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Can Oxygen Set Thermal Limits in an Insect and Drive Gigantism?

Wilco C. E. P. Verberk*, David T. Bilton

Abstract

Background: Thermal limits may arise through a mismatch between oxygen supply and demand in a range of animal taxa. Whilst this oxygen limitation hypothesis is supported by data from a range of marine fish and invertebrates, its generality remains contentious. In particular, it is unclear whether oxygen limitation determines thermal extremes in tracheated arthropods, where oxygen limitation may be unlikely due to the efficiency and plasticity of tracheal systems in supplying oxygen directly to metabolically active tissues. Although terrestrial taxa with open tracheal systems may not be prone to oxygen limitation, species may be affected during other life-history stages, particularly if these rely on diffusion into closed tracheal systems. Furthermore, a central role for oxygen limitation in insects is envisaged within a parallel line of research focussing on insect gigantism in the late Palaeozoic.

Methodology/Principal Findings: Here we examine thermal maxima in the aquatic life stages of an insect at normoxia, hypoxia (14 kPa) and hyperoxia (36 kPa). We demonstrate that upper thermal limits do indeed respond to external oxygen supply in the aquatic life stages of the stonofly Dinocras cephalotes, suggesting that the critical thermal limits of such aquatic larvae are set by oxygen limitation. This could result from impeded oxygen delivery, or limited oxygen regulatory capacity, both of which have implications for our understanding of the limits to insect body size and how these are influenced by atmospheric oxygen levels.

Conclusions/Significance: These findings extend the generality of the hypothesis of oxygen limitation of thermal tolerance, suggest that oxygen constraints on body size may be stronger in aquatic environments, and that oxygen toxicity may have actively selected for gigantism in the aquatic stages of Carboniferous arthropods.

Introduction

To predict species responses to global warming trends, it is paramount to understand the causal mechanisms underlying thermal limits. The idea of oxygen limitation as a mechanism setting upper thermal limits in animals was first expounded by Winterstein [1] and has since been greatly expanded by Portner and colleagues [2-5]. Recent work has extended this principle to lower thermal limits, and sees both upper and lower critical temperatures ($CT_{max}$ and $CT_{min}$) being coupled by the common mechanism of temperature-dependent oxygen limitation. As ectotherms warm the demand for oxygen in their tissues increases faster than the rate at which oxygen can be provided by cardiac and ventilatory processes, leading to a drop in whole-animal aerobic scope and a shift from aerobic to anaerobic metabolism. Similarly on cooling, metabolism slows, leading to insufficient ATP production in ventilatory muscles, reducing oxygen supply to tissues. Deleterious thermal effects are hypothesized to set in first at the whole-animal level, rather than lower hierarchical levels [3]. Thus, temperature-dependent oxygen limitation first lowers whole-animal aerobic scope (and hence performance), followed by the onset of anaerobic metabolism, finally resulting in heat damage to proteins, membranes and cells, and ultimately death [2,3,6,7].

Although argued to hold for ectotherms in general [3], most of the evidence for oxygen limitation at thermal extremes to date comes from a variety of marine taxa, including fish, crustaceans, bivalves, and annelids [3, 5 and references therein]. Recent studies of terrestrial isopods and beetles [7,8] question the generality of this mechanism, suggesting that upper and lower limits are decoupled in terrestrial arthropods, and showing no increase in critical thermal maximum ($CT_{max}$) with hyperoxia. Additionally, whilst hypoxia decreased $CT_{max}$ in isopods, an effect was only seen at extreme hypoxia in beetles, casting doubt on the degree to which oxygen limitation sets upper thermal limits in tracheates. From a functional perspective, both the fact that the tracheal system constitutes a single-step gas exchange system and their high efficiency of oxygen delivery, would indeed seem to preclude oxygen limitation as a major mechanism setting upper thermal limits in terrestrial insects [8]. In addition, terrestrial insects can maintain relatively constant internal oxygen levels, across steep clines in external supply, by adjusting ventilation rates or through compensatory developmental changes in tracheal length, diameter or branching [9,10].
Not all insect species and life-stages may be equally sensitive to temperature-dependent oxygen limitation however and there remains a need for additional studies across the range of morphologies and lifestyles seen in extant insects [11]. In particular, many insects have aquatic larval stages, where the lower oxygen content and diffusion rates in water compared to air dramatically reduce available oxygen [12,13], making oxygen limitation more likely. In addition, larger species or individuals may be more prone to temperature-dependent oxygen limitation because of longer diffusion pathways [4]. Similarly, if body size ultimately constrains an animal’s capacity to supply its tissues with oxygen, this would explain why animals attain larger sizes in the cold, where metabolic rates are reduced [13,14]. Such size based oxygen limitation is also central to the leading hypothesis regarding insect gigantism in the late Palaeozoic [13].

