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RESEARCH ARTICLE

Oxygen safety margins set thermal limits in an insect model system

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ABSTRACT

A mismatch between oxygen availability and metabolic demand may constrain thermal tolerance. While considerable support for this idea has been found in marine organisms, results from insects are equivocal and raise the possibility that mode of gas exchange, oxygen safety margins and the physico-chemical properties of the gas medium influence heat tolerance estimates. Here, we examined critical thermal maximum (CT_{max}) and aerobic scope under altered oxygen supply and in two life stages that varied in metabolic demand in *Bombyx mori* (Lepidoptera: Bombycidae). We also systematically examined the influence of changes in gas properties on CT_{max}. Larvae have a lower oxygen safety margin (higher critical oxygen partial pressure at which metabolism is suppressed relative to metabolic demand) and significantly higher CT_{max} under normoxia than pupae (53°C vs 50°C). Larvae, but not pupae, were oxygen limited with hypoxia (2.5 kPa) decreasing CT_{max} significantly from 53 to 51°C. Humidifying hypoxic air relieved the oxygen limitation effect on CT_{max} in larvae, whereas variation in other gas properties did not affect CT_{max}. Our data suggest that oxygen safety margins set thermal limits in air-breathing invertebrates and the magnitude of this effect potentially reconciles differences in oxygen limitation effects on thermal tolerance found among diverse taxa to date.

INTRODUCTION

The mechanisms underlying thermal tolerance are central to predicting climate change impacts at the individual level (Pörtner and Farrell, 2008). One major hypothesis proposed to explain the mechanism of thermal tolerance of ectotherms is the theory of oxygen- and capacity-limited thermal tolerance (OCLTT; Pörtner, 2001), which argues that thermal tolerance may be constrained by oxygen limitation and the mismatch between oxygen supply and demand at thermal extremes (i.e. at temperatures outside thermal optima). Evidence supporting OCLTT has been found in marine organisms and aquatic insects, yet results from terrestrial air-breathing invertebrates are equivocal (discussed in W. C. E. P. Verberk, J. Overgaard, R. Ern, M. Bayley, T. Wang, L.B. and J.S.T., submitted). Differences in breathing apparatus (gills versus occludible tracheae), and in the complexity of gas exchange systems (e.g. two-stage gas exchange, discussed in Klok et al., 2004), the respiratory milieu (e.g. Giomi et al., 2014; reviewed in Hsia et al., 2013) and the degree to which an animal is capable of regulating its gas exchange at rest, especially in the face of

changing oxygen availability, may explain the difference between marine invertebrates that show support for OCLTT and air-breathing invertebrates that generally do not (Verberk and Bilton, 2013). For example, Verberk and Bilton (2013) showed across four insect orders that the degree of respiratory control within a particular species determined the extent of the oxygen limitation [measured as a reduction in critical thermal maximum (CT_{max}) with hypoxia]. Therefore, the mode of gas exchange and ability to precisely regulate oxygen consumption or extract oxygen from the respiratory medium may be crucial factors in determining thermal limits.

Properties of the oxygen-carrying gas or liquid medium might also directly affect thermal limits. All organisms need to remove respiratory by-products (carbon dioxide) and meet tissue-specific oxygen demands while avoiding oxygen toxicity, regardless of whether the respired oxygen is extracted from dense, viscous water with low oxygen content or air (Verberk and Atkinson, 2013). However, water and air are fundamentally different in terms of oxygen diffusivity, conductance, permeability, density and heat capacity (Hsia et al., 2013; Verberk and Atkinson, 2013). One or several of these different properties could influence the differences in thermal tolerance under altered oxygen conditions. Indeed, breathing gas mixtures of reduced density reduces ventilatory resistance (Brubakk and Neumann, 2003). Therefore, altering gas mixture composition can be used as a proxy to investigate differences in key physical properties between air and water oxygen-delivery systems.

Broadly, there are three predictions that can be argued to provide support for the OCLTT hypothesis: (1) as organisms approach thermal limits, anaerobic metabolites accumulate; (2) decreased oxygen supply should reduce thermal tolerance, whereas increased oxygen supply should increase thermal tolerance; (3) at temperatures just before critical thermal limits, aerobic scope decreases (discussed in W. C. E. P. Verberk, J. Overgaard, R. Ern, M. Bayley, T. Wang, L.B. and J.S.T., submitted). In general, most studies test the prediction that oxygen supply (ambient oxygen availability) affects thermal limits. While hypoxia often decreases CT_{max} (e.g. Klok et al., 2004, reviewed in W. C. E. P. Verberk, J. Overgaard, R. Ern, M. Bayley, T. Wang, L.B. and J.S.T., submitted), hyperoxia has only been documented to increase heat tolerance in aquatic, gill-breathing stonefly *Dinocras cephalotes* nymphs (Verberk and Bilton, 2011). Among several taxa examined, this insect is the only one in which clear support for at least two OCLTT predictions has been found: lower (and higher) CT_{max} under hypoxia (and hyperoxia) and anaerobic metabolism close to thermal limits (Verberk and Bilton, 2011; Verberk et al., 2013). As air-breathing insects typically do not become oxygen limited above 5–6 kPa (Komai, 1998; Marshall et al., 2013) and since some insects are able to maintain low and constant internal ranges of P_{O_2} (e.g. Hetz and Bradley, 2005; Harrison et al., 2006; but see Matthews et al., 2012), insects have probably evolved to withstand reductions in oxygen supply through a suite of biochemical adaptations.

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List of symbols and abbreviations

ADS	absolute difference sum
CT_{max}	critical thermal maximum
OCLTT	oxygen- and capacity-limited thermal tolerance
P_{crit}	critical oxygen partial pressure
PMV	pre-mortal valley
P_{O_2}	oxygen partial pressure
Q_{10}	thermal sensitivity of metabolic rate
STP	standard temperature and pressure
TLR	thermolimit respirometry
T_c	critical temperature
$T(\dot{V}_{CO_2,max})$	temperature at which maximum metabolic rate occurred
$\dot{V}_{CO_2}$	rate of carbon dioxide release
$\dot{V}_{CO_2,max}$	maximum metabolic rate

Most studies test OCLTT in insects by altering oxygen supply through changes in ambient gas concentration, and thereby largely assume that the major factor influencing thermal tolerance is variation in oxygen supply. While such changes are indeed likely to affect organisms through a number of potential cellular mechanisms (reviewed in Harrison and Haddad, 2011), their impacts could differ depending on species-specific critical oxygen levels before metabolism is depressed (critical oxygen partial pressure, P_{crit}), and the maximum delivery ability of a specific gas exchange system (respiratory conductance), which can vary substantially both within and among species (e.g. Greenlee and Harrison, 2004; Lease et al., 2006; Klok et al., 2010). The effects of oxygen supply have been investigated during ontogeny in the context of limitations on body size (e.g. Callier and Nijhout, 2011, 2012; Kaiser et al., 2007; Klok et al., 2009; Harrison et al., 2010; and see review by Harrison and Haddad, 2011), but few other physiological parameters pertinent to OCLTT have been reported. However, recent examinations of OCLTT have found that oxygen delivery (availability) does not limit upper thermal tolerance in several insect species (including flies, cockroaches, crickets and beetles) or in the tropical eurythermal crustacean *Macrobrachium rosenbergii* (Mölich et al., 2012; McCue and De Los Santos, 2013; Ern et al., 2014).

