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RESEARCH ARTICLE

Effects of body size on the oxygen sensitivity of dragonfly flight

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ABSTRACT

One hypothesis for the small size of insects relative to vertebrates, and the existence of giant fossil insects, is that atmospheric oxygen levels constrain insect body sizes because oxygen delivery is more challenging in larger insects. This study tested this hypothesis in dragonflies by measuring the oxygen sensitivity of flight metabolic rates and behavior during hovering for 11 species of dragonflies that ranged in mass by an order of magnitude. We measured flight times and flight metabolic rates in seven oxygen concentrations ranging from 30% to 2.5% to assess the sensitivity of their flight to atmospheric oxygen. We also assessed the oxygen sensitivity of flight in low-density air (nitrogen replaced with helium) in order to increase the metabolic demands of hovering flight. Lowered atmospheric densities did induce higher flight metabolic rates. Flight behavior was more sensitive to decreasing oxygen levels than flight metabolic rate. The oxygen sensitivity of flight metabolic rates and behaviors were not correlated with body size, indicating that larger insects are able to maintain an oxygen supply-to-demand balance even during flight.

KEY WORDS: Dragonfly, Flight, Hyperoxia, Hypoxia, Insect, Respirometry

INTRODUCTION

Why are insects so small compared with vertebrates? Several possible explanations have been proposed. Smaller body sizes may be adaptive as a result of competition with or predation by birds, reptiles and mammals (Damuth, 1981; Blackburn and Gaston, 1994; Clapham and Karr, 2012). Alternatively, some non-adaptive mechanistic constraints on insect size may occur. The exoskeletons of insects may not be able to support large bodies because of scaling problems (Price, 1997), and/or the lack of anaerobic capacities may enforce an upper size limit because of reduced maximal power output (Marden, 1994). In recent years, it has been shown that insect gigantism in the late Paleozoic coincided with atmospheric hyperoxia; this finding stimulated the hypothesis that insect size is constrained by the ability to supply sufficient oxygen to active tissues (Graham et al., 1995). Recent examination of over 10,000 insect fossils demonstrates that maximum insect size correlates strongly with atmospheric oxygen level during the major oxygen rise and fall that occurs in the Carboniferous and Permian, but is then independent of atmospheric oxygen later in evolutionary time, with decreases in size associated with the evolution of flying competitors [birds and bats (Clapham and Karr, 2012)]. The mechanisms responsible for oxygen-linked changes in insect body size appear to be complex and variable across species (Harrison et al., 2010). One possible mechanism is that oxygen delivery could be more challenging for larger insects; therefore, a higher partial pressure of oxygen in the atmosphere would allow larger insects to exist and function (Graham et al., 1995). Insects deliver oxygen in the gas phase through the tracheal system. Diffusion is an important component of tracheal oxygen delivery, and thus larger size could be associated with increasing diffusive limitations on oxygen delivery, unless augmented by convection.

The experiments that have been conducted to test the hypothesis that insect body size is constrained by oxygen availability have yielded mixed results. Some single- (Greenberg and Ar, 1996; Frazier et al., 2001; Peck and Maddrell, 2005; Klok et al., 2010) and multi-generation studies (Henry and Harrison, 2004; Klok et al., 2010; Harrison and Haddad, 2011) have shown that there can be a positive correlation between insect mass and rearing oxygen level, although the most common finding is that hypoxia reduces size and hyperoxia has little effect. Such experiments examining the effect of atmospheric oxygen on growth and size have not explicitly addressed whether the growth of larger insects is more sensitive to oxygen relative to smaller insects. However, the observation that the size of 1 mg *Drosophila* decreases strongly with hypoxia (Peck and Maddrell, 2005; Harrison and Haddad, 2011) while the size of 1 g *Schistocerca* does not (Harrison et al., 2006) provides some evidence against this hypothesis.

Direct tests of whether the physiological and behavioral functions of larger insects are more sensitive to declining oxygen levels have been negative for resting grasshoppers (Greenlee and Harrison, 2004; Greenlee et al., 2007), hopping grasshoppers (Kirkton et al., 2005), resting beetles (Lease et al., 2012) and feeding caterpillars (Greenlee and Harrison, 2005). However, these prior tests for an increase in oxygen sensitivity with body size can be criticized on the basis that they did not examine insects during high rates of aerobic metabolism when the oxygen delivery system is operating near its maximal capacity. During flight, oxygen consumption rates of insects increase 5- to 100-fold, and the safety margin for oxygen delivery decreases (Rascón and Harrison, 2005; Harrison et al., 2006), suggesting that the ideal period in which to test for an increasing sensitivity of larger insects to oxygen would be during flight. Although power output and metabolic rate during flight can vary with speed, load lifting and turning (Dudley, 2002), hovering flight does elicit very high rates of aerobic metabolism relative to rest, and comparisons of flight metabolism and kinematics across species of different sizes have been very useful in discerning size-related patterns of flight physiology (Marden, 1994; Dudley, 2002; Schilder and Marden, 2004; Darveau et al., 2005).