Here we provide the first formal test of oxygen limitation in a freshwater trachete, the stonefly *Dinacras cephalotes* (Curtis, 1827). Their large, aquatic larvae rely predominantly on tegument respiration, making this an ideal species to study the principle of oxygen limitation. We examine the effects of hypoxia and hyperoxia on CT_{max} and test whether variation in CT_{max} amongst individual larvae is related to their oxygen consumption. Given the central role of oxygen in hypotheses on limits to both thermal tolerance and body size [6,13,15–17], we also discuss how the results of our study may improve our understanding of the role of oxygen in setting body size limits in arthropods. A second reason to discuss insect gigantism, stems from the fact that many insects exhibiting gigantism [18] apparently had aquatic larvae, although establishing larval lifestyle from fossil data can be difficult [19,20]. We do not suggest that insect gigantism is restricted to insects with early aquatic life stages. The now exact order of Palaeodictyoptera contained several species that displayed marked gigantism and available fossil evidence suggests a terrestrial larval stage [21,22]. However, gigantic insects do seem to have been predominantly aquatic; the aquatic larval stage is held to be a common ancestral ground plan [20] for the giant griffenflies (Pterygota, Ephemeroptera) of the late Palaeozoic as well as the stoneflies (Plecoptera) arising in the Permian. Additionally the gigantic Carboniferous myriapods, the arthopleurids, are usually considered amphibiotic, where the early life stages are aquatic [20].

To date, attempts to understand insect gigantism in the late Palaeozoic have mainly been approached from the perspective of (fossilized) terrestrial adults. Yet, if oxygen limitation at thermal extremes operates differently for aquatic larvae and terrestrial adults, approaching the problem of historical gigantism from a larval perspective instead may similarly shed new light on the morphologies and lifestyles seen in extant insects [11]. In particular, many insects have aquatic larval stages, where the lower oxygen content and diffusion rates in water compared to air dramatically reduce available oxygen [12,13], making oxygen limitation more likely. In addition, larger species or individuals may be more prone to temperature-dependent oxygen limitation because of longer diffusion pathways [4]. Similarly, if body size ultimately constrains an animal’s capacity to supply its tissues with oxygen, this would explain why animals attain larger sizes in the cold, where metabolic rates are reduced [13,14]. Such size based oxygen limitation is also central to the leading hypothesis regarding insect gigantism in the late Palaeozoic [13].

Results

Critical thermal maximum (CT_{max}) differed significantly across oxygen treatments (Table 1) being raised by 1.53°C in hypoxia and lowered by 2.92°C in hypoxia (Fig. 1A). Individual larvae differed in thermal sensitivity for oxygen consumption rates (expressed by Q_{10} values), which significantly affected their CT_{max} (Table 1). Thermal maxima were approximately 1°C lower for individuals with a high thermal sensitivity (Q_{10} value of 3) compared to individuals with a low thermal sensitivity (Q_{10} value of 1) (Fig. 1B). While we did not find a direct relationship between body mass and CT_{max} (Table 1; Fig. 1C), larger instars had higher Q_{10} values in comparison to smaller instars (t-test, t_{16} = 2.19, P=0.038). This suggests that potential effects of body mass are mediated primarily through increased thermal sensitivity, something which seems most apparent at hypoxia (Fig. 1C).