Variation in oxygen demand has not been a major focus of tests of OCLTT to date. One notable exception is the reduction in CT_{max} reported with increasing thermal sensitivity to oxygen consumption (Q_{10}) in *D. cephalotes* nymphs, suggesting that a greater thermal sensitivity of oxygen demand made these aquatic insects more vulnerable to heat stress (Verberk and Bilton, 2011). Similarly, *Calopteryx virgo* nymphs were shown to increase heat tolerance after mild heat stress, irrespective of oxygen supply conditions, which the authors attributed to rapid physiological adjustments in oxygen demand (Verberk and Calosi, 2012). Indeed, the role of oxygen safety margins (i.e. the difference between P_{crit} and ambient P_{O_2}) has not been previously considered in experimental assessments of OCLTT, yet it is relatively well established that resting metabolic rate (demand) of many species varies significantly among life stages or throughout development, usually in tandem with changes in oxygen supply capacity (reviewed in Chown and Nicolson, 2004; Harrison et al., 2012). For example, mass-specific $\dot{V}_{CO_2}$ of the silk moth *Bombyx mori* is 2.5-fold lower in pupae than fifth instar larvae (0.24±0.03 vs 0.64±0.04 ml CO_2 g^{-1} h^{-1}; Blossman-Myer and Burggren, 2010a) and is typical of Lepidoptera species [e.g. *Samia cynthia* (Hetz, 2007)]. Furthermore, morphology can change dramatically as insects develop, thereby creating greater (or smaller) oxygen safety

margins. For example, larger grasshoppers with higher mass-specific metabolic rates had lower critical P_{O_2} values than juveniles (Greenlee and Harrison, 2004), whereas caterpillars have a reduced oxygen safety margin (higher critical P_{O_2}) when nearing moult (Greenlee and Harrison, 2005; Lease et al., 2006; Callier and Nijhout, 2012).

Using thermolimit respirometry (TLR; Lighton and Turner, 2004), we investigated the two predictions of OCLTT relating to oxygen supply and aerobic scope in larvae of the silk moth *B. mori* (Lepidoptera: Bombycidae) at thermal extremes. In addition, by changing the oxygen supply in two life stages with different metabolic demands (larvae versus pupae), we also tested the relative roles of oxygen supply versus demand as a framework within which to test several key OCLTT predictions. We furthermore aimed to use differences in oxygen safety margins (Callier and Nijhout, 2011, 2012) to reconcile differences in conclusions reached among the diverse taxa examined to date. Lastly, we assessed the possible role of gas physico-chemical properties for their influence on thermal tolerance as a possible explanation for variation in the outcomes of tests of OCLTT among arthropod species examined to date (see supplementary material Table S1 for details).

Specifically, we investigated whether oxygen availability altered acute high temperature tolerance by performing TLR on larvae under normoxia, hypoxia and hyperoxia, predicting a general positive association between oxygen level and CT_{max}. To test the effects of supply versus demand, we compared CT_{max} of larvae and pupae with known differences in mass-specific metabolic rate under normoxia and hypoxia. Given that larvae have higher metabolic demand than pupae (Blossman-Myer and Burggren, 2010a), we predicted that larvae would be more oxygen limited than pupae if oxygen demand is an important factor in the heat tolerance of insects, assuming that the maintenance of metabolic rate reflects oxygen safety margins. To investigate the effects of the properties of the delivery medium on OCLTT, we assessed CT_{max} in larvae using humidified hypoxia and normoxia air, and two 'trimix' gases at 21 kPa oxygen containing helium. Lowering the oxygen concentration, adding moisture to any gas mixture or changing the composition of the gas will affect one or several aspects of the physical chemistry including the heat capacity, viscosity, density or diffusivity of the gas (supplementary material Table S1), which may influence organismal heat tolerance. Assuming that a significant part of gas exchange is diffusive (but see discussions in Wobschall and Hetz, 2004; Socha et al., 2010; Groenewald et al., 2012), increases in viscosity or density of gas mixture are predicted to increase the ventilatory workload, whereas an increase in diffusivity (e.g. trimix gas composition, supplementary material Table S1) is likely to enhance the conductance of the tracheae.

