fitness are generally set by a decline in aerobic scope and oxygen delivery capacity rather than by a decline in cellular level function. In ectotherms, as temperatures shift towards threshold levels (referred to as the pejus in OCLTT) the demand for oxygen in tissues increase at a rate greater than what can be supplied by cardiac and ventilatory processes, leading to a drop in whole-animal aerobic scope and a

shift into anaerobic metabolism (Pörtner, 2001, 2012). The capacity to maintain sufficient oxygen delivery beyond optimal temperature conditions ( $T_{opt}$ ) is, therefore, thought to determine an animals' thermal tolerance and performance (Bjelde et al., 2015; Chou et al., 2017).

The OCLTT hypothesis has been argued to apply to all animals, with support found across animal phyla including Chordata (Reptilia, Osteichthyes) and Arthropoda (Insecta, Crustacea) (Frederich and Pörtner, 2000; Malmendal et al., 2006; Michaud and Denlinger, 2007; Michaud et al., 2008; Frederich et al., 2009; Owerkowicz et al., 2009; Jost et al., 2012; Jensen et al., 2017; Teague et al., 2017). However, there is a growing body of evidence suggesting that the thermal tolerance of some species might not be limited by oxygen transport capacity (Stevens et al., 2010; Mölich et al., 2012; Overgaard et al., 2012; McCue and De Los Santos, 2013; Verberk et al., 2016; Kim et al., 2017; Youngblood et al., 2019). Wang et al. (2014) found that exposing European sea bass, *Dicentrarchus labrax*, to anaemia at upper critical temperatures did not alter their oxygen uptake, indicating that performance declines at upper critical temperatures observed in *D. labrax* were not due to an inability of the cardiorespiratory system to meet increasing oxygen demands. Oxygen exposures of 5 and 35 kPa in the fingered limpet, *Lottia digitalis*, have also been shown to have no effect on upper thermal tolerance (Bjelde et al., 2015). Furthermore, work on the Atlantic halibut, *Hippoglossus hippoglossus*, found that aerobic scope and cardiac performance increased at elevated temperatures, but this increase did not correspond with an increase in growth, demonstrating that aerobic scope was not responsible for limiting growth performance at high temperatures (Gräns et al., 2014).

Notwithstanding the importance of these studies in contributing to our understanding of OCLTT, very few have attempted to directly test multiple predictions of the hypothesis at relevant ecological upper and lower thermal limits (Stevens et al., 2010; Walczyńska et al., 2015; Boardman et al., 2016; Hoefnagel and Verberk, 2017; Pörtner et al., 2017). Short-term performance responses are also commonly used despite the OCLTT hypothesis emphasising the importance of long-term sustained performance over acute tolerance (Pörtner, 2014). A large gap may exist between an animals' relevant ecological thermal limits and its critical thermal limits, so investigating short-term responses under critical temperatures may be inappropriate for testing the OCLTT hypothesis (Verberk et al., 2016).

The present study aimed to address these limitations using moderate- to long-term performance indicators to test two key predictions of the OCLTT hypothesis: (1) hyperoxic environments increase thermal tolerance and hypoxic environments decrease thermal tolerance, such that changing oxygen availability manipulates performance at temperatures below and above  $T_{opt}$ , and (2) oxygen transport performance and aerobic scope decline as temperatures shift away from  $T_{opt}$  (Verberk et al., 2016; Pörtner et al., 2017).

Centre for Geometric Biology, School of Biological Sciences, Monash University, Clayton, VIC 3800, Australia.

\*Author for correspondence (emily.lombardi@monash.edu)

 E.J.L., 0000-0002-6502-6995; C.R.W., 0000-0002-0200-2187

Received 3 June 2019; Accepted 17 April 2020

To test the first prediction of the OCLTT hypothesis, we determined the effect of developmental oxygen concentration on the rate of growth and running performance (measured as running duration; a proxy for aerobic performance) in the speckled cockroach, *Nauphoeta cinerea* (Olivier 1789), at the species'  $T_{opt}$ , as well as upper and lower temperature limits. Growth rate can be used as a long-term performance indicator and running performance can provide insight into an animals' oxygen delivery capacity (Full and Tullis, 1990). The presence of anaerobic metabolites was not determined, however, so a direct link between running performance and aerobic performance cannot be made in the present study. The upper and lower temperature limits were defined as the temperatures associated with a 32% reduction in growth performance relative to growth at  $T_{opt}$ , rather than at critical thermal temperatures. If whole-animal oxygen limitation sets thermal tolerance and performance, as proposed by the OCLTT hypothesis, then it is predicted that the decline in growth and running performance observed at temperatures below and above  $T_{opt}$  will be reduced in hyperoxic environments and enhanced in hypoxic environments.

The second prediction of the OCLTT hypothesis was tested indirectly by measuring the rate of carbon dioxide production (a proxy for metabolic rate) and tracheal morphology in cockroaches developed under the same oxygen and temperature conditions as mentioned above. Routine metabolic rate (RMR) is defined as the metabolic rate of resting post-absorptive animals below which function is impaired. It therefore sets the baseline for energetic costs of living and is of significant functional importance. RMR can provide some insight into an organisms' aerobic scope, although aerobic scope is a function of both maximum metabolic rate (MMR) and RMR. Therefore, RMR can only provide a partial understanding of aerobic scope. The tracheal system is responsible for delivering oxygen to sites of respiration and removing carbon dioxide from the tissues to the atmosphere (Chown and Nicolson, 2004), and in some species, an outgrowth of the tracheal network in response to hypoxia has been observed (Ghabrial et al., 2003). This outgrowth can allow for a greater capacity of the tracheal system to meet cellular oxygen demand by altering an animal's oxygen transport performance (Ghabrial et al., 2003). If oxygen transport performance declines as temperatures shift away from  $T_{opt}$ , as suggested by the OCLTT hypothesis, then we would expect to see similar compensatory changes in the tracheal system at temperatures beyond  $T_{opt}$ , allowing for insects to compensate for the limited oxygen availability. Experimental manipulation of ambient oxygen during development might also cause a change in tracheal morphology that will alter oxygen delivery in normoxia, with potential consequences for aerobic capacity and sustained locomotion. Specifically, an outgrowth of the tracheal network might be observed under hypoxic conditions to ensure that cellular oxygen demand is met by the tracheal system. Conversely, conditions of hyperoxia may lead to reductions in tracheal dimensions in order to avoid hyperoxic damage.

*Nauphoeta cinerea* is a suitable study organism for testing these predictions in the laboratory owing to its ease of maintenance and ability to readily breed in captivity (Schimpf et al., 2012b, 2013).

## MATERIALS AND METHODS

### Determining the thermal performance curve for growth

Juvenile speckled cockroaches (mean±s.e.m mass=9.75±0.16 mg) were obtained from a breeding population maintained at 23±2°C at Monash University, Clayton, Victoria, Australia. Fifty individuals were randomly assigned to individual 20 ml plastic centrifuge tubes maintained at constant temperatures of 10, 14, 18, 22, 25, 30, 33 or

36°C (±0.3°C), in incubators (Versatile environmental test chambers, MLR-352H series, Panasonic Healthcase Co., Ltd). All containers were maintained under normoxic conditions at 21% O<sub>2</sub> in N<sub>2</sub> with a 12 h:12 h light:dark cycle. The bottom of each centrifuge tube was removed and replaced with mesh wiring to provide airflow, and black tape was placed halfway up each tube to provide refuge. Cockroaches were maintained on a diet of carrot and dry cat food. Fresh food was provided every second day to avoid effects of desiccation and the moulding of food. Water was not supplied as the carrot provided all required moisture.

Each week for 5 weeks, the mass of every individual in each of the temperature treatments was measured to the nearest 0.01 mg using a microbalance (XS105DU, Mettler Toledo, Port Melbourne, Victoria, Australia; linearity ±0.2 mg). Measurements coincided with the replacement of new food to minimise time spent outside treatments. After a period of 5 weeks, no individual had survived at 10°C and data for this treatment were discarded. Owing to mortality across each week, only those who survived the entire 5 weeks were used for analysis (Table S1).

Rates of mass gain were calculated as percentage of mass gained per day, on average, from day 7 to day 35. Owing to potential effects of stress from moving cockroaches from their stock populations, and issues with moulding of food in the first week, the mass gained in the first 7 days was not included in calculations. The  $T_{opt}$  was determined as the temperature at which growth was maximised, and the lower and upper developmental temperatures for subsequent developmental oxygen manipulation experiments (see below) were defined as the temperatures below and above  $T_{opt}$ , respectively, at which growth was reduced by 32% relative to the rate at  $T_{opt}$ . This reduction in growth rate was chosen because it ensured temperatures remained within critical thermal limits, but were sufficiently distinct for changes in performance to be observed across treatment temperatures given the stability of the incubators of ±0.3°C.