Discussion

Here we find strong evidence for the idea that a mismatch between external oxygen supply and internal oxygen demand can set thermal limits in aquatic insects [13]; hypoxia lowered thermal maxima, whilst hyperoxia increased them (Fig. 1A). At the same time, individuals that strongly increased oxygen consumption at elevated temperatures had lower thermal maxima (Fig. 1B). These results contrast with previous investigations on terrestrial adult insects, and provide a proof of principle that oxygen limitation can set upper thermal limits in aquatic insect larvae. Drawing on differences in ontogeny (closed trachea) and ecology (aquatic habitat), two explanations could be made for the apparent mismatch between oxygen supply and demand in the stonefly nymphs, causing the onset of oxygen limitation at thermal extremes.

First, oxygen delivery may be impeded in stonefly nymphs because of their closed tracheae and because of the lower oxygen content and diffusion rates in water compared to air. At higher temperatures, more oxygen is available to an aquatic organism because of the higher diffusivity of oxygen, yet scope for aerobic metabolism is nevertheless reduced as increases in organismal oxygen demand exceed increases in oxygen supply [13]. Although a closed tracheal system still represents a one-stage oxygen delivery system, oxygen delivery is more likely to become rate limiting at higher temperatures because of the additional step of oxygen diffusion across the epithelium. The absence of air sacs in larvae may further limit oxygen delivery rates by increasing the relative importance of diffusive rather than convective movement of oxygen in the trachea [9], although the compression and expansion of the trachea themselves [24] may in some cases

**Table 1. Statistical analysis of critical thermal maxima in relation to ambient oxygen levels, larval oxygen consumption and body mass.**

| Source                          | SS (Type III) | d.f. | F ratio | P-value     |
|---------------------------------|---------------|------|---------|-------------|
| Oxygen treatment                | 131.897       | 2    | 45.338  | <0.0001     |
| Q_{10} oxygen consumption rates | 12.132        | 1    | 8.341   | 0.0062      |
| Body mass (mg dry weight)       | .356          | 1    | .245    | 0.6234      |

*ANOVA* statistics on critical thermal maxima. Significant results are indicated in bold. Thermal maxima were highest for hyperoxia (36 kPa) and lowest for hypoxia (14 kPa). In addition thermal maxima were lowest for larvae which consumed more oxygen at higher temperatures. (SS = Sum of squares; d.f. = degrees of freedom).

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thermal maxima were lower for individuals which strongly increased their oxygen consumption rates at higher temperatures (high Q_{10} values). Each bar represents the average (± s.e.) of 15 nymphs. Letters indicate significant differences (P<0.05; Tukey HSD post hoc test following an ANOVA including only oxygen treatment: F_{2,41} = 44.06, P<0.001).

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Figure 1. Differences in critical thermal maxima (CT\textsubscript{max}) in the stonfly Dinocras cephalotes at three different levels of oxygen (a), the relationship between CT\textsubscript{max} of the stonfly nymphs and their thermal sensitivity in oxygen consumption (b) and their body mass (c). Differences in CT\textsubscript{max} were consistent with the mechanism of oxygen limitation: hypoxia lowered CT\textsubscript{max} while hyperoxia increased CT\textsubscript{max} and thermal maxima were lower for individuals which strongly increased their oxygen consumption rates at higher temperatures (high Q_{10} values). Each bar represents the average (± s.e.) of 15 nymphs. Letters indicate significant differences (P<0.05; Tukey HSD post hoc test following an ANOVA including only oxygen treatment: F_{2,41} = 44.06, P<0.001).