RESULTS
Oxygen limitation in larvae

CT_{max} differed significantly among oxygen treatments in larvae (Fig. 1; Table 1). Hypoxia (2.5 kPa O_2) significantly reduced CT_{max} in larvae compared with both 21 and 40 kPa O_2 (activity CT_{max} and $\dot{V}_{CO_2}$ CT_{max}: $F_{2,28}=18.84$, $P<0.001$ and $F_{2,39}=6.72$, $P=0.003$ respectively, Fig. 1A; supplementary material Table S3). Neither thermal oxygen sensitivity (measured as Q_{10} between 25 and 35°C) nor body mass had a significant effect on either activity CT_{max} [ANCOVA: Q_{10} (25–35°C): $F_{1,23}=3.94$, $P=0.06$; mass: $F_{1,23}=0.14$, $P=0.72$; oxygen treatment: $F_{2,23}=49.48$, d.f.=2, $P<0.001$] or $\dot{V}_{CO_2}$ CT_{max} [ANCOVA: Q_{10} (25–35°C): $F_{1,35}=1.13$, $P=0.30$; mass: $F_{1,35}=1.40$, $P=0.25$; oxygen treatment: $F_{2,35}=7.65$, $P=0.002$; Fig. 1]. ANCOVAs using higher Q_{10} ranges (Table 1)
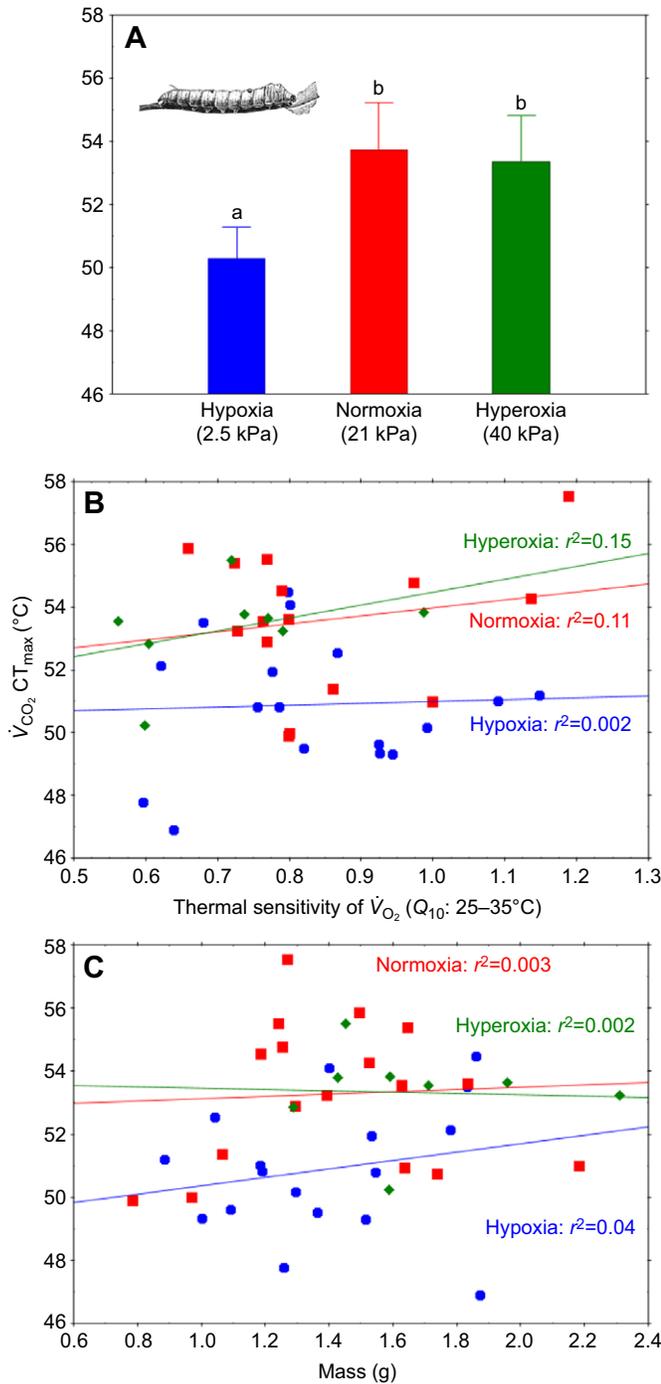
Fig. 1. Effect of oxygen availability on $\dot{V}_{CO_2}$ critical thermal maximum in *Bombyx mori* larvae. (A) Decreasing oxygen availability decreases CT_{max} of $\dot{V}_{CO_2}$. The relationship between CT_{max} and thermal sensitivity of $\dot{V}_{O_2}$ (B; measured as Q_{10} between 25°C and 35°C) and body mass (C) is shown. Bars indicate means with 95% confidence interval. Different letters indicate statistically different oxygen treatments. Each data point is one individual.

showed that for both activity and $\dot{V}_{CO_2}$ CT_{max}, only oxygen treatment was significant.

In comparison to normoxia and hyperoxia, mean $\dot{V}_{CO_2}$ at 25°C in larvae was significantly lower under hypoxia ($F_{2,34}=21.45$, $P<0.001$; Fig. 2A). Hypoxia also reduced minimum, mean and maximum metabolic rates during heating over the course of TLR relative to normoxia (Fig. 2C,D). Maximum $\dot{V}_{CO_2}$ during heating ($\dot{V}_{CO_2}$,max) was also lower under hypoxia compared with both

Table 1. Summary data for each of the experimental gas conditions and life stages assessed in *Bombyx mori*

Gas mixture	Mean mass (g)	COV at 25°C	Activity CT_{max} (°C)	$\dot{V}_{CO_2}$ CT_{max} (°C)	Absolute aerobic scope (ml h^{-1})	Factorial aerobic scope	Q_{10} (25–35°C)	Q_{10} (30–40°C)	Q_{10} (35–45°C)	Q_{10} (40–50°C)
Larvae										
Hypoxia	1.39±0.08 (17)	0.29±0.02 (17)	44.44±1.20 (9)	50.90±0.51 (17)	0.36±0.07 (17)	2.57±0.62 (17)	0.83±0.04 (17)	1.22±0.08 (17)	1.38±0.09 (17)	1.45±0.14 (17)
Hypoxia (humidified)	1.54±0.15 (7)	0.18±0.03 (7)	47.44±2.46 (6)	51.28±1.19 (6)	0.50±0.10 (7)	2.50±0.97 (7)	0.59±0.06 (7)	1.18±0.12 (7)	1.44±0.14 (7)	**1.58±0.22** (7)
Normoxia	1.42±0.08 (17)	0.24±0.02 (17)	52.99±0.33 (11)	53.27±0.55 (17)	1.17±0.07 (14)	6.71±0.69 (14)	0.85±0.04 (15)	1.41±0.08 (15)	**1.92±0.10** (15)	**1.81±0.15** (15)
Normoxia (humidified)	1.37±0.26 (9)	0.15±0.03 (9)	52.33±1.80 (9)	52.28±1.89 (9)	1.00±0.10 (7)	4.50±0.97 (7)	0.75±0.06 (7)	1.16±0.12 (7)	**1.77±0.14** (7)	**2.65±0.22** (7)
Trimix (21/16)	1.65±0.10 (8)	0.22±0.03 (8)	53.03±0.43 (8)	54.42±0.45 (8)	1.15±0.10 (7)	6.94±0.97 (7)	0.91±0.05 (8)	1.25±0.11 (8)	**1.79±0.13** (8)	**1.95±0.20** (8)
Trimix (21/63)	1.32±0.12 (11)	0.31±0.02 (11)	52.15±1.04 (11)	54.62±1.04 (11)	0.98±0.09 (9)	10.69±0.86 (9)	0.78±0.05 (11)	1.27±0.10 (11)	**1.81±0.11** (11)	**2.34±0.17** (11)
Hyperoxia	1.67±0.12 (8)	0.32±0.03 (8)	52.45±0.28 (8)	53.33±0.52 (8)	1.32±0.10 (7)	11.59±0.97 (7)	0.71±0.06 (7)	1.10±0.12 (7)	**1.88±0.14** (7)	**2.20±0.22** (7)
Pupae										
Hypoxia	0.78±0.05 (6)	0.21±0.03 (5)	50.27±0.88 (6)	50.17±0.75 (6)	0.002±0.11 (6)	2.16±1.05 (6)	0.67±0.07 (5)	1.14±0.14 (5)	1.18±0.17 (5)	1.26±0.26 (5)
Normoxia	0.84±0.07 (5)	0.23±0.03 (5)	51.93±0.36 (5)	50.29±0.35 (5)	0.004±0.14 (4)	5.62±1.29 (4)	0.99±0.08 (4)	**1.68±0.16** (4)	**1.86±0.19** (4)	1.28±0.29 (4)