### Developmental oxygen manipulation

To investigate whether oxygen delivery will influence thermal performance at the upper and lower developmental temperatures, 80 juvenile speckled cockroaches (mean±s.e.m mass=20.45±0.20 mg) were randomly assigned to individual 20 ml centrifuge tubes maintained at factorial combinations of one of three levels of ambient O<sub>2</sub> (10, 21 or 40% O<sub>2</sub> in N<sub>2</sub>), and one of three temperatures ( $T_{opt}$ , upper and lower developmental temperatures), as determined from the thermal performance curve for growth; a total of nine treatment groups. Atmospheric air is 21% oxygen, and oxygen levels of 10% (hypoxic treatment) and 40% (hyperoxic treatment) were selected because previous work has shown that these are sufficient to result in developmental changes in tracheal morphology in *N. cinerea* without adverse effects on maturation (Bartrim et al., 2014). Cockroaches were obtained from the same laboratory breeding population at Monash University and maintained under the same photoperiod and feeding conditions as for measurement of the thermal performance curve for growth. Each treatment group consisted of a clear plastic storage container (J. Burrows 15 L Storage Container, Clayton, VIC, Australia), to maintain oxygen concentrations, with four racks of centrifuge tubes each containing a juvenile cockroach (20 individuals per rack). Owing to temperature fluctuations of 0.3°C within incubators, racks were randomly rotated within each container every 2 days, and containers were randomly rotated within each incubator every 4 days. Rotations coincided with the replacement of new food to minimise time spent outside treatments.

Oxygen concentrations within the 40% O<sub>2</sub> treatment containers were maintained at the selected O<sub>2</sub> level using a ROXY-4 gas regulation system (Sable Systems, Las Vegas, NV, USA) that measured O<sub>2</sub> levels within each container using O<sub>2</sub> fuel cell sensors fitted to the middle of each container lid. The O<sub>2</sub> was then delivered to the housing containers as required. The 10% O<sub>2</sub> treatments were maintained by delivering atmospheric air at a constant rate of 50 ml min<sup>-1</sup> and nitrogen at a constant rate of 50 ml min<sup>-1</sup> into a 100 ml glass mixing chamber, and then into the animal container. Atmospheric air delivered at a constant rate of 100 ml min<sup>-1</sup> was used to maintain the 21% O<sub>2</sub> treatments. Flow rates were regulated by mass flow controllers (Aalborg GFC17, Stanton Scientific, Byron Bay, NSW, Australia).

A single input hole was drilled into each container, and six output holes were drilled on opposite ends to provide for greater air flow. Holes that were required for the cell sensor and input hole were covered with modelling putty to minimise leakage of the input gases. Humidifiers were constructed from 500 ml glass jars filled to 350 ml with water; however, they were removed within the first week because of issues of high water vapour moulding the food.

### Growth rate

Growth rate was calculated using the same technique as for the measurement of the thermal performance curve for growth. Each week for 5 weeks, the mass of every individual in each treatment group were measured to the nearest 0.01 mg using a microbalance. The percentage of mass gained per treatment per day, on average, using the data from day 7 to day 35, was calculated and used for analysis. Owing to mortality across each week, only those who survived the entire 5 weeks were used in analysis (Table S2).

### Running performance

Juvenile cockroaches ( $n$ =approximately 11 per treatment) were randomly selected from each treatment group and run on a modified treadmill (LE8708TS, Harvard Apparatus, Holliston, MA, USA) under normoxia until exhaustion. Prior to measurement, cockroaches were weighed to the nearest 0.01 mg. The treadmill was maintained at a constant speed of 2 cm s<sup>-1</sup> and small plastic cups (measurement chambers) lined with flouon were placed over individuals to prevent escape during measurements (Movie 1). Exhaustion was determined when cockroaches refused to continue running after three nudges of encouragement within 1 min. The total time spent continuously running was used in analysis to determine running performance (a proxy for aerobic performance). Each cockroach was run on the treadmill once at each of the three developmental temperatures in a temperature-controlled room (or once at the lower developmental temperature and  $T_{opt}$ , depending on survival). These temperatures were randomly set in the order: (1) lower developmental temperature, (2)  $T_{opt}$  and (3) upper developmental temperature. Temperatures were recorded prior to measurements and temperature fluctuations within the temperature-controlled room were  $\pm 0.5^\circ\text{C}$ . At least 1 week was provided between each temperature measurement to allow for recovery, although mortality did occur within some treatments. Only individuals that survived were included in statistical analysis.

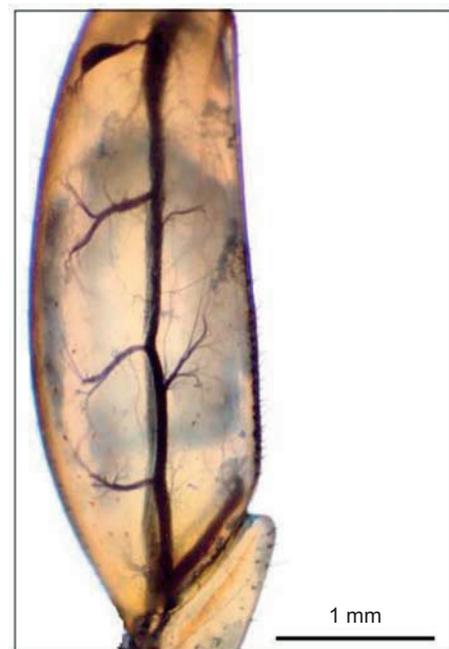
### Tracheal morphology

Tracheal morphology was measured using a staining technique modified from Weis-Fogh (1964), Kanwisher (1966) and Bartrim et al. (2014). An ink solution was prepared from 1 g gelatine, 20 ml water, 4 ml liquid detergent and 25 ml black ink (Quink, Parker Pens, Clayton, VIC, Australia). Cockroaches ( $n$ =approximately 11

per treatment) were cold-euthanised for 1 h in a  $-20^\circ\text{C}$  freezer and were removed and allowed to defrost. Cockroaches were then submerged into the ink solution in a pressurised bulb-shaped flask attached to a tubed tonometer composed of two stop-cocks (see Bartrim et al., 2014). The flask was then slowly re-pressurised by sequentially opening and closing the two stop-cocks. The stop-cocks were opened twice every 5 min for the first 30 min, and once every 1 min thereafter until the flask returned to atmospheric pressure. The stop-cocks then remained open for an additional 5 min to ensure infiltration of the ink solution into the tracheal network. Care was taken not to leave cockroaches inside the ink solution for more than 55 min, as this would result in the staining of tissues, obscuring the tracheal system. Dimensions of the tracheal system were taken using a microscope-mounted digital camera attached to a dissecting microscope (Olympus SZ61, Olympus Australia, Mount Waverley, Australia). Images were captured from the camera and analysed on a PC using the Olympus micro imaging software cellSens platform. The legs of each cockroach were imaged because this area is aerobically important for locomotion, and because the tracheal network can be viewed externally without dissection. The femur segment of the right and left metathoracic legs of each cockroach were imaged on a 1 mm grid for scale (Fig. 1). The length of each femur as well as the diameter of the main trunk, calculated from estimated thirds of the femur, were recorded. The average femur length and tracheal diameter for each cockroach were then calculated and used for analysis.

### Routine metabolic rate

Juvenile cockroaches ( $n$ =approximately 19 per treatment) were randomly selected from each treatment group and subjected to standard flow-through respirometry (Withers, 2001; Lighton, 2008) to measure rate of carbon dioxide production as a proxy for RMR. Prior to respirometry, cockroaches were fasted for 24–82 h, which is



**Fig. 1. Stained tracheal system of the femur in the back right leg of *Nauphoeta cinerea* developed at 21% O<sub>2</sub> at 28.3°C.** Developmental oxygen concentration (10, 21 and 40%) had no significant effect on the tracheal morphology in cockroaches developed at 28.3, 32.3 and 34.5°C.

sufficient to yield a post-absorptive state in adult *N. cinerea* (Schimpf et al., 2012a) and weighed to the nearest 0.01 mg. Background CO<sub>2</sub> concentrations were measured using an empty 10 ml respirometry chamber until the CO<sub>2</sub> trace was stable (~15 min). This empty chamber was then replaced with a chamber containing a cockroach. The CO<sub>2</sub> production for each cockroach was measured for 3 h in darkness under normoxia at each of the three developmental temperatures (1 h at each temperature, with an initial 20 min settling period to induce resting behaviour). The order of measurement temperature was randomised, and shifts between each temperature occurred over a period of 20 min. These 20 min periods were not included in the 1 h measurement period at each temperature. The first and last 10 min of each 1 h recording at each temperature were discarded to remove the effects of handling stress. The activity of cockroaches was not monitored, so activity may have occurred during the measurement period. We therefore refer to this trait as RMR to account for low levels of spontaneous activity. After the total measurement period, cockroaches were removed from the chambers, and the background CO<sub>2</sub> concentration was measured again for 15 min using empty chambers. The mean of the lowest 30 min of CO<sub>2</sub> production measured over the 1 h period at each temperature was used for RMR calculations. This 30 min measurement duration ensured that potential effects of discontinuous gas exchange and differences in gas exchange patterns across treatments were accounted for. The background CO<sub>2</sub> concentrations were subtracted from the CO<sub>2</sub> concentrations obtained from the 30 min recording of each cockroach (see Alton et al., 2017), and standard equations (see Lighton, 2008) were used to calculate final rates of CO<sub>2</sub> production. Temperatures were controlled using a temperature cabinet, and approximately 2 m of tubing was looped inside the cabinet to ensure the air entering the chamber had equilibrated with the specified measurement temperature. Dry atmospheric air scrubbed of water vapour and CO<sub>2</sub> was delivered through the chamber at a constant rate of 25 ml min<sup>-1</sup> and then passed through an Li-840A CO<sub>2</sub> analyser (Li-Cor, Lincoln, NE, USA), which was interfaced with a computer running Lab-Chart 7 (ADInstruments, Bella Vista, NSW, Australia). The CO<sub>2</sub> analysers were calibrated with N<sub>2</sub> and certified gas mixers (11.4±0.5 ppm mol CO<sub>2</sub> in N<sub>2</sub>, and 50.3±1.0 ppm mol CO<sub>2</sub> in N<sub>2</sub>, Air Liquide, North Sunshine, VIC, Australia). Mass flow controllers (Aalborg GFC17, Stanton Scientific, Byron Bay, NSW, Australia) were used to regulate the flow rate into the chamber and were calibrated using a Gilian Gilibrator-2 NIOSH Primary Standard Air Flow Calibrator.