generate substantial convective movement [25]. An increased difficulty of oxygen delivery in an aquatic environment fits with the fact that CT\textsubscript{max} at normoxia is generally reported to be higher for terrestrial than aquatic arthropod life stages [7,8,26–29]. As CT\textsubscript{max} is reached at lower temperatures in aquatic taxa, this is less likely to be a result of thermal damage at the cellular level such as the disruption of membrane structure and problems associated with protein folding [30,31], which would make oxygen limitation more decisive in setting thermal limits in aquatic life stages, rather than one of several factors as suggested for terrestrial insects [7,8]. One assumption here is that aquatic taxa have not considerably altered their membrane fluidity and protein activity in response to the more stable, often lower thermal regime in aquatic habitats (see below). If oxygen is more important at less extreme thermal maxima relative to other factors (membrane fluidity and protein stability), it would explain why CT\textsubscript{max} increased with hyperoxia in the stonfly nymphs studied here (Fig. 1A) and in aquatic mayfly nymphs studied by Whitney [26], but not in the terrestrial tracheates with open tracheal systems studied so far [7,8].

Second, changes in external oxygen levels may have stronger impacts on internal oxygen levels in aquatic invertebrates, and hence thermal tolerance, if their oxygen regulatory capacity is more limited than that of terrestrial invertebrates. Insects are forced to regulate internal oxygen levels within a fairly narrow range, balancing the risk of asphyxiation with that of oxygen toxicity [10,32,33]. Whereas terrestrial insects can simply open or close spiracles to regulate oxygen uptake, such regulation is more limited than that of terrestrial invertebrates. Insects are predicted to be more prone to oxygen limitation, such size dependency may only be evident under certain conditions [15,17], given that larger individuals may have modified respiratory structures, and change their respiratory behaviour to compensate for reductions in oxygen supply capacity associated with larger size [10,15]. Costs associated with such compensatory changes may include tracheal hypertrophy [9,23], or increased thermal sensitivity (see results). These costs may be reflected in the
long term, underlying observed variation in body size across environmental gradients of temperature or oxygen availability [13,14]. Here we found support for size related performance in the hypoxic treatment only (Fig. 1C). A possible explanation for this is that the tracheal network of the larger individuals, which developed under normoxia in the field, was of insufficient capacity when larvae approached their thermal limits under hypoxia. At higher levels of oxygen, other limits may have set in that were less dependent on size, involving the failure of oxygen delivery systems operating at the cellular level [14].

A fundamental problem with a passive oxygen ceiling constraining maximum body size is that it is inferred from extant taxa having physiologies optimized to normoxic conditions and therefore may not reflect the evolutionary limit of tracheal breathing [37]. The second explanation of limited oxygen regulatory capacity recasts oxygen as an active driver of gigantism by focussing on the risks of having too much oxygen, rather than too little. In aquatic larvae with closed tracheal systems and limited ability to regulate internal oxygen levels, internal oxygen levels would be expected to closely track environmental oxygen levels. While many species are likely to have experienced and evolved responses to cope with periods of hypoxia [38], the same may not apply for hyperoxia, putting aquatic larvae at greater risk of oxygen poisoning than terrestrial adults [39]. If large body sizes are more sensitive to hypoxia and asphyxiation, they may equally confer protection from oxygen toxicity [40], constituting an antioxidant response [41].

In support of oxygen as an active driver of increasing body size, Loudon [9] found that beetle larvae increased in body mass when they were transferred from hypoxia to normoxia during their development. The logic is that larvae which started their development in hypoxia increased their tracheal size, but could not decrease them again upon returning to normoxia, as the new trachea are built around the old trachea of the previous larval instar. Although increasing body mass entails costs, body mass results from the net effect of many different factors [14]. During development, internal hypoxia acts as a signal and may stimulate tissue differentiation instead of growth, thus affecting the size reached. Similarly hyperoxia may trigger an increase in body size as a readily available way to effectively escape oxygen toxicity, possibly enabled through lower costs in ventilation or tracheal investments [10,40,42]. Hence hyperoxia may actively drive evolutionary increases in body mass, even in small insects [40]. Direct evidence for oxygen toxicity in a range of freshwater invertebrate species is provided by Fox & Taylor [32] who found that smaller juvenile stages are more sensitive to hyperoxic conditions than their larger aquatic adults.