Means±s.e.m. are shown for coefficient of variation of $\dot{V}_{CO_2}$ (COV) at 25°C, activity CT_{max}, $\dot{V}_{CO_2}$ CT_{max}, aerobic scope and Q_{10} values over different temperature ranges. Sample sizes are given in brackets. Significant differences in COV at 25°C, activity CT_{max}, $\dot{V}_{CO_2}$ CT_{max} and absolute and relative aerobic scope are shown in supplementary material Fig. S2. Q_{10} values not significantly different from 2.0 are highlighted in bold. Additional summary data can be found in supplementary material Table S2.
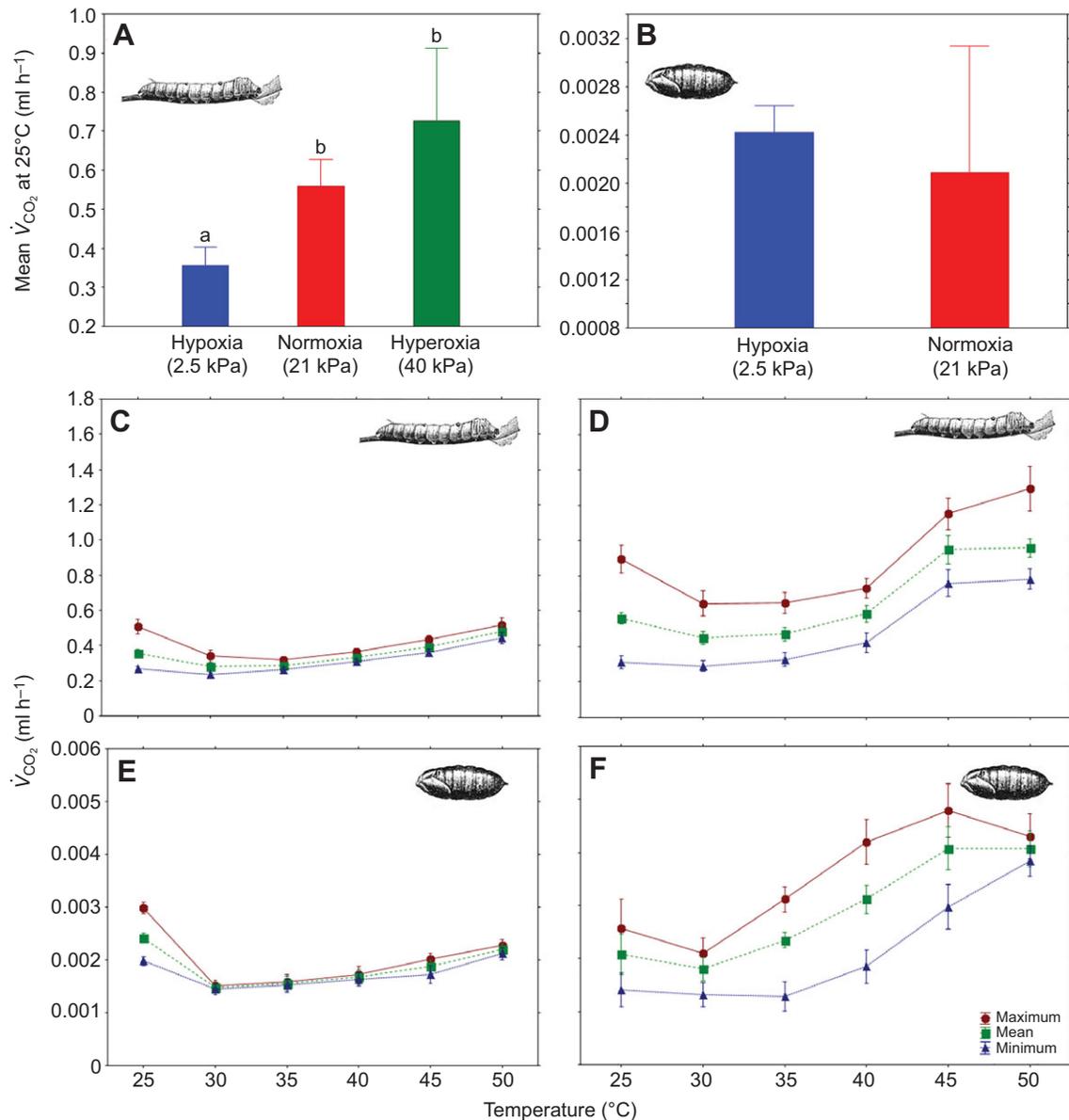
Fig. 2. Effect of temperature on $\dot{V}_{CO_2}$ in *Bombyx mori* larvae and pupae. Larvae (A) have a lower P_{crit} than pupae (B) because a reduction in oxygen causes a significant decrease in $\dot{V}_{CO_2}$ only in the larvae. $\dot{V}_{CO_2,max}$ (red circles), $\dot{V}_{CO_2,mean}$ (green squares) and $\dot{V}_{CO_2,min}$ (blue triangles) at the different temperatures during thermolimit respirometry under hypoxia (C,E) and normoxia (D,F) in both larvae (C,D) and pupae (E,F). Bars indicate means with 95% confidence intervals. Points are means±s.e.m.

normoxia and hyperoxia ($P<0.001$; supplementary material Table S3; Fig. 2C,D, supplementary material Fig. S3E).

Absolute and factorial aerobic scopes were significantly reduced under hypoxia ($P<0.001$; supplementary material Table S3; Fig. S2C and D). The exposure to hypoxia initially increased spiracle activity (indicated by the coefficient of variation of $\dot{V}_{CO_2}$, COV) at 25°C (Table 1, supplementary material Table S3; Fig. S2E). However, at higher temperatures, COV decreased and resulted in smooth $\dot{V}_{CO_2}$ traces, indicating less tightly controlled spiracle behaviour (supplementary material Tables S2 and S3; Fig. 3C, supplementary material Fig. S2E,F).