### Statistical methods

The  $T_{opt}$ , lower and upper developmental temperatures were determined in the measurement of the thermal performance curve for growth using the Akaike information criterion (AIC) weighted mean of three models fitted to the data: Baker (Baker et al., 1991), Sharpe–Schoolfield (Schoolfield et al., 1981) and cubic polynomial. A linear model was used to test for significant effects of developmental oxygen and developmental temperature on growth. Linear mixed-effect models were applied to the running performance, tracheal morphology and RMR data to determine whether the developmental oxygen and temperature treatments had a significant effect on these performance traits. Following Quinn and Keough (2002), random effects were retained in models only if a likelihood ratio test yielded *P*-values greater than 0.25 for the random effect.

Cockroach mass, measurement temperature, developmental temperature and developmental oxygen were included as categorical fixed effects in the model for running performance,

with cockroach mass included as a continuous fixed effect. Order of experimental measurement (run), measurement chamber and cockroach identification number (ID) were included as random categorical effects in the model. Measurement chamber was found to explain very little of the variation, so was removed from the final model. Developmental temperature and developmental oxygen were included as categorical fixed effects in the model for tracheal morphology, with average femur length included as a continuous fixed effect. The run and order of tracheal staining were included as random categorical effects in the model for tracheal morphology but were later removed because they explained very little. The linear mixed-effect model for RMR controlled for the categorical fixed effects of measurement temperature, developmental temperature, and developmental oxygen, with cockroach mass and fasting duration included as continuous fixed effects in the model. Run, measurement chamber, temperature order and cockroach ID were also included as categorical random effects. Measurement chamber and temperature order were found to explain very little of the variation, so were removed from the final RMR model.

In order to meet assumptions of normality and homoscedasticity, a natural log transformation was applied to the response variables of running performance, average tracheal diameter and RMR in each of the three linear mixed-effect models. Cockroach mass was also log transformed in the running performance and RMR measures, and average femur length was log transformed in the tracheal morphology measure. Interactions of the main effects were removed from all linear models if they were not significant. Pairwise comparisons using Tukey's honest significant difference were conducted to investigate significant differences among sample means.

Data analysis was performed using R version 3.2.3 (2015-12-10) and Rstudio version 0.99.891. All significance testing was at the 0.05 level.

## RESULTS

### Thermal performance curve for growth

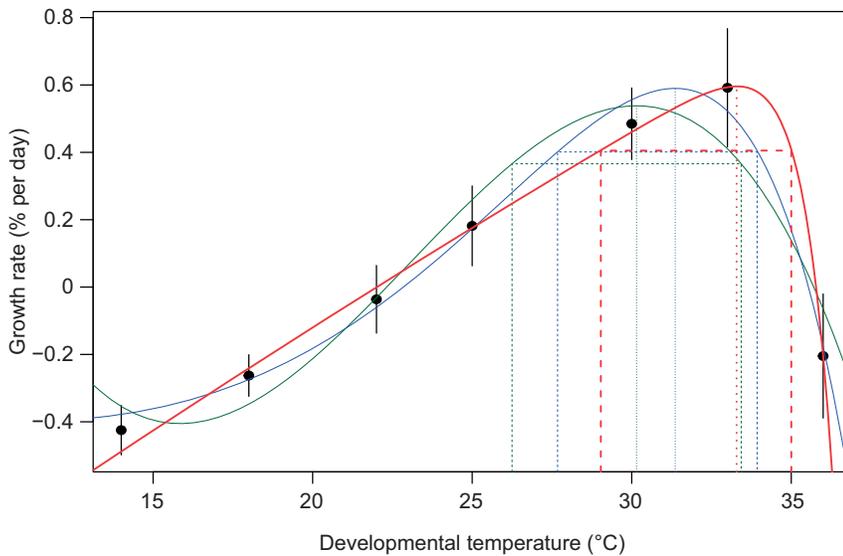
The AIC weighted values of the Baker, Sharpe–Schoolfield and cubic polynomial models indicated an optimal developmental temperature for growth at 32.3°C, a lower developmental temperature at which growth was reduced by 32% at 28.3°C, and an upper developmental temperature at which growth was reduced by 32% at 34.5°C (Fig. 2).

### Growth rate

There was a significant interaction of the main effects of developmental temperature and developmental oxygen on the rate of growth in *N. cinerea* ( $F_{4,572}=3.69$ ,  $P=0.006$ ). In contrast to the OCLTT hypothesis, hyperoxia did not significantly increase growth and hypoxia did not significantly reduce growth at both the upper (34.5°C) and lower (28.3°C) developmental temperature treatments relative to normoxia at those temperatures. Interestingly, individuals developed under hyperoxia had significantly lower rates of growth relative to normoxia, at 34.5°C (Fig. 3). At  $T_{opt}$  (32.3°C), developmental hypoxia did lower rates of growth; however, there was no significant effect of developmental hyperoxia on growth rate, further contradicting OCLTT expectations (Fig. 3). Significant differences across temperatures were only observed in the hyperoxic and normoxic treatments, with growth declining at the upper developmental temperature of 34.5°C (Fig. 3).

### Running performance and tracheal morphology

In contrast to OCLTT, developmental oxygen concentration had no significant effect on running performance and tracheal morphology



**Fig. 2. The thermal performance curve for growth in *N. cinerea* over a 5 week developmental period.** Points show the mean $\pm$ s.e.m. mass gained at each temperature, calculated as percentage of mass gained per day, on average, from day 7 to day 35. Coloured curves indicate three models fitted to the data (blue, Baker model; red, Sharpe–Schoolfield model; green, cubic polynomial model; optimum temperatures predicted by each model are shown as dotted lines, and upper and lower temperatures at which growth was reduced by 32% are shown as dashed lines). Sample sizes are as follows: 14°C,  $n=20$ ; 18°C,  $n=40$ ; 22°C,  $n=41$ ; 25°C,  $n=38$ ; 30°C,  $n=36$ ; 33°C,  $n=31$ ; 36°C,  $n=18$ .

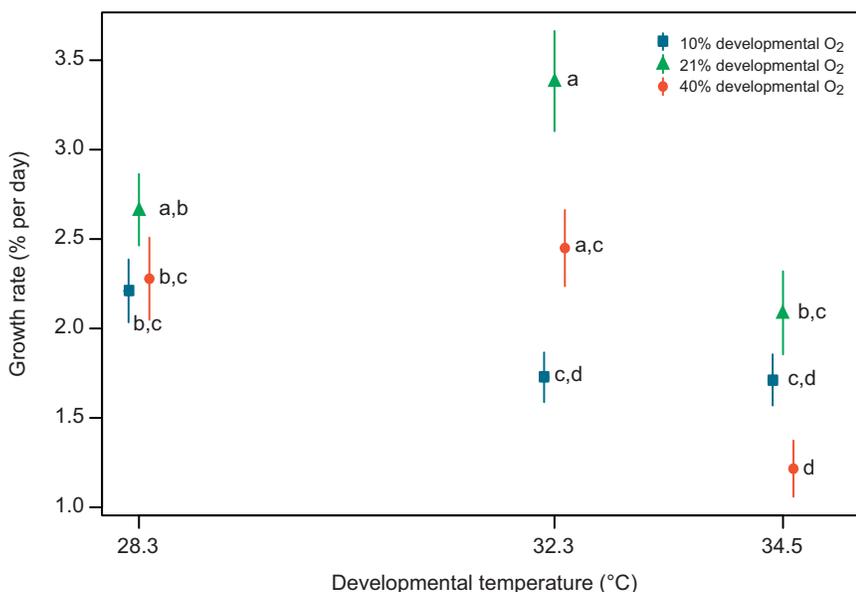
across all three temperature treatments (Figs 4, 5). There was no significant interaction of the fixed effects of cockroach mass, measurement temperature, developmental temperature and developmental oxygen on running performance ( $P>0.05$  in all cases). In controlling for the effect of cockroach mass and measurement temperature, there was no significant difference in running performance across all three oxygen treatments (10, 21 and 40%) at each of the three developmental temperatures (34.5, 32.3 and 28.3°C). Similarly, there was no significant interaction of the fixed effects of average femur length, developmental temperature and developmental oxygen on tracheal morphology ( $P>0.05$  in all cases). In controlling for the effect of average femur length, tracheal morphology did not significantly differ across all three oxygen treatments (10, 21 and 40%) at each of the three developmental temperatures (34.5, 32.3 and 28.3°C).