Thus, a larval view of Paleozoic hyperoxia-enabled gigantism in insects may be very informative. We suggest the aquatic larval stage as a route to gigantism, explaining the predominance of aquatic life stages amongst extinct gigantic insects. In aquatic juvenile stages, impeded oxygen delivery may have resulted in stronger constraints on body size, whilst larger body sizes may have been actively selected for to avert oxygen toxicity. The widespread gigantism in marine species further supports this hypothesis, with examples of gigantism [47,48] found predominately at times when oxygen levels were perhaps not higher than current ones, but rising [49], effectively putting the animals at risk of oxygen poisoning. Each of the two explanations for a larger role of oxygen in aquatic stages yields testable predictions concerning the relative rates of evolution of larger and smaller body sizes, and the shifting or broadening size spectra.

Materials and Methods

Animals were collected from the River Dart, Devon, UK and maintained in the lab at 10±1°C in a 12 L:12 D regime. They were kept in a flow-through aquarium (10 L min\(^{-1}\)), fed with artificial pond water [50], buffered and diluted to reflect the pH and conductivity of the field site (pH 6.4–6.6, 70–150 µS cm\(^{-1}\)) and fed chironomid larvae. Oxygen consumption was measured for each larva at 10°C and 15°C using closed glass respiration chambers of 67.5–68.5 ml. The chambers were immersed in a temperature controlled bath (±0.1°C) and stirred using underwater magnetic stirrers to ensure mixing of water. Respiration chambers were fitted with a fine nylon mesh forming a false bottom to prevent contact between the larvae and the magnetic stirrer bar. Individuals were allowed to acclimate for 10 min before the chambers were closed and left for 60 min. Oxygen content was measured before and after using an O\(_2\) electrode (1302 Oxygen Electrode, Strathkelvin Instruments) that was connected to a calibrated meter (Oxygen Meter 929, Strathkelvin Instruments). On average, larvae depleted oxygen levels only by 5% (with a maximum of 18%) and oxygen consumption was expressed as µg O\(_2\) (mg wet mass\(^{-1}\)) h\(^{-1}\). Oxygen consumption of larvae at 10°C was significantly correlated with their consumption at 15°C [Partial correlation, corrected for differences in body mass: \(r = 0.511, P<0.001\)], justifying the calculation of Q\(_{10}\) values for individuals. One individual was removed from further analysis as abnormally low oxygen consumption measured at 10°C resulted in an unrealistic Q\(_{10}\) value of 99. The Q\(_{10}\) values calculated for each specimen were correlated with the \(CT_{\text{max}}\) values of the exact same individuals.

To assess \(CT_{\text{max}}\) at normoxia, animals were placed in small flow-through chambers (70×70×30 mm; flowrate 0.016 L s\(^{-1}\)) fed with water from a 25 L header tank passing through a tubular counter current heat exchanger. Water in the header tank was of the same composition as that used to maintain animals, and was bubbled with a mixture of 20% oxygen and 80% nitrogen, obtained using a gas-mixing pump (Wosthoff, Bochum, Germany), and covered with 18 mm thick expanded polystyrene sheeting to prevent equilibration with the atmosphere. Before the start of the experiment, animals were left to acclimate for 1 h at the starting temperature of 10°C, after which temperature in the experimental chambers was increased at 0.25°C min\(^{-1}\), using a Grant R5 water bath with a GP200 pump unit (Grant Instrument (Cambridge) Ltd, UK), connected to the heat exchanger. Temperatures were logged using a HH806AU digital thermometer (Omega Engineering Inc., USA). \(CT_{\text{max}}\) was recorded as the point at which animals no longer showed any body movement or muscular spasms.