Changes in oxygen demand under normoxia (comparison of larvae versus pupae)
Under normoxia, larvae had a significantly higher CT_{max} (both activity CT_{max} and $\dot{V}_{CO_2}$ CT_{max}: 53°C vs 50°C, $P<0.04$ in both

cases; supplementary material Fig. S2A,B; Table 1, supplementary material Table S3) and a 260-fold higher mean $\dot{V}_{CO_2}$ at 25°C than pupae (Fig. 2). Spiracle activity (COV) was similar between larvae and pupae until the temperature reached 45°C. Between 45°C and 50°C, spiracle activity was significantly reduced in pupae (supplementary material Tables S2 and S3; Fig. S2F). Pupae also had a lower aerobic range across all measurement temperatures (supplementary material Tables S2 and S3).

Oxygen limitation in pupae
Activity CT_{max} was significantly reduced under hypoxia ($Z=1.00$, $P=0.0045$), although $\dot{V}_{CO_2}$ CT_{max} was not significantly different between oxygen levels within the pupal life stage ($t_9=0.14$, $P=0.89$). In contrast to larvae, mean $\dot{V}_{CO_2}$ at 25°C was not significantly decreased under hypoxia (Fig. 2B), indicating that pupae have a lower P_{crit} than larvae. $\dot{V}_{CO_2,max}$ during heating, factorial aerobic
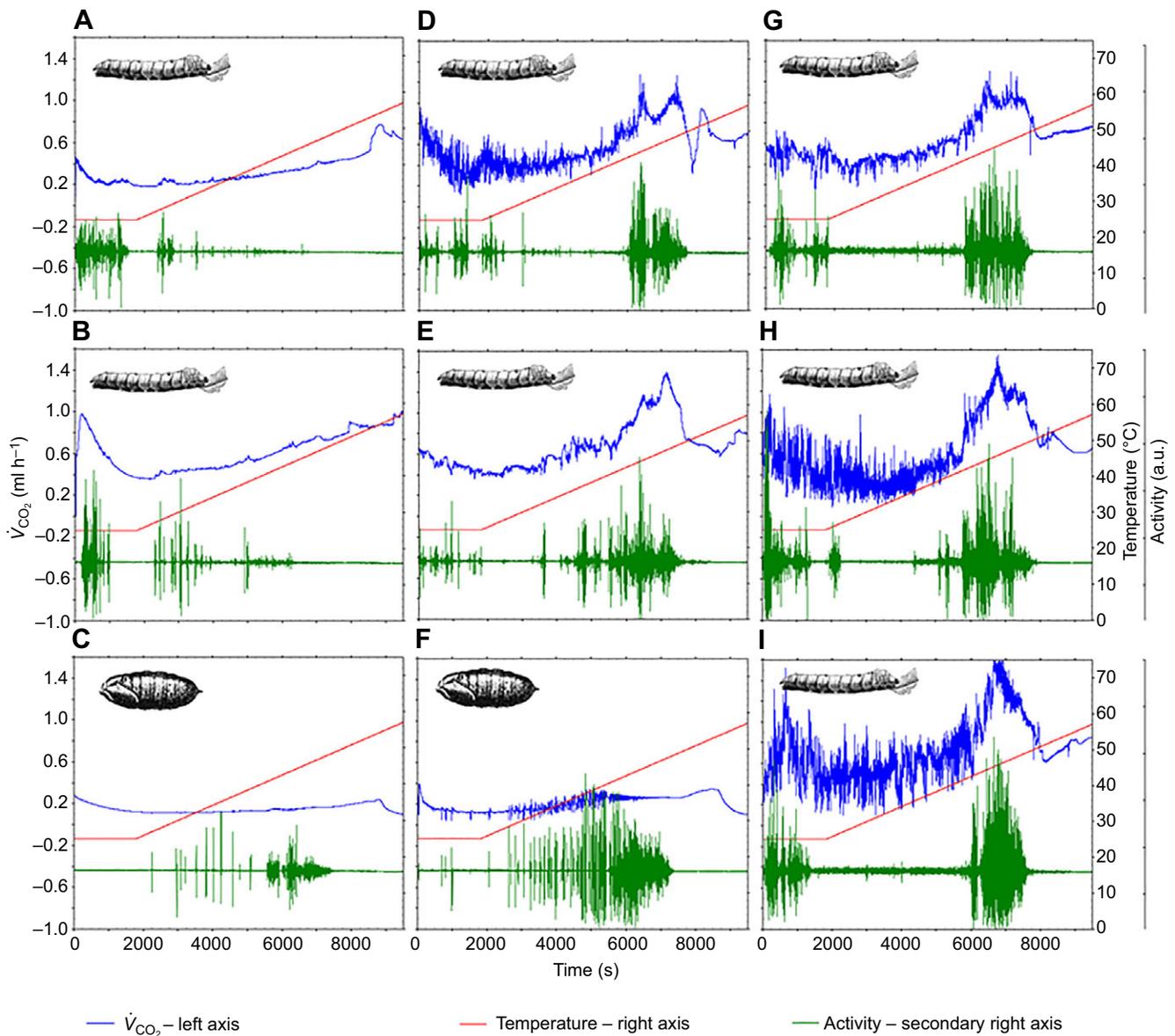
Fig. 3. Examples of thermolimit respirometry recordings of *Bombyx mori*. Larvae were analysed under hypoxia (A,B) and normoxia (D,E), with both dry (A,D) and humidified air (B,E). In addition, larvae were analysed under two Trimix gases (G,H) as well as hyperoxia (I). Pupae were analysed under hypoxia (C) and normoxia (F). See details in supplementary material Table S1. Graphs show $\dot{V}_{CO_2}$ (blue line, left axis), temperature (red line, right axis) and activity (green line, secondary right axis). In all cases, flow rate was 200 ml min^{-1}. Masses of the insects in these examples were: A, 1.04 g; B, 1.67 g; C, 0.79 g; D, 1.29 g; E, 1.30 g; F, 0.73 g; G, 1.48 g; H, 1.52 g; I, 1.71 g.

scope, spiracle activity at 25°C and between 45°C and 50°C, and aerobic ranges at all temperatures were significantly decreased under hypoxia in pupae (supplementary material Table S3).