Tests for effects of body size on oxygen sensitivity may differ between intra- and interspecific studies. In intraspecific studies, size variation occurs because of ontogeny (which can have developmental effects that are independent of body size) and variation among individuals (with usually much smaller variance than occurs across species). Interspecific comparisons are the best comparisons for testing for possible physico-chemical constraints of body mass on maximal size, as across species there has been sufficient time for evolution to select for high oxygen delivery rates that match aerobic needs regardless of size. To date, only one study has tested whether larger insects are more oxygen sensitive using a

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comparative approach, and that study examined the size sensitivity of resting metabolic rates (Greenlee et al., 2007).

The present interspecific study looks at the effects of body size on the sensitivity of dragonfly hovering flight to atmospheric oxygen level. The only prior study of oxygen delivery in a flying dragonfly found that hyperoxia stimulated flight metabolic rate, suggesting that dragonflies are highly sensitive to oxygen (Harrison and Lighton, 1998). Ventilation during flight of dragonflies is thought to occur primarily by autoventilation (Weis-Fogh, 1967). Dragonflies have air sacs surrounding the flight muscles; these are deformed by muscle motion during wing strokes, causing air movement. Because autoventilation is strictly coupled to wing-beats and lacks the adjustability of ventilation mechanisms such as abdominal pumping, perhaps dragonflies are more sensitive to changes in atmospheric oxygen than groups that rely heavily on abdominal pumping. The giant Protodonata of the late Paleozoic were morphologically very similar to modern dragonflies (Grimaldi and Engel, 2005), thus it is plausible that extant Odonata and the Paleozoic Protodonata had similar oxygen sensitivities.

We measured behavioral and physiological responses to changes in ambient oxygen concentrations using multiple species of dragonflies that varied by an order of magnitude in body size. Using flow-through respirometry, the flight metabolic rates and hovering durations of individuals were measured in 2.5, 5, 7.5, 10, 15, 21 and 30% O_2, balanced with N_2 (nitrox). The same individuals were also flown in variable oxygen atmospheres in hypodense air using helium as a balance gas (heliox). The use of hypodense air increases the power requirements and metabolic rates during flight (Dudley, 1995; Roberts et al., 2004), and thus might increase the oxygen sensitivity of flight. If larger dragonfly species are more sensitive to changing oxygen levels, then we predicted that the flight performance (metabolic rate, flight duration) of larger species would be more negatively affected by declining oxygen.

RESULTS

Effect of flight bout duration on CO_2 emission rates

Many of the flight bouts were quite short (a few seconds), raising the question of whether the CO_2 emission rates approximated steady-state conditions. To assess this question, we tested the relationship between flight bout duration and the measured CO_2 emission rate associated with that peak for individual animals flown in 21% nitrox. Although CO_2 emission rates were more variable when flight bout durations were short, they were independent of flight bout duration, suggesting that metabolic rates during very short flight bouts of 1–2 s approximate the rates during steady-state flight (Fig. 1).

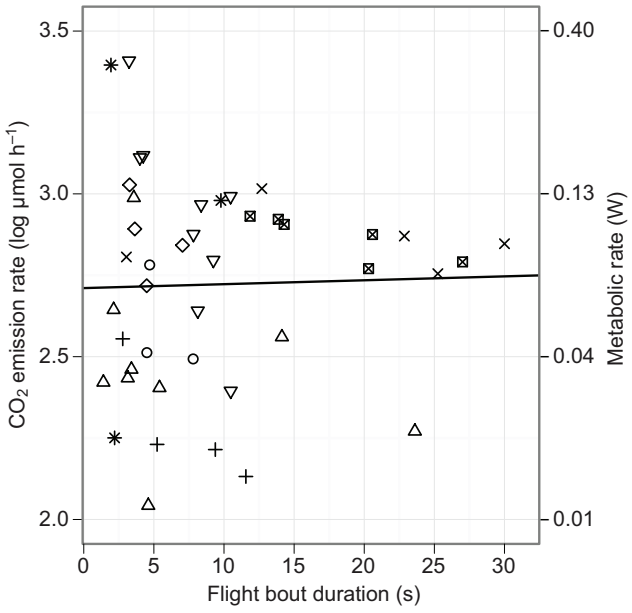

Fig. 1. Carbon dioxide emission rate was independent of the duration of flight bouts in dragonflies. To simplify visualization, only eight animals are shown. Each unique symbol represents a single animal. The regression (using all animals) was not significantly different from zero (a=0.001, b=2.688, $F_{1,606}$=0.153, P=0.696, R^2=0.0002).