Animals which were at this point transferred to fully oxygenated...
water of 10°C recovered with no apparent lasting damage. Below critical maxima, larvae initiated in repeated swimming behaviour (around 29°C), interpreted as attempts to escape experimental conditions and fell upon their backs (around 30°C) and an onset of spasms was observed (around 31°C). We used a dynamic method (acute exposure to changing temperatures) in contrast to most work performed on marine taxa, which used static methods (chronic exposure to constant temperatures [3, 5 but see 2]). As faster rates of warming can result in higher critical thermal maxima [51, 52], we employed the same rate of warming as employed in previous studies on terrestrial insects to minimize confounding effects arising from methodological differences in a direct comparison of our results with theirs. To assess CTmax at hypoxia (5% O2, 95% N2) and hyperoxia (60% O2, 40% N2), the gas mixture was adjusted 10 min after placing the animals in the small flow-through chambers. In this way animals were gradually exposed to hypoxic and hyperoxic conditions during acclimation.

In several test runs, oxygen levels were measured after adjusting the gas mixture to determine when and at which value oxygen levels stabilized. From these measurements the relative contributions of the gas mixture and normal air to the (hypoxic or hyperoxic) test water could be determined. The oxygen levels stabilised after 1 hour at 36 kPa (34–38) and 14 kPa (13.3–14.8) for the hyperoxia and hypoxia treatment, respectively.

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Author Contributions

Conceived and designed the experiments: WV DB. Performed the experiments: WV. Analyzed the data: WV. Wrote the paper: WV DB.

References

1. Winterstein H (1905) Wärmelähmung und Narkose. Z Allg Physiol 5: 323–350.
2. Frolovich M, Portner HO (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.
3. Portner HO (2002) Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. Comp Biochem Physiol A 132: 739–761. (doi:10.1016/S0956-4633(02)00045-4).
4. Portner HO (2006) Climate-dependent evolution of Antarctic ectotherms: An integrative approach. Deep-Sea Res II 53: 1071–1104. (doi:10.1016/j.dsr2.2006.02.015).
5. Portner HO (2010) Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. J Exp Biol 213: 801–893. (doi:10.1242/jeb.057525).
6. Portner HO (2001) Climate change and temperature-dependent biogeography: oxygen limitation of thermal tolerance in animals. Naturwissenschaften 88: 137–146. (doi:10.1007/s001140010026).
7. Stevens MM, Jackson S, Bester SA, Terblanche JS, Chown SL (2010) Oxygen limitation and thermal tolerance in two terrestrial arthropod species. J Exp Biol 213: 2209–2218. (doi:10.1242/jeb.041700).
8. Klok CJ, Sinclair BJ, Chown SL (2004) Upper thermal tolerance and oxygen limitation in terrestrial arthropods. J Exp Biol 207: 2361–2370. (doi:10.1242/jeb.01023).
9. Loudon C (1989) Tracheal hypertrophy in mealworms: Design and plasticity in response to ionic content of the water. Aquat Ecol 37: 151–158. (doi:10.1023/A:102292050259).
10. Hetz SK, Bradley TJ (2005) Insects breathe discontinuously to avoid oxygen limitation and thermal tolerance: Evolving and Ecological Physiology. Annu Rev Physiol 67: 237–261. (doi:10.1146/annurev.physiol.67.051204.160159).
11. Calosi P, Bilton DT, Spicer JI, Atfield A (2008) Thermal tolerance and geographical range size in the Agabus brunneus group of European diving beetles (Coleoptera: Dytiscidae). J Bioenerg Biometabol 35: 31–45. (doi:10.1007/s00114-006-9706-8).
12. Forstner K, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and Ecological Physiology. Annu Rev Physiol 61: 243–262. (doi:10.1146/annurev.physiol.61.1.243).
13. Somero GN (2004) Adaptation of enzymes to temperature: searching for basic ‘strategies’. Comp Biochem Physiol B 139: 321–333. (doi:10.1016/j.cbpb.2004.05.003).
14. Fox HM, Taylor AER (1955) The tolerance of oxygen by aquatic invertebrates. Proc R Soc Lond B 143: 214–225. (doi:10.1098/rspb.1955.0006).
15. Klok CJ, Kaiser A, Lighton JRB, Harrison JF (2010) Critical oxygen partial pressures and maximal tracheal conductances for Drosophila melanogaster reared for multiple generations in hypoxia or hyperoxia. J Insect Physiol 56: 461–469. (doi:10.1016/j.jinsphys.2009.01.004).
16. Stuart MT, Hessen D (2005) Life stage-related differences in hardening and acclimation of thermal tolerance traits in the kelp fly, Parnara brunnea (Diptera, Helcomyzidae). J Insect Physiol 51: 336–343. (doi:10.1016/j.jinsphys.2005.11.016).
17. Forbes BD, Kalff J (1992) Oxygen, animals and oceanic ventilation: an alternative view. Geobiology 1: 7–17. (doi:10.1143/1.4724699.2000.00118.x).
18. Hoback WW, Stanley DW (2001) Insects in hypoxia. J Insect Physiol 47: 533–542. (doi:10.1016/S0022-1910(00)01533-0).
19. Van Voorhis WA (2009) Metabolic function in Drosophila melanogaster in response to hypoxia and pure oxygen. J Exp Biol 211: 3409–3420. (doi:10.1242/jeb.031179).
20. Klok CJ, Hub AJ, Harrison JF (2009) Single and multigenerational responses of body mass to atmospheric oxygen concentrations in Drosophila melanogaster.
41. Lane N (2002) Oxygen: The Molecule that made the World. Oxford: Oxford University Press.