Effects of gas properties on thermal limits

Larval activity CT_{max} was lower under humidified hypoxia than humidified normoxia (t_{13}=2.32, P=0.04; supplementary material Fig. S2A; Table 1, supplementary material Table S3). In contrast, $\dot{V}_{CO_2}$ CT_{max} was not significantly different (t_{13}=0.81, P=0.43; supplementary material Fig. S2B; Table 1, supplementary material Table S3). Both humidified normoxia and hypoxia decreased the spiracle activity (COV) at 25°C relative to their dry gas counterparts (P<0.006 in both cases; Table 1, supplementary material Table S3; Fig. S2E). Adding helium to the gas mixture had no effect on CT_{max} (Table 1, supplementary material Table S3; Fig. S2A,B). In

comparison to normoxia, Trimix (21/63) significantly increased the spiracle activity at 25°C and the factorial aerobic scope (P=0.04 and P=0.03; Table 1, supplementary material Table S1).

DISCUSSION

While OCLTT has been proposed to explain pejus temperatures and the physiological breakdown of mechanisms occurring around these thresholds, tests at the extremes and other critical threshold temperatures (such as T_cII) remain important validations of key predictions since these form a continuum and exceeding the pejus is likely to lead to – and be correlated with – the critical temperatures. Furthermore, acute heat stress, such as that applied here was used to develop the theory (e.g. Frederich and Pörtner, 2000) and can be informative of mechanisms. Moreover, recent meta-analysis of marine organisms has shown that temperature and oxygen tolerance

limits are governed by biological complexity and the evolutionary limits of the plasticity of each species (Storch et al., 2014). Thus, investigating thermal limits, even if they fall close to thermal extremes as in the case of CT_{max}, is relevant for exploring the role of oxygen in setting thermal limits.

Despite extensive interest in OCLTT, the relative importance of supply- and demand-related changes influencing upper thermal limits of insects have not been well explored (but see McCue and De Los Santos, 2013). Here, we provide several findings that are of broader interest to understanding thermal limits of air-breathing ectothermic animals, in addition to providing novel thermal limit and gas exchange data previously unreported for this model organism. Perhaps the most important finding is that we demonstrate that oxygen supply may be playing a role in setting thermal limits in larvae, but not pupae, of *B. mori*.

In most cases activity CT_{max} and $\dot{V}_{CO_2}$ CT_{max} were positively correlated (Table 1). Critical thermal maxima estimated from $\dot{V}_{CO_2}$ CT_{max} are likely the most relevant measure from thermolimit respirometry for investigating OCLTT as the theory has been used to explain critical thermal limits and not only the physical activity limits (i.e. activity CT_{max}) of the organism, although the extent to which these are correlated is typically not reported. The $\dot{V}_{CO_2}$ CT_{max}, which indicates the loss of spiracle control closely approximates death (Vorhees and Bradley, 2012), whereas activity CT_{max} commonly used in OCLTT studies may be influenced by the organism's innate behaviour and voluntary activity levels under heat stress. Estimates of CT_{max} from dynamic (ramping) assay or through thermolimit respirometry (for review of methodology, see Terblanche et al., 2011) for *B. mori* have not been previously reported. However, fifth instar larvae of *B. mori* have an upper lethal temperature of 44–45°C after a 1–2 h exposure (Hsieh et al., 1995; Chavadi et al., 2006) and heat-shock mortality after 47°C is similar for fifth instar larvae (10 min) and pupae (8 min) (Joy and Gopinathan, 1995). Here, we found that larvae and pupae had CT_{max} of 53°C and 50°C, respectively. These CT_{max} estimates are higher than the upper lethal temperatures previously determined for *B. mori* using static protocols, which is probably a consequence of the dynamic ramping rate methodology employed here, and the overall shorter, more acute exposures under our assay conditions (Hsieh et al., 1995; Chavadi et al., 2006; Joy and Gopinathan, 1995; reviewed in Terblanche et al., 2011).

Our results for *B. mori* larvae showed that hypoxia reduced CT_{max}, yet hyperoxia did not increase CT_{max} (Fig. 1). While generally expected that hyperoxia should increase CT_{max}, this may not necessarily be the case if the respiratory cascade is limited by a different feature(s) during hyperoxia than the mechanism(s) operating under hypoxia. In other words, oxygen diffusion may set the limit to delivery under hypoxia, directly affecting upper thermal tolerance and in line with OCLTT predictions, but tracheal conductance could set the upper limit to oxygen supply under normoxia and hyperoxia (e.g. Kaiser et al., 2007). This means that hyperoxia is not simply the inverse of hypoxia for the insect, and it may therefore be inappropriate to consider both as equal tests of OCLTT predictions.

The reduction in heat tolerance with hypoxia found in larvae but not pupae suggests a stage-specific effect of OCLTT on CT_{max} that has not been previously documented. These differences between life stages in *B. mori* may be attributed to their relative differences in scope for metabolic demand and metabolic regulation and respiratory conductance. This is perhaps not surprising given that *Drosophila melanogaster* pupae are not oxygen limited and the effects of temperature are less pronounced on metabolic rate in this life stage (Merkey et al., 2011, but see Odell, 1998). Lepidopteran insect species

are renowned for their ability to maintain tracheal P_{O_2} independent of ambient conditions (Hetz and Bradley, 2005; Terblanche et al., 2008; Boardman et al., 2012). Larvae may be able to use abdominal pumping and tracheal compression to maintain tissue oxygenation in the face of decreasing ambient P_{O_2} and increasing temperature (Greenlee et al., 2013), whereas pupae are likely to be more constrained and rely more on diffusion-based gas exchange. Although initially tested by comparison of breathing modes among species (Verberk and Bilton, 2013), our results also lend support to the idea that respiratory control determines thermal limits. If respiratory control is defined as the ability to precisely regulate oxygen uptake in the face of external changes in oxygen availability at rest, one might expect *B. mori* larvae to have a higher oxygen safety margin than pupae because they have more behavioural options (i.e. whole-body movement, tracheal convection) available to assist ventilation than the more immobile pupae. Alternatively, if tracheal volume scales isometrically or hypermetrically with body mass across instars (Callier and Nijhout, 2011; Snelling et al., 2011) and can be extrapolated to mid-way through the pupal stage, this, together with the pupae's lower mass, could mean that they have excess capacity for oxygen delivery (see discussion in Blossman-Myer and Burggren, 2010a). *Manduca sexta* pupae were found to have reserve or excess aerobic potential that was only utilised upon emergence, which is indicative of a high oxygen safety margin (Odell, 1998). In addition, pupae are likely to be less susceptible to changes in P_{O_2} because of their low resting metabolic rate; however, it is unlikely that size is the sole determining factor because P_{crit} has been found to be largely independent of body size in beetles (Lease et al., 2012) and dragonflies (Henry and Harrison, 2014), and perhaps the same is true in our Lepidoptera model here. As holometabolous insects, *B. mori* undergo a significant morphological transformation during their pupal stage. Variation in pupal age should have an effect on metabolic rate, oxygen availability and the associated temperature dependence of these traits, with responses over time often resembling a U-shaped curve (Odell, 1998; Merkey et al., 2011). Previous work on allometric scaling in *B. mori* has shown that mass-specific metabolic rate is lowest during the pupal stage, but the age of pupae used in that study was not reported (Blossman-Myer and Burggren, 2010a). Here, pupae were 8–12 days old, which is likely the nadir of the U-shaped curve of responses (Odell, 1998) because pupation in *B. mori* usually lasts 14–21 days (Hsueh and Tang, 1944). While this would require further investigation, the only proxy we have for age would be mass because pupal mass decreases from completion of the cocoon to time of emergence (Hsueh and Tang, 1944). As mass is not significantly different between pupae treated with normoxia and hypoxia (Mann–Whitney U, $Z=0.65$, $P=0.65$), and pupae were assigned at random to the gas treatments, age is unlikely to have significantly biased the outcomes of our study.