#### Routine metabolic rate

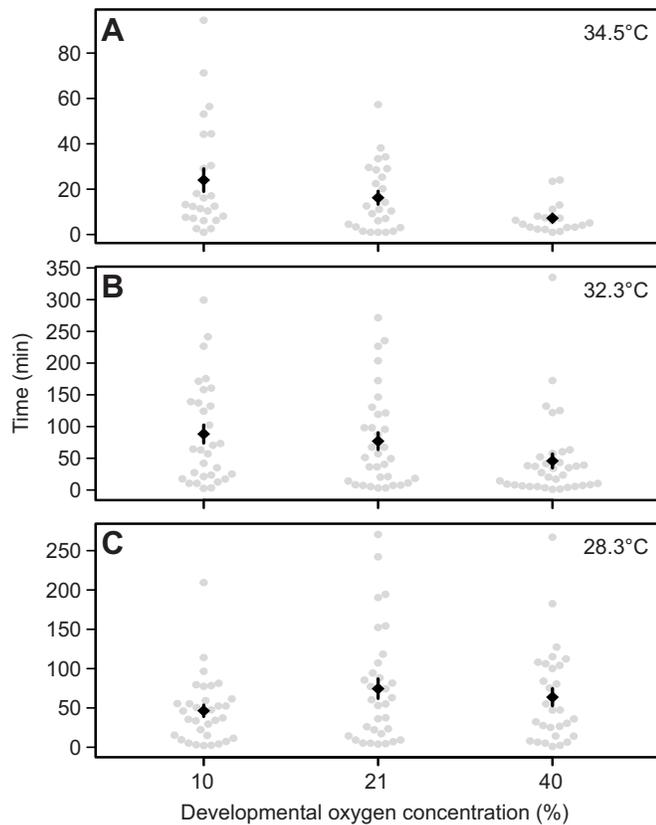
There was a significant interaction of the main effects of developmental temperature and developmental oxygen on RMR

( $\chi^2_4=11.17$ ,  $P=0.025$ ), as well as a significant main effect of ln-transformed mass ( $\chi^2_1=1137.27$ ,  $P<0.001$ ). Measurement temperature exhibited no significant interactions with developmental temperature or developmental oxygen ( $P>0.05$  in both cases), so these interactions were removed from the minimum adequate model, leaving the significant main effects of measurement temperature ( $\chi^2_1=1340.38$ ,  $P<0.001$ ) and fasting duration ( $\chi^2_1=4.28$ ,  $P=0.038$ ). Because the effect of measurement temperature did not differ among treatments and the effect of measurement temperature was strictly positive over all measurement temperatures, for presentation, all RMR data were adjusted to a common temperature ( $T_{opt}$ ) as well as a common body mass (equal to the mean of natural log-transformed mass, 67.67 mg), using the parameter estimates for measurement temperature (mean $\pm$ s.e.m.=0.078 $\pm$ 0.002, equivalent to a  $Q_{10}$  of 2.18) and mass (mean $\pm$ s.e.m.=0.999 $\pm$ 0.030).

Developmental oxygen concentration had no significant effect on RMR at the upper (34.5°C) and lower (28.3°C) developmental temperature relative to normoxia under those temperatures (Fig. 6).



**Fig. 3. The effect of oxygen concentration during development on the growth rate of *N. cinerea* at three developmental temperatures: a lower developmental temperature of 28.3°C, an optimal developmental temperature of 32.3°C and an upper developmental temperature of 34.5°C.** Points show the mean $\pm$ s.e.m. mass gained in individuals developed at each oxygen and temperature treatment, calculated as percentage of mass gained per day, on average, from day 7 to day 35. All points that share a letter are not significantly different from each other. Blue squares, 10% O<sub>2</sub>; green triangles, 21% O<sub>2</sub>; red circles, 40% O<sub>2</sub>. Sample sizes are as follows: 10% O<sub>2</sub> at 34.5°C,  $n=60$ ; 21% O<sub>2</sub> at 34.5°C,  $n=64$ ; 40% O<sub>2</sub> at 34.5°C,  $n=56$ ; 10% O<sub>2</sub> at 32.3°C,  $n=68$ ; 21% O<sub>2</sub> at 32.3°C,  $n=61$ ; 40% O<sub>2</sub> at 32.3°C,  $n=65$ ; 10% O<sub>2</sub> at 28.3°C,  $n=68$ ; 21% O<sub>2</sub> at 28.3°C,  $n=70$ ; 40% O<sub>2</sub> at 28.3°C,  $n=69$ .



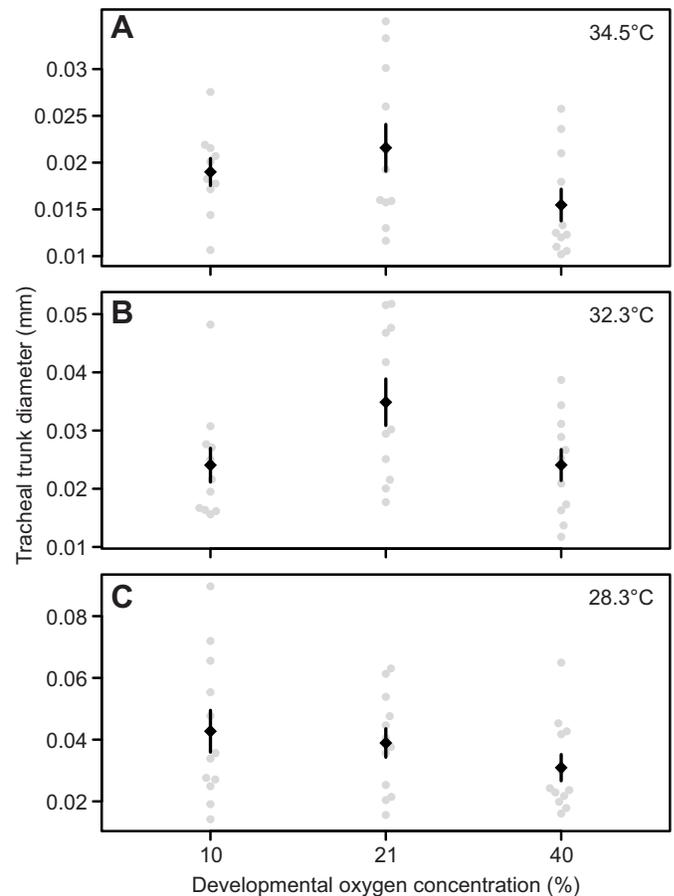
**Fig. 4. The effect of developmental oxygen concentration on the running performance of *N. cinerea* at three developmental temperatures.** (A) The upper developmental temperature of 34.5°C, (B) the optimal developmental temperature of 32.3°C and (C) the lower developmental temperature of 28.3°C. Running performance (proxy for aerobic performance) was calculated as the total time (min) spent continuously running on a treadmill under normoxia (21% O<sub>2</sub>) at a speed of 2 cm s<sup>-1</sup>. Total time spent continuously running for each individual are shown as grey dots. Black diamonds show the mean ± s.e.m. for each treatment. Cockroaches were run once at each of the three developmental temperatures (or once at 28.3°C and 32.3°C, depending on survival). Developmental oxygen concentration had no significant effect on running performance across all three temperature treatments. Sample sizes are as follows: 10% O<sub>2</sub> at 34.5°C, *n*=11; 21% O<sub>2</sub> at 34.5°C, *n*=11; 40% O<sub>2</sub> at 34.5°C, *n*=9; 10% O<sub>2</sub> at 32.3°C, *n*=11; 21% O<sub>2</sub> at 32.3°C, *n*=11; 40% O<sub>2</sub> at 32.3°C, *n*=12; 10% O<sub>2</sub> at 28.3°C, *n*=11; 21% O<sub>2</sub> at 28.3°C, *n*=12; 40% O<sub>2</sub> at 28.3°C, *n*=11.

No significant differences were found in RMR in the hyperoxic and normoxic treatment between the upper and lower developmental temperature and  $T_{opt}$  (Fig. 6). However, cockroaches developed under hypoxia did show significantly higher RMR at the lower developmental temperature of 28.3°C and significantly lower RMR at the upper developmental temperature of 34.5°C, when compared with those developed under hypoxia at  $T_{opt}$ , indicating a decrease in RMR with an increase in developmental temperature (Fig. 6).

