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Oxygen limitation is not the cause of death during lethal heat exposure in an insect

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Oxygen- and capacity-limited thermal tolerance (OCLTT) is a controversial hypothesis claiming to explain variation in, and mechanistically determine, animal thermal limits. The lack of support from Insecta is typically argued to be a consequence of their high-performance respiratory systems. However, no studies have reported internal body oxygen levels during thermal ramping so it is unclear if changes in ambient gas are partially or fully offset by a compensatory respiratory system. Here we provide such an assessment by simultaneously recording haemolymph oxygen (pO_2) levels—as an approximation of tissue oxygenation—while experimentally manipulating ambient oxygen and subjecting organisms to thermal extremes in a series of thermolimit respirometry experiments using pupae of the butterfly *Pieris napi*. The main results are that while *P. napi* undergo large changes in haemolymph pO_2 that are positively correlated with experimental oxygen levels, haemolymph pO_2 is similar pre- and post-death during thermal assays. OCLTT predicts that reduction in body oxygen level should lead to a reduction in CTmax. Despite finding the former, there was no change in CTmax across a wide range of body oxygen levels. Thus, we argue that oxygen availability is not a functional determinant of the upper thermal limits in pupae of *P. napi*.

1. Introduction

Oxygen limitation is proposed as a major mechanistic determinant of animal thermal tolerance and, hence, vulnerability to climate change. However, the applicability of the broader theory of oxygen- and capacity-limited thermal tolerance (OCLTT, *sensu* Pörtner [1]) to the highly diverse and functionally important Insecta is particularly controversial [2–5], mainly since they possess highly efficient respiratory systems, can readily transition between diffusive- or convection-dominated gas exchange [6] and can show pronounced metabolic responses to their environmental conditions (e.g. when maintaining flight [7]). Insects are also renowned for their survival of prolonged hypoxia or anoxia [8]. How such physiological mechanisms and responses may influence the extent of support for oxygen as a determinant of thermal tolerance in insects remains unclear.

Several key testable predictions can be made for the OCLTT to be considered a viable mechanism setting animal thermal niches. At the core lies the issue of how well regulated an insect's internal body oxygen levels are and whether these levels are tightly regulated to achieve homeostasis (e.g. through spiracle behaviour, or transitions between convection and diffusion). We hypothesized that (i) if body oxygen levels are carefully regulated through spiracle behaviour to achieve homeostasis of tissue pO_2, as argued from observations in other lepidopteran pupae [9], then changing ambient oxygen levels should not alter internal haemolymph pO_2 (i.e. indicating that pupae are oxyregulating). (ii) If, on the

other hand, pO_2 variability can be experimentally induced, indicating some degree of oxyconforming, according to OCLTT, lower internal pO_2 should be coupled with decreased CTmax. (iii) Finally, haemolymph pO_2 falls prior to death by high temperature stress because cellular oxygen demand rises faster than it can be provided by the oxygen supply systems owing to a supply–demand mismatch. We test these hypotheses using pupae of the butterfly *Pieris napi* (Linnaeus). To date, no studies of the Insecta have recorded haemolymph oxygen levels while experimentally manipulating ambient oxygen and subjecting organisms to upper lethal temperatures, despite the obvious importance thereof.

2. Material and methods

(a) Study animals and rearing conditions

We used *P. napi* originating from adults collected from two sites (approx. 20 km apart) in Skåne, southern Sweden (Kullaberg; 56°18′ N, 12°27′ E and Vejbystrand; 56°18′ N, 12°46′ E) and brought to the laboratory during summer 2017. Butterflies from both sites and of both sexes were kept in cages (0.8 × 0.8 × 0.5 m) and provided with *Armoracia rusticana* Gaertner, Meyer & Scherbius for oviposition at 25°C under long day conditions (next to large windows and under 400 W metal halide lamps) and fed sugar water. Their offspring were mass-reared into diapause on *A. rusticana* at short day conditions (10 L:14 D, 20°C) and kept in dark conditions at 2°C. For the experiments, we used pupae that had been in diapause for 6 months, a time sufficient for diapause termination [10]. Pupae were removed from the 2°C conditions and warmed to 20°C for 3–5 days, during which time they were used.