In the present study, the mean $\dot{V}_{CO_2}$ at 25°C under normoxia and hypoxia (Fig. 2) suggests that pupae have a lower P_{crit} than larvae and are less sensitive to changes in oxygen availability, thus indicating a higher oxygen safety margin. In addition, CT_{max} estimates suggest that pupae are less susceptible to changes in ambient oxygen because hypoxia had no effect on CT_{max}. These findings support the notion that insects with greater oxygen safety margin show a smaller effect of hypoxia on CT_{max} (Verberk and Bilton, 2013). Unlike in *D. cephalotes* (Verberk and Bilton, 2011), thermal sensitivity of oxygen consumption rates (Q_{10}) are not correlated with CT_{max} in *B. mori* (Fig. 1). This may be caused by the low Q_{10} in *B. mori* (0.5 to 1.2 between 25 and 35°C). These values are only statistically indistinguishable from the broad theoretical expectation of 2.0 at higher temperatures of 30–45°C in pupae and 35–50°C in

larvae. Collectively, this suggests that oxygen safety margins, and not necessarily thermal sensitivity, are a key factor determining the potential for oxygen limitation to influence thermal limits.

Testing the generality of this idea using current literature is limited. The effects of oxygen on both CT_{max} and P_{crit} have only been investigated in two species, *Drosophila melanogaster* and *Tenebrio molitor*, and to our knowledge no studies are available that have simultaneously investigated these responses. However, since CT_{max} in both species is reduced under hypoxia (Lighton, 2007; Stevens et al., 2010) and both species have $P_{crit} < 3$ kPa (Klok et al., 2010; Van Voorhies, 2009; Lease et al., 2012), these species are too similar to infer whether oxygen safety margins are a more general feature in determining the extent of oxygen limitation and its relationship to upper thermal limits among terrestrial insects. Both CT_{max} and P_{crit} require caution when drawing comparisons between studies as each trait can be significantly influenced by seemingly subtle differences in methodological approach (see e.g. Marshall et al., 2013; discussion in W. C. E. P. Verberk, J. Overgaard, R. Ern, M. Bayley, T. Wang, L.B. and J.S.T., submitted). Thus, we propose that the best way to test the generality of the effects of oxygen safety margins (and, by association, life stage) on thermal limits would be to use organisms that show clear differences in oxygen safety margins throughout ontogeny [e.g. *Schistocerca americana* (Greenlee and Harrison, 2004)]. Correlating these within- and between-life-stage changes in oxygen safety margins with changes in CT_{max}, should test the universality of the concept, especially if several insect orders can be examined. Further studies should also be mindful of the pronounced effect that age and life stage may have on estimates of thermal limits, metabolic rates and oxygen safety margins.

Another key finding from this study is that gas physico-chemical properties can play a role in tests of OCLTT, but are unlikely to be responsible for the relatively large differences in support for the theory between invertebrate taxa found to date in the literature. For example, while activity and $\dot{V}_{CO_2}$ CT_{max} are broadly similar under either dry or humidified normoxia, or dry or humidified hypoxia, humidifying gas relieves oxygen supply issues in larvae and reduced the oxygen limitation effect on $\dot{V}_{CO_2}$ CT_{max} (humidified normoxia versus humidified hypoxia) while Trimix (21/63) clearly influences estimates associated with metabolic rate but not thermal limits (supplementary material Table S3). Humidified air may have increased the delivery of oxygen to the tissues under hypoxia, alleviating the oxygen supply constraint. Trimix increases the diffusivity of oxygen in nitrogen and thus enhances the conductance of the tracheae – assuming that a significant part of gas exchange is diffusive (but see discussions in Wobschall and Hetz, 2004; Socha et al., 2010; Groenewald et al., 2012) – which thus allowed an increase in spiracle activity and aerobic scope.

These results support OCLTT for upper thermal tolerance for larvae of *B. mori*, but reveals that responses to altered oxygen availability may be life-stage specific. The differences between life stages and their relative susceptibility to high temperature stress with changes in oxygen suggest that further investigation into life-stage-related oxygen safety margins is urgently needed. In particular, there is much scope for studies of the scaling of tracheal morphology with ontogeny, coupled with changes in sensitivity of respiratory metabolism to hypoxia, and with due consideration of innate variation in thermal tolerance for terrestrial arthropods.

MATERIALS AND METHODS
Insects
Bombyx mori Linnaeus 1758 (Lepidoptera: Bombycidae) larvae were reared from eggs at 22°C, 50% relative humidity (RH, iButton, DS1923, accuracy ±0.5°C, 5% RH, Dallas Semiconductors, Dallas, TX, USA) under natural photoperiod (approximately 13 h:11 h, L:D) and fed mulberry leaves (*Morus* sp.). Fifth instar larvae were fasted for at least 17 h prior to respirometry (Blossman-Myer and Burggren, 2010a) to avoid specific dynamic action effects. All respirometry was conducted during daytime to limit the confounding effects of diurnal hormonal changes (Blossman-Myer and Burggren, 2010a). Pupae used in this study all pupated within 3 days of one another, and were used for experiments within 8–12 days of pupation. On the day of each experiment, four pupae were assigned at random to either the normoxia or hypoxia gas treatment. Although cocoons of *B. mori* have no effect on oxygen diffusion (Blossman-Myer and Burggren, 2010b), pupae were removed from cocoons prior to respirometry to increase analyser response times and minimize potential interactions of gases with the cocoon.