## DISCUSSION

### Growth rate

Few studies have investigated the effect of chronic exposures to different oxygen concentrations on a species' thermal performance, with even fewer investigating long-term performance indicators, such as growth rate or reproductive success, particularly at lower temperatures (Frazier et al., 2001; Owerkowicz et al., 2009; VandenBrooks et al., 2012; Bartrim et al., 2014). Those studies that have investigated the effect of oxygen on performance at cooler



**Fig. 5. The effect of developmental oxygen concentration on the tracheal morphology of *N. cinerea* at three developmental temperatures.** (A) The upper developmental temperature of 34.5°C, (B) the optimal developmental temperature of 32.3°C and (C) the lower developmental temperature of 28.3°C. The average tracheal trunk diameters in the femurs of each cockroach were quantified and used as indicators of changes in tracheal morphology. The average tracheal trunk diameter for each individual is shown as a grey dot. Black diamonds show the mean ± s.e.m. for each treatment. Developmental oxygen concentration had no significant effect on tracheal morphology across all three temperature treatments. Sample sizes are as follows: 10% O<sub>2</sub> at 34.5°C, *n*=10; 21% O<sub>2</sub> at 34.5°C, *n*=11; 40% O<sub>2</sub> at 34.5°C, *n*=11; 10% O<sub>2</sub> at 32.3°C, *n*=11; 21% O<sub>2</sub> at 32.3°C, *n*=11; 40% O<sub>2</sub> at 32.3°C, *n*=11; 10% O<sub>2</sub> at 28.3°C, *n*=12; 21% O<sub>2</sub> at 28.3°C, *n*=12; 40% O<sub>2</sub> at 28.3°C, *n*=12.

temperatures have found evidence in support of the OCLTT hypothesis, which suggests that hypoxic environments should reduce the rate of growth and hyperoxic environments should increase the rate of growth relative to normoxia. Hoefnagel and Verberk (2017) found a decline in final body size in the great pond snail, *Lymnaea stagnalis*, when reared at 17 and 22°C in hypoxia when compared with those reared in normoxia. An effect of body size and fecundity in response to temperature and oxygen was also found in the rotifer *Lecane inermis* (Walczyńska et al., 2015). The fitness (measured as fecundity and growth) of larger sized rotifers was more sensitive to changes in temperature than to oxygen, and vice versa for smaller sized individuals, indicating that the observed decline in the scope for growth upon cooling in smaller individuals was attributed to a lower supply of energy, whereas the observed increase in the scope for growth upon cooling in larger individuals was attributed to lower demands of oxygen (Walczyńska et al., 2015).

The non-significant effect of developmental oxygen on growth in cooler temperatures in the present study, as opposed to the significant effects found for the fitness of *L. stagnalis* and *L. inermis*, may be the result of an effect of different respiratory media and respiratory systems across the species. In contrast to *L. stagnalis* and *L. inermis*, *N. cinerea* makes use of a tracheal respiratory system composed of fine branches that reach deep into the tissues, providing a gas exchange pathway that has a much higher diffusive capacity than blood or water (Verberk and Bilton, 2011). This high capacity for oxygen delivery of the tracheal system may allow *N. cinerea* to better meet oxygen demands even under hypoxic conditions. Indeed, short-term investigations into the effects of ambient oxygen concentrations ranging from 2.5 to 40 kPa in other tracheated species have found no significant effects on critical thermal minimums (Klok et al., 2004; Stevens et al., 2010; Boardman et al., 2016). These results suggest that oxygen availability in tracheated species is not a limiting factor contributing to performance declines at cooler temperatures (Klok et al., 2004; Verberk et al., 2016).

In contrast to the OCLTT predictions, the present study further found no significant effect of developmental hypoxia on the rate of growth in *N. cinerea* at the upper developmental temperature of 34.5°C. Individuals developed under hyperoxia at 34.5°C also had significantly reduced rates of growth when compared with those developed in normoxia. The effect of hypoxia on growth at upper thermal limits in previous studies have garnered mixed support for the OCLTT hypothesis. For instance, developmental hypoxia at warmer temperatures has been shown to reduce growth and final body size in the aquatic isopod *Asellus aquaticus*, as well as in *L. stagnalis* (Hoefnagel and Verberk, 2015, 2017). Conversely, oxygen availability was found not to be responsible for setting the upper thermal tolerance for growth in *Macrobrachium rosenbergii* (Ern et al., 2014). Experimental rearing of *M. rosenbergii* at different temperatures found that the oxygen transport capacity in the species could be maintained well beyond the temperatures required for growth, indicating that other mechanisms, such as protein dysfunction, may be responsible for the loss of performance at elevated temperatures (Ern et al., 2014). Evidence for increased growth rates under hyperoxic conditions at warmer temperatures is also mixed, although most studies generally examine the effect of hyperoxia under standard or optimal temperatures rather than at temperatures above the optimal temperature for growth. Some studies have found hyperoxia to increase the rate of growth (Frazier et al., 2001; Owerkowicz et al., 2009; Verberk et al., 2013), while others have found negative relationships (McCue and De Los Santos, 2013; Bartrim et al., 2014), such as the one shown in the present study. Other studies have found no effect of hyperoxia on growth rate (Gräns et al., 2014; Bjelde et al., 2015).

The observation that hyperoxia reduces growth rate only at the upper developmental temperature, however, is consistent with the observation that hyperoxia and temperature-induced oxidative stress can increase the production of reactive oxygen species (Sohal et al., 1993; Abele et al., 2002; Boardman et al., 2012; Kipp and Boyle, 2013; Schieber and Chandel, 2014). A coupling effect of heat stress and hyperoxic damage could explain the significant decline in growth seen at 34.5°C but not at 28.3 or 32.3°C in the 40% oxygen treatment (Fig. 4).

The present study further shows an effect of hypoxia on growth at  $T_{opt}$ , which suggests that oxygen delivery is important for growth. In contrast to the OCLTT hypothesis, these results suggest that ensuring adequate oxygen delivery is most important at temperatures where growth is maximal ( $T_{opt}$ ) and that oxygen

delivery is less important at temperatures below and above  $T_{opt}$ , where growth rates are slower. Such an observation suggests that, at least in *N. cinerea*, factors other than oxygen delivery are responsible for the decline in growth performance observed at temperatures below and above  $T_{opt}$ .

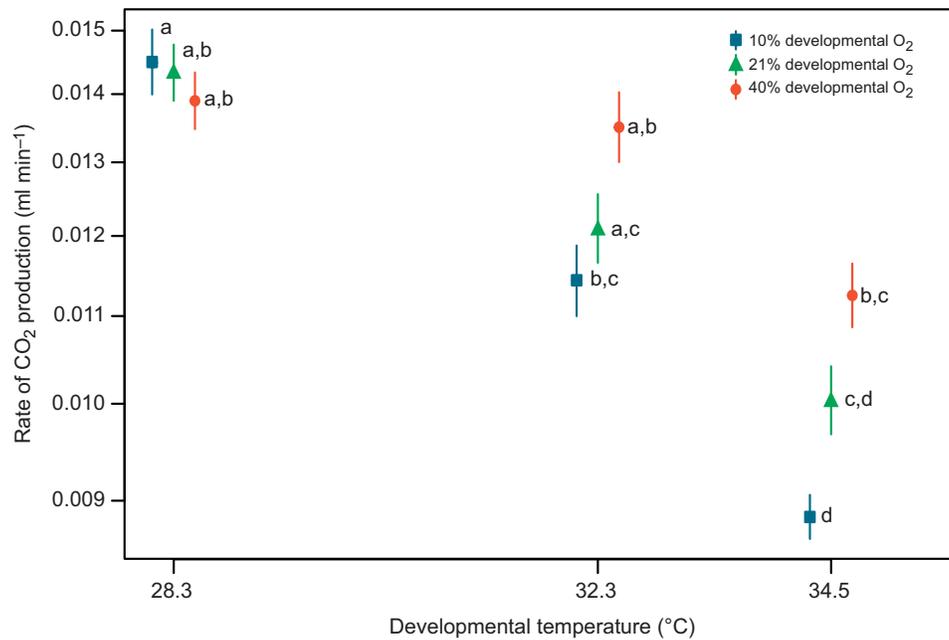
### Running performance and tracheal morphology

Modulation of oxygen diffusive capacity through compensatory changes of the tracheal system may be an important mechanism by which insects adjust to varying oxygen availability. Because conditions of hyperoxia can lead to increased oxidative stress owing to higher tissue oxygen levels, modulation of the tracheal network may allow for sufficient tissue oxygen levels to be maintained, without the accumulation of oxygen radicals. An outgrowth of the tracheal system under hypoxic conditions may also increase the capacity of the tracheal system to meet increasing cellular oxygen demand. Such changes in tracheal morphology have the potential to reduce the negative effects of hypoxia and hyperoxia on fitness traits (such as growth rate and aerobic performance) and may enhance an animal's oxygen transport capacity (Harrison et al., 2006, 2010). Compensatory changes of the tracheal system have been observed in *Drosophila melanogaster* larvae in response to increased atmospheric oxygen (Henry and Harrison, 2004). *Blattella germanica* have also been shown to reduce their tracheal dimensions in response to 40% oxygen concentrations, and to increase their tracheal dimensions in response to 12% oxygen concentrations (VandenBrooks et al., 2012). There is evidence for similar compensatory changes in the limb tracheal diameter of adult speckled cockroaches when reared under 10% oxygen (Bartrim et al., 2014). If oxygen transport capacity declines as temperatures shift away from  $T_{opt}$ , as proposed by the OCLTT hypothesis, then we might expect to see changes in the tracheal system at temperatures beyond  $T_{opt}$ , allowing for insects to compensate for the varying oxygen availability.