Air density effects on flight metabolism and behavior

We first tested for significant interactions between air density, body mass and oxygen (both three-way and pair-wise) on CO_2 emission rate and all of our flight behavior variables. None of these interactions were significant for any of these variables; therefore, these interaction terms were excluded from subsequent statistical analyses.

Dragonflies consistently produced carbon dioxide at a higher rate when hovering in hypodense atmospheres (heliox mixtures; Fig. 2, Table 1). CO_2 emission rates averaged approximately 10% higher when the dragonflies were flown in heliox, but in 2.5% oxygen atmospheres, CO_2 emission rates were 75% higher than in nitrox. The flight bouts were also significantly more frequent in heliox gas mixtures (Fig. 3, Table 1). However, there was no consistent or significant effect of air density on flight bout duration or total flight duration (Figs 4, 5, Table 1).

Oxygen effects on flight metabolism and behavior

There was an overall effect of oxygen concentration on CO_2 emission rates (Table 1). However, at oxygen concentrations equal to or greater than 5%, carbon dioxide production remained relatively constant (*a priori* paired *t*-tests versus 21%: for all oxygen concentrations greater than 5%, P>0.05); while at 2.5% oxygen, metabolic rates dropped (*a priori* paired *t*-tests: nitrox, t=–5.02, d.f.=11.61, P<0.001; heliox, t=–2.91, d.f.=11.10, P=0.01; Fig. 2, Table 1). All measures of flight behavior were highly significantly affected by oxygen level (Table 1), with a general positive correlation between flight behavior and oxygen level (Figs 3–5).

Body mass effects

Within models containing body mass, oxygen level and air density as independent factors, there were significant linear effects of body mass on the number of flight bouts, bout duration and the total flight duration (Table 1), though no significant effect of body mass on CO_2 emission rates during flight (Table 1). To analyze these body mass effects more fully, and independently of the effects of air density and atmospheric oxygen level, we assessed the effect of body mass on each of these parameters in 21% oxygen, in normal density atmosphere (nitrox).

CO_2 emission scaled non-significantly with mass to the 0.44 power. Though the regression model was a poor fit, this slope did not significantly differ from the 2/3 power relationship between mass and metabolic rate typically seen in insects (*t*-test on slopes: t=0.071, d.f.=9, P>0.05).

Using a general linear model, the mass of the dragonfly species did not significantly affect carbon dioxide production during flight, but it did affect measures of flight behavior (Table 1). Though not statistically significant, larger dragonflies generally had longer flight bout durations and longer total flight durations per trial (in oxygen levels greater than 10%) and fewer flight bouts per trial than smaller species (across all oxygen levels).

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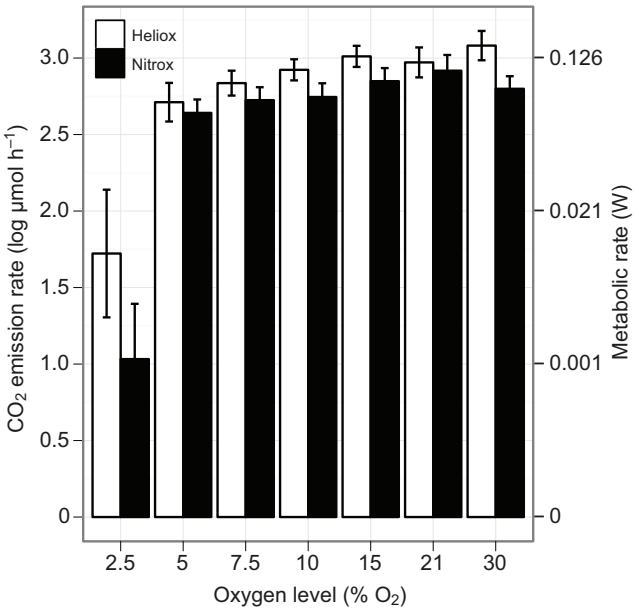

Fig. 2. Carbon dioxide emission versus oxygen level in dragonflies. White bars represent mean emission rates in hypodense air and black bars represent mean emission rates in normodense air (averaged across all species). There were significant effects of air density and oxygen on CO_2 emission rates and metabolic rates (Table 1). Assuming that respiratory quotients remained constant at 1.0, metabolic rates were calculated using an energy equivalence of 1 ml O_2=20.1 J. Data are means ± s.e.m.