Thermolimit respirometry
Thermolimit respirometry (TLR) (Lighton and Turner, 2004) was performed to determine CT_{max} from activity and metabolic rate data (activity CT_{max} and $\dot{V}_{CO_2}$ CT_{max}, respectively) under different oxygen treatments (Fig. 3). All individuals were weighed pre-fasting, post-fasting (just prior to respirometry) and post-respirometry on a microbalance (to 0.1 mg; AB104-S/Fact, Mettler Toledo International, Inc., Columbus, OH, USA). Normoxia was obtained using room air from a pump, while other mixtures were obtained from commercially available compressed cylinders (Air Products, South Africa): 2.5 kPa O_2, balance N_2 (hypoxia); 40 kPa O_2, balance N_2 (hyperoxia); 21 kPa O_2, 63 kPa He, balance N_2 (Trimix 21/63); 21 kPa O_2, 16 kPa He, balance N_2 (Trimix 21/16). All gases were passed through a set of scrubber columns containing soda lime and 50:50 silica gel:Drierite (WA Hammond Drierite Company Ltd., OH, USA) to remove CO_2 and water from the airstream, respectively. Flowrate, regulated to 200 ml min^{-1} (STPD) by a flow control valve (Sidetrak, Sierra International, USA), was pushed into a calibrated Li-7000 infra-red CO_2/H_2O analyser and standard LiCor software (LiCor, Lincoln, NE, USA) which recorded CO_2 production differentially ($\dot{V}_{CO_2}$) in ppm. Activity of larvae in 5 ml custom-built cuvettes (3 ml for pupae) was monitored using infra-red activity detectors (AD-2, Sable Systems International, Las Vegas, NV, USA). Baseline recordings were taken before and after each respirometry run to correct for potential analyser drift, but which was generally non-existent. When necessary, air was humidified by placing a bubbler of water containing 1 mol l^{-1} NaOH (Stevens et al., 2010) upstream of the cuvette and humidity was visually confirmed by observing condensation in Bev-A-Line tubing. The temperature of individuals was controlled using a programmable circulating and refrigeration bath filled with water (CC410wl, Huber, Berching, Germany). The bath was set to hold at 25°C for 15 min to allow the individual to equilibrate and settle at this temperature, before temperature was increased to 60°C at a rate of 0.25°C min^{-1} (observed ramp rate: 0.248°C min^{-1}).

Data extraction and analysis
Respirometry files were corrected for baseline drift and data transformed and extracted using Expedata (version 1.1.25, Sable Systems). The pre-mortal valley [PMV, defined as the decrease in $\dot{V}_{CO_2}$ before the post-mortal peak (see Lighton and Turner, 2004)], maximum metabolic rate during heating ($\dot{V}_{CO_2,max}$) and temperature at which $\dot{V}_{CO_2,max}$ occurred [$T(\dot{V}_{CO_2,max})$] were calculated (Lighton and Turner, 2004; Stevens et al., 2010). PMV and $T(\dot{V}_{CO_2,max})$ data were not available for TLR under hypoxia or humidified air. As hypoxia and moist air resulted in smooth $\dot{V}_{CO_2}$ traces, CT_{max} was calculated from $\dot{V}_{CO_2}$ using a modification of published methods (Lighton and Turner, 2004). $\dot{V}_{CO_2}$ was transformed to differentiate per second. The period where the differentials were reduced to 0 was marked. The entire channel was then transformed to the absolute difference sum (ADS). The period between the markers on the ADS trace was selected and the linear regression of $\dot{V}_{CO_2}$ ADS over this period obtained. The peak of the residuals – indicating the breakpoint in the $\dot{V}_{CO_2}$ channel where spiracles switched from high to low variability and spiracle control was lost – was recorded as $\dot{V}_{CO_2}$ ADS CT_{max} (referred to as '$\dot{V}_{CO_2}$ CT_{max}'). The loss of muscular control was determined from the activity ADS in the same way from the activity channel (already recorded as differential) and reported as 'activity CT_{max}' (supplementary material Fig. S1).

In addition to these standard TLR variables, a number of other respirometry-based variables were also extracted. To measure the degree of spiracle activity, the coefficient of variation of $\dot{V}_{CO_2}$ (COV) was calculated at 25°C as the standard deviation divided by mean $\dot{V}_{CO_2}$ (Lighton and Lovegrove, 1990). COV at 25°C was calculated from the first 15 min of the recording, while COV 40–45°C and 45–50°C were calculated from all the values spanning these respective temperature ranges. $\dot{V}_{CO_2,max}$ $\dot{V}_{CO_2,mean}$ $\dot{V}_{CO_2,min}$ at 25°C was extracted from the final 10 min of the initial 15 min equilibrium period at 25°C. $\dot{V}_{CO_2,max}$ $\dot{V}_{CO_2,mean}$ $\dot{V}_{CO_2,min}$ at 30°C, 35°C, 40°C, 45°C and 50°C were extracted from the central 2 min of the TLR recordings as the temperature ramped up and these target temperature were achieved. Aerobic range (ml h^{-1}) at each of these temperatures was calculated as $\dot{V}_{CO_2,max}$ minus $\dot{V}_{CO_2,min}$. Absolute aerobic scope was calculated as the highest $\dot{V}_{CO_2,max}$ for the trace minus the lowest $\dot{V}_{CO_2,min}$ across the entire trace, while factorial aerobic scope was calculated as $\dot{V}_{CO_2,max}$ divided by $\dot{V}_{CO_2,min}$ across the entire trace for each individual recording. Q_{10} values between 25–35°C, 30–40°C, 35–45°C and 40–50°C were calculated from $\dot{V}_{CO_2,mean}$ data.

Statistics

In order to test our hypotheses, comparisons between two or three groups were made (see supplementary material Table S3) using either independent t-tests or ANOVA. Assumptions of normality and equal variance were checked, and when violated, non-parametric Mann–Whitney U or Kruskal–Wallis ANOVA tests were used. Mass was not significantly different between larval treatment groups ($H_{6,77}$=10.7, P=0.10) and was thus excluded from analyses. Comparisons between larvae and pupae were also analysed with both standard tests as well as ANCOVA with post-fasting body mass as the covariate. For analyses involving Q_{10}, aerobic scope and aerobic range parameters, data that were three times larger than the group mean were excluded from analyses. Non-overlapping 95% confidence intervals were used to determine significantly different groups. Statistical analyses were undertaken in Statistica software (Statsoft Inc., Tulsa, OK, USA).

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