(b) Thermolimit respirometry

Thermolimit respirometry (TLR) was performed using a custom-built set-up described thoroughly previously [11]. Briefly, individual pupae were placed in a 10 ml flow-through chamber that was housed within an isolated styrofoam box temperature-controlled by a water bath (Huber, Germany). The water bath started at 30°C for 10 min, and then ramped up to 60°C at 0.25°C min^{-1}. Airflow was maintained at 200 ml min^{-1} (controlled by a mass control valve (Sidetrak, Sierra International, USA)) into a calibrated Li-7000 infra-red CO_2/H_2O analyser and using standard LiCor software (LiCor, Lincoln, USA). CO_2 production was recorded differentially ($\dot{V}CO_2$) in ppm. Baseline recordings were taken before and after each run to correct for potential drift, although largely non-existent. Pupal temperature was monitored with a T-type thermocouple using a TC-08 (Pico Technology, Eaton Socon, United Kingdom). Thermal limits were assessed from $\dot{V}CO_2$ under hypoxia (10 kPa O_2, balance N_2), normoxia (21 kPa O_2) and hyperoxia (30 kPa O_2, balance N_2) as described previously [11]. Briefly, $\dot{V}CO_2$ was calculated by first drift-correcting the measurement and then multiplying the fractional CO_2 amount with STPD-adjusted flowrate in Expedata 1.9.10 (Sable Systems). Critical temperature (CTmax) was assessed using the absolute differential sum (ADS) function in Expedata to define the point at which $\dot{V}CO_2$ switched from high to low variability indicating the loss of spiracle control [12]. Pilot experiments were performed to ensure respiration was continuous in the pupae at 30°C and above.

(c) Oxygen measurements

As a proxy of body oxygenation levels, we measured haemolymph pO_2 during the TLR-experiment using oxygen calibrated microsensors (NTH-PSt1, PreSens, Regensburg, Germany). A 0.25 × 0.5 cm window was cut in the respirometry chamber and the pupa fastened ventrally, using dental adhesive, sealing the opening.

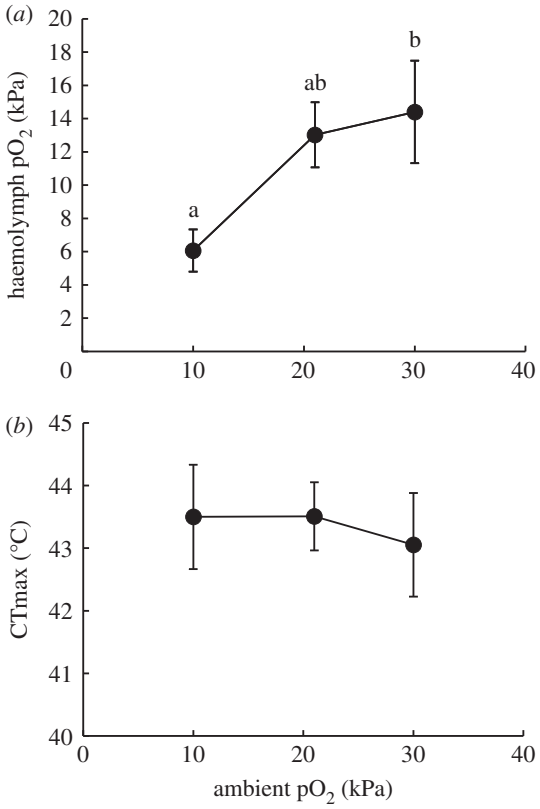

Figure 1. The effect of ambient pO_2 on two physiological traits in pupae of the butterfly *Pieris napi* (mean ± s.e.): (*a*) baseline haemolymph pO_2 measured during 10 min at 30°C, and (*b*) CTmax defined through $\dot{V}CO_2$ ADS variability. Sample sizes at 10, 21 and 30 kPa O_2 are 5, 5 and 6 pupae, respectively. Letters denote significant differences among groups ($p < 0.05$).