In our study of *N. cinerea*, tracheal diameter was not significantly different at 28.3°C and 34.5°C. There were also no significant differences observed in running performance (proxy for aerobic performance) across treatment groups. It should be noted, however, that aerobic performance was determined from the running duration of cockroaches on a treadmill. Average running duration was high across treatments (15 to 80 min) and so it is plausible that the performance trait was fuelled aerobically. Nonetheless, the presence of anaerobic metabolites was not tested in individuals and anaerobic metabolism might have played a role in the performance between treatments. Therefore, care should be taken when drawing comparisons in aerobic performance results between those observed in this study and other published work.

The lack of change in tracheal morphology may indicate that oxygen is not a limiting factor contributing to performance declines at temperatures beyond  $T_{opt}$  in *N. cinerea*. However, multiple compensatory mechanisms that share identical functional outcomes (i.e. to reduce oxidative stress) may exist within a species. For instance, *D. melanogaster* are able to increase their tracheal diffusive capacities in response to hypoxia by either increasing the length of the main dorsal tracheae without a change in diameter (Beitel and Krasnow, 2000) or increasing the diameter of the main dorsal trachea without a change in length (Henry and Harrison, 2004; Harrison et al., 2006). The absence of a change in tracheal diameter in the present study may therefore not necessarily indicate a limitation of oxygen.

Possible effects of ontogeny are also likely. Bartrim et al. (2014) found that adult speckled cockroaches reared in hypoxic



**Fig. 6. The effect of developmental oxygen concentration and temperature on the rate of carbon dioxide production ( $\text{ml min}^{-1}$ ) (proxy for routine metabolic rate) of *N. cinerea*.** Points show the mean  $\pm$  s.e.m. of mass- and measurement temperature-adjusted  $\text{CO}_2$  production for cockroaches developed at each treatment [data were adjusted to the mean of ln-transformed mass (67.67 mg) and to  $T_{\text{opt}}$  ( $32.3^\circ\text{C}$ )]. All points that share a letter are not significantly different from each other. Blue squares, 10%  $\text{O}_2$ ; green triangles, 21%  $\text{O}_2$ ; red circles, 40%  $\text{O}_2$ . Sample sizes are as follows: 10%  $\text{O}_2$  at  $28.3^\circ\text{C}$ ,  $n=16$ ; 21%  $\text{O}_2$  at  $28.3^\circ\text{C}$ ,  $n=17$ ; 40%  $\text{O}_2$  at  $28.3^\circ\text{C}$ ,  $n=21$ ; 10%  $\text{O}_2$  at  $32.3^\circ\text{C}$ ,  $n=18$ ; 21%  $\text{O}_2$  at  $32.3^\circ\text{C}$ ,  $n=20$ ; 40%  $\text{O}_2$  at  $32.3^\circ\text{C}$ ,  $n=21$ ; 10%  $\text{O}_2$  at  $34.3^\circ\text{C}$ ,  $n=24$ ; 21%  $\text{O}_2$  at  $34.3^\circ\text{C}$ ,  $n=20$ ; 40%  $\text{O}_2$  at  $34.3^\circ\text{C}$ ,  $n=15$ .

atmospheres had significantly greater limb tracheal diameters and branching than those reared in normoxia. This suggests that oxygen delivery in adults may be more constrained than in the juveniles examined in the present study. The mechanisms responsible for ontogenetic effects in respiratory systems and respiratory capacities across species are not clearly understood. It has been suggested that larger insects invest more of their body volume in the tracheal system, which could potentially lead to greater effects of atmospheric partial pressures of oxygen on larger insects (Harrison et al., 2010). This has been supported in the tenebrionid beetle *T. molitor* (Kaiser et al., 2007). These observations, however, are not universal, and the mechanisms responsible for ontogenetic effects in respiratory capacities are, therefore, likely to be species specific (Wegener and Moratzky, 1995; Kirkton et al., 2012; Boardman and Terblanche, 2015). In *Schistocerca americana*, for example, first instars were more sensitive to hypoxia than later instars, possibly owing to reduced development in their air sacs leading to lower ventilation capacities (Greenlee and Harrison, 2004; Greenlee et al., 2009). These results indicate that the impact of temperature and atmospheric oxygen level on insect tracheal development and function is complex, and that an absence of compensatory changes in the tracheal system may not indicate a limitation of oxygen on fitness.

#### Routine metabolic rate

A negative relationship was found between RMR and developmental temperature in the hypoxic treatment, with lower RMR found for individuals developed at the upper temperature of  $34.5^\circ\text{C}$  and higher RMR found for individuals developed at the lower temperature of  $28.3^\circ\text{C}$ . Such declines in RMR with increased developmental temperature may act to maintain or increase aerobic scope in hypoxic conditions as metabolic demand increases with temperature (Gräns et al., 2014; Norin et al., 2014; Claesson et al., 2016). In the normoxic group, in contrast, RMR at the upper and lower developmental temperature did not significantly differ from that at  $T_{\text{opt}}$ . These trends, however, are based on routine metabolism whereas aerobic scope is a function of both MMR and RMR. MMR and RMR have been known to experience different constraints and

levels of selection allowing them to shift independently from each other (Wone et al., 2015; Pettersen et al., 2018). Without a clear understanding of temperature- and oxygen-induced changes in MMR, it is difficult to draw clear conclusions regarding the role of changes in RMR for aerobic scope and the OCLTT hypothesis. Notwithstanding this limitation, RMR is still an important indicator of performance and reflects the baseline energetic cost of living, so comparisons across treatments are still informative.

The difference between the acclimation responses of RMR at high temperatures in normoxia and hypoxia is suggestive of a role for oxygen limitation as a contributor to the metabolic acclimation response. Such an interpretation does not support the OCLTT hypothesis, however, which states that physiological performances, such as growth, should decline in concert with aerobic scope (Pörtner et al., 2017), because growth declines at  $34.5^\circ\text{C}$  in normoxia, but RMR does not show an acclimation response. Taken together, these data suggest that although oxygen delivery performance may be important for metabolic acclimation, it may not be responsible for the decline in growth seen in juvenile speckled cockroaches developed at high temperature.

#### Conclusions

Despite incorporating long-term performance indicators and less extreme upper and lower temperatures into experimentation in the present study, the results did not support an effect of oxygen on the thermal performance for growth in *N. cinerea*. Development under hypoxia did not significantly reduce growth rate or running performance, and development under hyperoxia did not significantly increase growth rate or running performance. The non-significant effect of developmental temperature and oxygen on the tracheal morphology in *N. cinerea* might further suggest that oxygen delivery capacity is not the primary driver shaping thermal tolerance in juveniles of this species. Furthermore, the RMR results indicate that oxygen delivery capacity is not the primary driver shaping thermal tolerance; however, owing to the absence of MMR data, these results warrant further investigation.

Collectively, these results suggest that the OCLTT hypothesis is not a 'unifying' model that can be used to explain thermal tolerance

across all levels and orders of biological organisation. Given the mixed support found for the OCLTT hypothesis, it is likely that a species' thermal tolerance is shaped by a range of factors (including cellular dysfunction) and encompasses interactive effects of ontogeny, locomotion requirements, respiratory media and capacities to regulate oxygen uptake. Given our limited understanding of the effect of oxygen on long-term performance traits, future studies should also consider incorporating traits such as growth rate and reproductive success into thermal performance measures.

#### Acknowledgements

The authors would like to thank two anonymous reviewers, whose comments substantially improved the paper.

#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: E.J.L., C.R.W.; Methodology: E.J.L., C.R.W.; Software: C.L.B.; Validation: E.J.L., C.L.B., C.R.W.; Formal analysis: E.J.L., C.R.W.; Investigation: E.J.L.; Resources: E.J.L., C.L.B., C.R.W.; Data curation: E.J.L., C.L.B., C.R.W.; Writing - original draft: E.J.L.; Writing - review & editing: E.J.L., C.R.W.; Visualization: E.J.L., C.R.W.; Supervision: E.J.L., C.L.B., C.R.W.; Project administration: E.J.L., C.R.W.; Funding acquisition: C.R.W.

#### Funding

This research was supported by the Australian Research Council (project DP180103925).

#### Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.208306.supplemental>

#### References

- Abele, D., Heise, K., Pörtner, H. O. and Puntarulo, S. (2002). Temperature-dependence of mitochondrial function and production of reactive oxygen species in the intertidal mud clam *Mya arenaria*. *J. Exp. Biol.* **205**, 1831-1841.
- Alton, L. A., Condon, C., White, C. R. and Angilletta, M. J., Jr (2017). Colder environments did not select for a faster metabolism during experimental evolution of *Drosophila melanogaster*. *Evolution* **71**, 145-152. doi:10.1111/evo.13094
- Baker, T. T., Lafferty, R. and Quinn, T. J. (1991). A general growth model for mark recapture data. *Fish. Res.* **11**, 257-281. doi:10.1016/0165-7836(91)90005-Z
- Bartrm, H., Matthews, P. G. D., Lemon, S. and White, C. R. (2014). Oxygen-induced plasticity in tracheal morphology and discontinuous gas exchange cycles in cockroaches *Nauphoeta cinerea*. *J. Comp. Physiol. B.* **184**, 977-990. doi:10.1007/s00360-014-0862-8
- Beitel, G. J. and Krasnow, M. A. (2000). Genetic control of epithelial tube size in the *Drosophila* tracheal system. *Development* **127**, 3271-3282.
- Bjelde, B. E., Miller, N. A., Stillman, J. H. and Todgham, A. E. (2015). The role of oxygen in determining upper thermal limits in *Lottia digitalis* under air exposure and submersion. *Physiol. Biochem. Zool.* **88**, 483-493. doi:10.1086/682220
- Boardman, L. and Terblanche, J. S. (2015). Oxygen safety margins set thermal limits in an insect model system. *J. Exp. Biol.* **218**, 1677-1685. doi:10.1242/jeb.120261
- Boardman, L., Terblanche, J. S., Hetz, S. K., Marais, E. and Chown, S. L. (2012). Reactive oxygen species production and discontinuous gas exchange in insects. *Proc. R. Soc. B.* **279**, 893-901. doi:10.1098/rspb.2011.1243
- Boardman, L., Sørensen, J. G., Košťál, V., Šimek, P. and Terblanche, J. S. (2016). Cold tolerance is unaffected by oxygen availability despite changes in anaerobic metabolism. *Sci. Rep.* **6**, 1-13. doi:10.1038/srep32856
- Chou, H., Pathmasiri, W., Deese-Spruill, J., Sumner, S. and Buchwalter, D. B. (2017). Metabolomics reveal physiological changes in mayfly larvae (*Neocloeon triangulifer*) at ecological upper thermal limits. *J. Insect. Physiol.* **101**, 107-112. doi:10.1016/j.jinsphys.2017.07.008
- Chown, S. L. and Nicolson, S. W. (2004). *Insect Physiological Ecology: Mechanisms and Patterns*. New York: Oxford University Press.
- Claësso, D., Wang, T. and Malte, H. (2016). Maximal oxygen consumption increases with temperature in the European eel (*Anguilla anguilla*) through increased heart rate and arteriovenous extraction. *Conserv. Physiol.* **4**, 1-8. doi:10.1093/conphys/cow027
- Ern, R., Huong, D. T. T., Phuong, N. T., Wang, T. and Bayley, M. (2014). Oxygen delivery does not limit thermal tolerance in a tropical eurythermal crustacean. *J. Exp. Biol.* **217**, 809-814. doi:10.1242/jeb.094169
- Frazier, M. R., Woods, H. A. and Harrison, J. F. (2001). Interactive effects of rearing temperature and oxygen on the development of *Drosophila melanogaster*. *Physiol. Biochem. Zool.* **74**, 641-650. doi:10.1086/322172
- Frederich, M. and Pörtner, H.-O. (2000). Oxygen limitation of thermal tolerance defined by cardiac and ventilatory performance in spider crab *Maja squinado*. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **279**, R1531-R1538. doi:10.1152/ajpregu.2000.279.5.R1531
- Frederich, M., O'Rourke, M. R., Furey, N. B. and Jost, J. A. (2009). AMP-activated protein kinase (AMPK) in the rock crab, *Cancer irroratus*: an early indicator of temperature stress. *J. Exp. Biol.* **212**, 722-730. doi:10.1242/jeb.021998
- Fry, F. E. J. (1947). *Effects of the Environment on Animal Activity*. Ontario: University of Toronto Press.
- Full, R. J. and Tullis, A. (1990). Capacity for sustained terrestrial locomotion in an insect: energetics, thermal dependence, and kinematics. *J. Comp. Physiol. B.* **160**, 573-581. doi:10.1007/BF00258985
- Ghabrial, A., Luschnig, S., Metzstein, M. M. and Krasnow, M. A. (2003). Branching morphogenesis of *Drosophila* tracheal system. *Annu. Rev. Cell. Dev. Bi.* **19**, 623-647. doi:10.1146/annurev.cellbio.19.031403.160043
- Gräns, A., Jutfelt, F., Sandblom, E., Jönsson, E., Wiklander, K., Seth, H., Olsson, C., Dupont, S., Ortega-Martinez, O., Einarsdottir, I. et al. (2014). Aerobic scope fails to explain the detrimental effects on growth resulting from warming and elevated CO<sub>2</sub> in Atlantic halibut. *J. Exp. Biol.* **217**, 711-717. doi:10.1242/jeb.096743
- Greenlee, K. J. and Harrison, J. F. (2004). Development of respiratory function in the American locust *Schistocerca americana*: II. Within-instar effects. *J. Exp. Biol.* **207**, 509-517. doi:10.1242/jeb.00766
- Greenlee, K. J., Henry, J. R., Kirkton, S. D., Westneat, M. W., Fezzaa, K., Lee, W. and Harrison, J. F. (2009). Synchrotron imaging of the grasshopper tracheal system: morphological and physiological components of tracheal hypermetry. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **297**, R1343-R1350. doi:10.1152/ajpregu.00231.2009
- Harrison, J. F., Frazier, M. R., Henry, J. R., Kaiser, A., Klok, C. J. and Rascón, B. (2006). Responses of terrestrial insects to hypoxia or hyperoxia. *Respir. Physiol. Neurobiol.* **154**, 4-7. doi:10.1016/j.resp.2006.02.008
- Harrison, J. F., Kaiser, A. and Vandenbrooks, J. M. (2010). Atmospheric oxygen level and the evolution of insect body size. *Proc. Royal. Soc. B.* **277**, 1937-1946. doi:10.1098/rspb.2010.0001
- Henry, J. R. and Harrison, J. F. (2004). Plastic and evolved responses of larval tracheae and mass to varying atmospheric oxygen content in *Drosophila melanogaster*. *J. Exp. Biol.* **207**, 3559-3568. doi:10.1242/jeb.01189
- Hoefnagel, K. N. and Verberk, W. C. E. P. (2015). Is the temperature-size rule mediated by oxygen in aquatic ectotherms? *J. Therm. Biol.* **54**, 56-65. doi:10.1016/j.jtherbio.2014.12.003
- Hoefnagel, K. N. and Verberk, W. C. E. P. (2017). Long-term and acute effects of temperature and oxygen on metabolism, food intake, growth and heat tolerance in a freshwater gastropod. *J. Therm. Biol.* **68**, 27-38. doi:10.1016/j.jtherbio.2016.11.017
- Jensen, D. L., Overgaard, J., Wang, T., Gesser, H. and Malte, H. (2017). Temperature effects on aerobic scope and cardiac performance of European perch (*Perca fluviatilis*). *J. Therm. Biol.* **86**, 162-169. doi:10.1016/j.jtherbio.2017.04.006
- Jost, J. A., Podolski, S. M. and Frederich, M. (2012). Enhancing thermal tolerance by eliminating the pejus range: a comparative study with three decapod crustaceans. *Mar. Ecol. Prog. Ser.* **444**, 263-274. doi:10.3354/meps09379
- Kaiser, A., Klok, C. J., Socha, J. J., Lee, W.-K. and Quinlan, M. C. (2007). Increase in tracheal investment with beetle size supports hypothesis of oxygen limitation on insect gigantism. *Proc. Natl. Acad. Sci. USA* **104**, 13198-13203. doi:10.1073/pnas.0611544104
- Kanwisher, J. W. (1966). Tracheal gas dynamics in pupae of the *Cecropia* silkworm. *Biol. Bull.* **130**, 96-105. doi:10.2307/1539956
- Kim, K. S., Chou, H., Funk, D. H., Jackson, J. K., Sweeney, B. W. and Buchwalter, D. B. (2017). Physiological responses to short-term thermal stress in mayfly (*Neocloeon triangulifer*) larvae in relation to upper thermal limits. *J. Exp. Biol.* **220**, 2598-2605. doi:10.1242/jeb.156919
- Kipp, E. and Boyle, M. (2013). The effects of heat stress on reactive oxygen species production and chlorophyll concentration in *Arabidopsis thaliana*. *Res. Plant. Sci.* **1**, 20-23.
- Kirkton, S., Hennessey, L., Duffy, B., Bennett, M., Lee, W.-K. and Greenlee, K. (2012). Intermolt development reduces oxygen delivery capacity and jumping performance in the American locust (*Schistocerca americana*). *J. Comp. Physiol.* **182**, 217-230. doi:10.1007/s00360-011-0615-x
- Klok, C. J., Sinclair, B. J. and Chown, S. L. (2004). Upper thermal tolerance and oxygen limitation in terrestrial arthropods. *J. Exp. Biol.* **207**, 2361-2370. doi:10.1242/jeb.01023
- Lighton, J. R. B. (2008). *Measuring Metabolic Rates: a Manual for Scientists*. Oxford: Oxford University Press.
- Malmendal, A., Overgaard, J., Bundy, J. G., Sørensen, J. G., Nielsen, N. C., Loeschcke, V. and Holmstrup, M. (2006). Metabolomic profiling of heat stress: hardening and recovery of homeostasis in *Drosophila*. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **291**, R205-R212. doi:10.1152/ajpregu.00867.2005