42. Owerkowicz T, Elsey RM, Hicks JW (2009) Atmospheric oxygen level affects growth trajectory, cardiopulmonary allometry and metabolic rate in the American alligator (Alligator mississippiensis). J Exp Biol 212: 1237–1247. (doi:10.1242/jeb.023945).

43. Grimaldi D, Engel MS (2005) Evolution of the Insects. New York: Cambridge University Press.

44. Nel AN, Fleck G, Garraste R, Gaud G (2008) The Odonatoptera of the Late Permian Lodeve Basin (Insecta). J Iberian Geol 34: 115–122.

45. Okajima R (2008) The controlling factors limiting maximum body size of insects. Lethaia 41: 423–430. (doi:10.1111/j.1502-3931.2008.00094.x).

46. Chapelle G, Peck LS (2004) Amphipod crustacean size spectra: new insights in the relationship between size and oxygen. Oikos 106: 167–175. (doi:10.1111/j.0030-1299.2004.12994.x).

47. Rudkin DM, Young GA, Elias RJ, Dobrzanski EP (2003) The world’s biggest trilobite – *Isotelus rex* new species from the Upper Ordovician of northern Manitoba, Canada. J Paleont 77: 99–112.

48. Braddy SJ, Poschmann M, Teide OE (2008) Giant claw reveals the largest ever arthropod. Biol Lett 4: 106–109. (doi: 10.1098/rsbl.2007.0491).

49. Berner RA, VanderBrooks JM, Ward PD (2007) Oxygen and Evolution. Science 316: 557–558.

50. ASTM (1980) Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians. In: American standard for testing and materials, Philadelphia, Pennsylvania, USA. pp 279–290.

51. Terblanche JS, Deere JA, Clusella-Trullas S, Janion C, Chown SL (2007) Critical thermal limits depend on methodological context. Proc R Soc Lond B 274: 2935–2942. (doi:10.1098/rspb.2007.0985).

52. Peck LS, Clark MS, Morley SA, Massey A, Rosetti H (2009) Animal temperature limits and ecological relevance: effects of size, activity and rates of change. Funct Ecol 23: 248–256. (doi: 10.1111/j.1365-2435.2008.01537.x).