Table 1. Generalized linear models investigating the effect of ambient pO_2 on physiological traits in pupae of the butterfly *Pieris napi*. Significant values ($p < 0.05$) are written in italics.

effect	Wald-χ^2	d.f.	*p*-values
(*a*) baseline pO_2			
intercept	9.756	1	*0.002*
mass (covariate)	4.826	1	*0.028*
treatment	8.901	2	*0.012*
(*b*) CTmax			
intercept	84.938	1	*<0.001*
mass (covariate)	1.278	1	0.258
treatment	0.213	2	0.899
(*c*) pO_2 before and after CTmax [1]			
intercept	25.059	1	*<0.001*
mass (covariate)	9.028	1	*<0.001*
treatment	78.340	2	*<0.001*
time-point (continuous)	3.810	1	0.051
(*d*) pO_2 before and after CTmax [2]			
intercept	30.809	1	*<0.001*
mass (covariate)	9.278	1	0.066
treatment	77.894	2	*<0.001*
time-point (categorical)	3.510	1	0.061

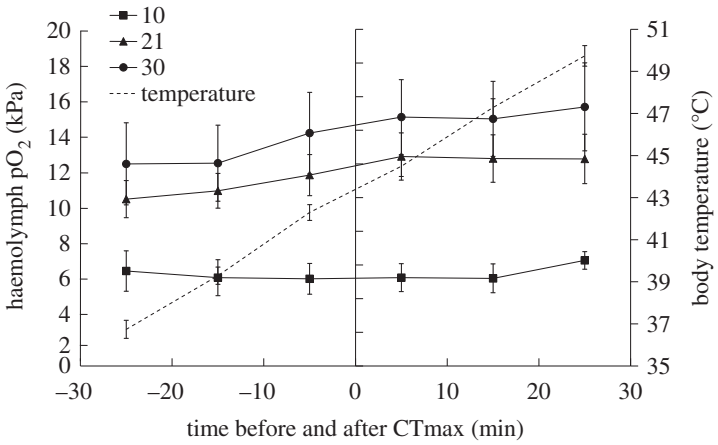

Figure 2. The effect of ambient pO_2 on haemolymph pO_2 before (25, 15 and 5 min) and after (same times) death through high temperature stress in pupae of the butterfly *Pieris napi* (mean ± s.e.). Note that the interaction between time-point and ambient pO_2 was non-significant ($p > 0.05$). Sample sizes at 10, 21 and 30 kPa O_2 are 5, 5 and 6 pupae, respectively.

A hole was punctured laterally in the first abdominal segment with a sterile needle and the oxygen probe inserted 1–2 mm below the surface with a micromanipulator. The measurements were temporally synchronized, and baseline haemolymph pO_2 (during the first 10 min) was extracted using Expedata (sample measurement in electronic supplementary material, figure S1) and converted to kPa using pupal temperature. To assess whether haemolymph clotting may have introduced a bias into the pO_2 readings, after each pupal measurement we re-measured the calibration solutions, and in all cases the precision and accuracy of oxygen readings were completely unaffected.

(d) Statistical analyses

All tests were performed using IBM SPSS statistics 23.0 (IBM SPSS Inc., Chicago, IL, USA). Generalized linear models using Gaussian distribution and an identity link function were used for all analyses. In all models, pupal mass (averaged before and after the measurement) was used as covariate. Model improvement was tracked through the Akaike Information Criterion (AIC). In case of significant main effects, univariate *post hoc* tests using Bonferroni corrections were used to test for among-group effects. (i) First, baseline haemolymph pO_2 and CTmax were added as dependent variables in individual tests, while ambient pO_2 was added as a categorical explanatory variable. (ii) Then haemolymph pO_2 values at 25, 15 and 5 min before and after CTmax were added as the dependent variable while ambient pO_2 was added as the categorical, and time-point as the continuous, explanatory variable. We tested whether haemolymph pO_2 changes prior to CTmax (i.e. as a negative slope), and assume that changes should become apparent within 25 min before CTmax [5,13]. (iii) The test was repeated using haemolymph pO_2 as the dependent variable, and ambient pO_2 as well as a grouping variable denoting a value as pre- or post-CTmax as categorical explanatory variables.