- McCue, M. D. and De Los Santos, R.** (2013). Upper thermal limits of insects are not the result of insufficient oxygen delivery. *Physiol. Biochem. Zool.* **86**, 257-265. doi:10.1086/669932
- Michaud, M. R. and Denlinger, D. L.** (2007). Shifts in the carbohydrate, polyol, and amino acid pools during rapid cold-hardening and diapause-associated cold-hardening in flesh flies (*Sarcophaga crassipalpis*): a metabolomic comparison. *J. Comp. Physiol. B.* **177**, 753-773. doi:10.1007/s00360-007-0172-5
- Michaud, M. R., Benoit, J. B., Lopez-Martinez, G., Elnitsky, M. A., Lee, R. E., Jr and Denlinger, D. L.** (2008). Metabolomics reveals unique and shared metabolic changes in response to heat shock, freezing and desiccation in the Antarctic midge, *Belgica antarctica*. *J. Insect Physiol.* **54**, 645-655. doi:10.1016/j.jinsphys.2008.01.003
- Mölich, A. B., Förster, T. D. and Lighton, J. R. B.** (2012). Hyperthermic overdrive: oxygen delivery does not limit thermal tolerance in *Drosophila melanogaster*. *J. Insect Sci.* **12**, 1-7. doi:10.1673/031.012.10901
- Norin, T., Malte, H. and Clark, T. D.** (2014). Aerobic scope does not predict the performance of a tropical eurythermal fish at elevated temperatures. *J. Exp. Biol.* **217**, 244-251. doi:10.1242/jeb.089755
- Overgaard, J., Andersen, J. L., Findsen, A., Pedersen, P. B. M., Hansen, K., Ozolina, K. and Wang, T.** (2012). Aerobic scope and cardiovascular oxygen transport is not compromised at high temperatures in the toad *Rhinella marina*. *J. Exp. Biol.* **215**, 3519-3526. doi:10.1242/jeb.070110
- Owerkowicz, T., Elsey, R. M. and Hicks, J. W.** (2009). Atmospheric oxygen level affects growth trajectory, cardiopulmonary allometry and metabolic rate in the American alligator (*Alligator mississippiensis*). *J. Exp. Biol.* **212**, 1237-1248. doi:10.1242/jeb.023945
- Pettersen, A. K., Marshall, D. J. and White, C. R.** (2018). Understanding variation in metabolic rate. *J. Exp. Biol.* **221**, jeb166876. doi:10.1242/jeb.166876
- Pörtner, H.-O.** (2001). Climate change and temperature-dependent biogeography: oxygen limitation of thermal tolerance in animals. *Naturwissenschaften* **88**, 137-146. doi:10.1007/s001140100216
- Pörtner, H.-O.** (2012). Integrating climate-related stressor effects on marine organisms: unifying principles linking molecule to ecosystem-level changes. *Mar. Ecol. Prog. Ser.* **470**, 273-290. doi:10.3354/meps10123
- Pörtner, H.-O.** (2014). How and how not to investigate the oxygen and capacity limitation of thermal tolerance (OCLTT) and aerobic scope: remarks on the article by Gräns et al. *J. Exp. Biol.* **217**, 4432-4433. doi:10.1242/jeb.114181
- Pörtner, H.-O., Bock, C. and Mark, F. C.** (2017). Oxygen- and capacity-limited thermal tolerance: bridging ecology and physiology. *J. Exp. Biol.* **220**, 2685-2696. doi:10.1242/jeb.134585
- Quinn, G. P. and Keough, M. J.** (2002). *Experimental Design and Data Analysis for Biologists*. Cambridge: Cambridge University Press.
- Schieber, M. and Chandel, N. S.** (2014). ROS function in redox signaling and oxidative stress. *Curr. Biol.* **24**, R453-R462. doi:10.1016/j.cub.2014.03.034
- Schimpf, N. G., Matthews, P. G. D. and White, C. R.** (2012a). Cockroaches that exchange respiratory gases discontinuously survive food and water restrictions. *Evolution* **66**, 597-604. doi:10.1111/j.1558-5646.2011.01456.x
- Schimpf, N. G., Matthews, P. G. D. and White, C. R.** (2012b). Standard metabolic rate is associated with gestation duration, but not clutch size, in speckled cockroaches *Nauphoeta cinerea*. *Biol. Open* **1**, 1185-1191. doi:10.1242/bio.20122683
- Schimpf, N. G., Matthews, P. G. D. and White, C. R.** (2013). Discontinuous gas exchange exhibition is a heritable trait in speckled cockroaches *Nauphoeta cinerea*. *J. Evolution. Biol.* **26**, 1588-1597. doi:10.1111/jeb.12093
- Schoolfield, R. M., Sharpe, P. J. H. and Magnuson, C. E.** (1981). Non-linear regression of biological temperature dependent rate models based on absolute reaction rate theory. *J. Theor. Biol.* **88**, 719-731. doi:10.1016/0022-5193(81)90246-0
- Sohal, R. S., Agarwal, S., Dubey, A. and Orr, W. C.** (1993). Protein oxidative damage is associated with life expectancy of houseflies. *Proc. Natl. Acad. Sci. USA* **90**, 7255-7259. doi:10.1073/pnas.90.15.7255
- Stevens, M. M., Jackson, S., Bester, S. A., Terblanche, J. S. and Chown, S. L.** (2010). Oxygen limitation and thermal tolerance in two terrestrial arthropod species. *J. Exp. Biol.* **213**, 2209-2218. doi:10.1242/jeb.040170
- Teague, C., Youngblood, J. P., Ragan, K., Angilletta, M. J., Jr and VandenBrooks, J. M.** (2017). A positive genetic correlation between hypoxia tolerance and heat tolerance supports a controversial theory of heat stress. *Biol. Letters*. **13**, 1-4. doi:10.1098/rsbl.2017.0309
- VandenBrooks, J. M., Munoz, E. E., Weed, M. D., Ford, C. F., Harrison, M. A. and Harrison, J. F.** (2012). Impacts of paleo-oxygen levels on the size, development, reproduction, and tracheal system of *Blatella germanica*. *Evol. Biol.* **39**, 83-93. doi:10.1007/s11692-011-9138-3
- Verberk, W. C. E. P. and Bilton, D. T.** (2011). Can oxygen set thermal limits in an insect and drive gigantism? *PLoS ONE* **6**, e22610. doi:10.1371/journal.pone.0022610
- Verberk, W. C. E. P., Sommer, U., Davidson, R. L. and Viant, M. R.** (2013). Anaerobic metabolism at thermal extremes: a metabolomic test of the oxygen limitation hypothesis in an aquatic insect. *Integr. Comp. Biol.* **53**, 609-619. doi:10.1093/icb/ict015
- Verberk, W. C. E. P., Overgaard, J., Ern, R., Bayley, M., Wang, T., Boardman, L. and Terblanche, J. S.** (2016). Does oxygen limit thermal tolerance in arthropods? A critical review of current evidence. *Comp. Biochem. Phys. A.* **192**, 64-78. doi:10.1016/j.cbpa.2015.10.020
- Walczyńska, A., Labacka, A. M., Sobczyk, M., Czarnoleski, M. and Kozłowski, J.** (2015). The temperature-size rule in *Lecane inermis* (Rotifera) is adaptive and driven by nuclei size adjustment to temperature and oxygen combinations. *J. Therm. Biol.* **54**, 78-85. doi:10.1016/j.jtherbio.2014.11.002
- Wang, T., Lefevre, S., Iversen, N. K., Findorf, I., Buchanan, R. and McKenzie, D. J.** (2014). Anaemia only causes a small reduction in the upper critical temperature of sea bass: Is oxygen delivery the limiting factor for temperature tolerance in fishes? *J. Exp. Biol.* **217**, 4275-4278. doi:10.1242/jeb.104166
- Wegener, G. and Moratzky, T.** (1995). Hypoxia and anoxia in insects: microcalorimetric studies on two species (*Locusta migratoria* and *Manduca sexta*) showing different degrees of anoxia tolerance. *Thermochim. Acta.* **251**, 209-218. doi:10.1016/0040-6031(94)02009-D
- Weis-Fogh, T.** (1964). Functional design of the tracheal system of flying insects as compared with the avian lung. *J. Exp. Biol.* **41**, 207-227.
- Withers, P. C.** (2001). Design, calibration and calculation for flow-through respirometry systems. *Aust. J. Zool.* **49**, 445-461. doi:10.1071/ZO00057
- Wone, B. W. M., Madsen, P., Donovan, E. R., Labocha, M. K., Sears, W. M., Downs, C. J., Sorensen, D. A. and Hayes, J. P.** (2015). A strong response to selection on mass-independent maximal metabolic rate without a correlated response in basal metabolic rate. *Heredity* **114**, 419-427. doi:10.1038/hdy.2014.122
- Youngblood, J. P., da Silva, C. R. B., Angilletta, M. J., Jr and VandenBrooks, J. M.** (2019). Oxygen limitation does not drive the decreasing heat tolerance of grasshoppers during development. *Physiol. Biochem. Zool.* **92**, 567-572. doi:10.1086/705439