3. Results

(a) Hypothesis 1—Ambient pO_2 directly influences haemolymph pO_2

Baseline pO_2 was positively correlated with ambient pO_2 (table 1*a* and figure 1*a*). Pairwise tests showed that the hypoxia treatment was associated with significantly lower haemolymph pO_2 than the hyperoxia treatment ($p = 0.014$), with the normoxia treatment being intermediate, and not differing significantly from either hypoxia or hyperoxia ($p > 0.05$). Baseline haemolymph pO_2 was negatively correlated with body mass of pupae (table 1*a*).

(b) Hypothesis 2—No effect of ambient pO_2 on CTmax

Ambient pO_2 did not affect CTmax (table 1*b*, figure 1*b*), which was 43.1 ± 0.5°C (mean ± s.e.) across the ambient pO_2 treatments as determined through the ADS of $\dot{V}CO_2$.

(c) Hypothesis 3—Haemolymph pO_2 is stable during CTmax

Haemolymph pO_2 was stable for 25 min before CTmax, and did not change significantly upon death through lethal high temperature (table 1*c*,*d* and figure 2). Furthermore, there was no significant interaction between time-point and ambient pO_2 ($p > 0.05$). There were however large differences in haemolymph pO_2 owing to ambient pO_2 during these time-points in both tests, as mentioned above. Haemolymph pO_2 was significantly lower in the hypoxia group than in the normoxia and hyperoxia groups (both $p < 0.001$), which marginally differed from each other ($p = 0.060$). While the treatment × time-point interaction was non-significant ($p > 0.05$), there were trends of increasing pO_2 with time in all treatments (table 1*c*,*d* and figure 2).

4. Discussion

The OCLTT theory provides an intuitively appealing mechanism explaining thermal limits, but evidence from air-breathing insects is equivocal (see discussion in [3,5,11]). Therefore, we undertook a comprehensive test of several key predictions of the OCLTT in pupae of the butterfly *P. napi*. We measured haemolymph pO_2 as proxy of body and tissue oxygen levels, and indeed our values (see figure 2) are in strong agreement with a previous study where pO_2 was measured directly from insect tissues [7]. Therefore, we believe our method provides a reasonable proxy of tissue oxygenation at a resolution high enough for the investigation of key predictions of OCLTT. The OCLTT theory predicts that upper lethal temperature (CTmax) occurs as a direct consequence of a mismatch between oxygen supply and oxygen demand. There was no evidence supporting such a view in our data for two main reasons. First, CTmax was stable across a diverse range of

ambient pO_2. Moreover, we were able to generate a wide range of haemolymph pO_2 through our experimental gas conditions that failed to elicit any marked variation in CTmax. These data put the current study in line with a number of previous investigations in terrestrial insect upper thermal limits [2,4], where only severe hypoxia (less than 2 kPa O_2) was linked to reductions in CTmax ([3] but see [11]) and may be explained by hypoxia-induced activity reduction rather than strictly setting upper thermal limit at the cellular or tissue level.

Second, a key causal prediction of OCLTT is that haemolymph pO_2 should decrease just prior to death by high temperature stress as the mismatch between supply and demand is likely most apparent at that point. While indeed there was a strong relationship between ambient pO_2 and haemolymph pO_2 for 30 min prior to death, haemolymph pO_2 remained stable through this period, as well as for the subsequent 30 min. This is likely owing to a range of plastic compensatory mechanisms operating over a large thermal range, most likely owing to highly effective convection-based gas exchange in the tracheal system [6].

In conclusion, we argue that oxygen limitation effects on CTmax are small even when detected and not deterministic of thermal limits in a causal sense. This is probably owing in part to the high conductance of the tracheal system [14] and transitions between diffusion- and convection-based gas exchange [6]. Here we find evidence that CTmax in *P. napi* is likely not set by cellular oxygen supply–demand relationships, assuming that tissue and haemolymph oxygen levels are correlated, and significantly advance understanding of insect pO_2 regulation and how it is affected by ambient gas concentration.

Data accessibility. Data are available at Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.18qv7ks [15].

Authors' contributions. P.L. and J.S.T. conceived the study, P.L. and M.J. performed the experiment, P.L. analysed the data, and all authors wrote and approved the paper. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Competing interests. We declare we have no competing interests.

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