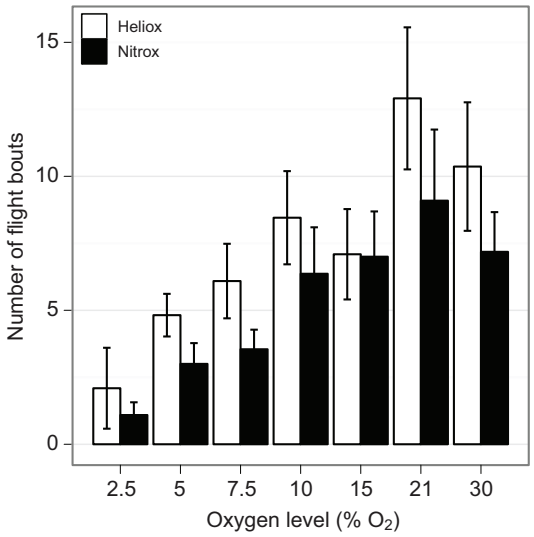

Fig. 3. Frequency of flight versus oxygen level in dragonflies. White bars represent results in hypodense air and black bars represent normodense air. Lower oxygen levels and higher air density both significantly reduced the number of flight bouts per trial (Table 1). Data are means ± s.e.m.

Perchers versus fliers

Perchers and fliers did not differ in their carbon dioxide production rates, mean flight bout durations, total flight time or the number of flight bouts (ANOVAs, all $P>0.05$). Perchers and fliers also did not differ in their oxygen sensitivity of CO_2 production (Fig. 6A), total flight duration per trial (Fig. 6B), number of flight bouts or mean flight bout duration (data not shown).

Effects of body mass on the oxygen sensitivity of flight metabolism and behavior

There were no significant oxygen × mass interactions in the general linear models testing for effects on CO_2 emission rate, number of flight bouts, mean flight bout duration or total flight duration (Table 1), suggesting that dragonflies of different masses responded similarly to oxygen. However, this approach has relatively low power because it does not consider the fact that each individual and species was assessed repeatedly in different oxygen levels. Therefore, to assess response to oxygen levels within species, we plotted the value for each dependent variable for that species versus oxygen and calculated the slope of a linear regression, assessing nitrox and heliox data separately (slopes for total flight duration are shown in Fig. 7). These slopes were then plotted versus mass to test whether larger animals were more responsive to varying oxygen level.

In both normobaria and hypobaria, there was no significant correlation between dragonfly mass and the oxygen sensitivity of each of the tested variables (Fig. 8). This conclusion did not change if we adjusted the analyses for phylogenetic relatedness.

DISCUSSION

Our study is the first to test for an effect of body mass on the oxygen sensitivity of metabolic rate and behavior during aerobic flight for

Table 1. ANOVA results demonstrating the impact of gas density, oxygen content or species body mass on $\log_{10}$ CO_2 emission rate, number of flight bouts ($\sqrt{N}$), mean flight duration ($\log_{10}$ s) and total flight duration ($\log_{10}$ s)

Dependent variable	Source of variation	d.f.	SS	MS	*F*	*P*
CO_2 emission rate ($\log_{10}$ µmol h^{-1})	Density	1	2.061	2.061	4.242	**0.041**
	Oxygen	1	14.897	14.897	30.663	**<0.001**
	Mass	1	0.167	0.167	0.344	0.558
	Total	149	72.386	0.486		
Number of flight bouts ($\sqrt{N}$)	Density	1	5.503	5.503	4.705	**0.032**
	Oxygen	1	46.86	46.86	40.06	**<0.001**
	Mass	1	9.951	9.951	8.507	**0.004**
	Total	150	175.462	1.17		
Mean flight duration ($\log_{10}$ s)	Density	1	0.002	0.002	0.412	0.522
	Oxygen	1	0.07	0.07	13.054	**<0.001**
	Mass	1	0.102	0.102	19.091	**<0.001**
	Total	134	0.714	0.005		
Total flight duration ($\log_{10}$ s)	Density	1	0.025	0.025	3.6937	0.057
	Oxygen	1	0.34	0.34	50.226	**<0.001**
	Mass	1	0.031	0.031	4.641	**0.033**
	Total	135	0.9	0.007		

There were no significant two-way or three-way interaction terms in any of these models. Bold indicates significance with $P<0.05$.

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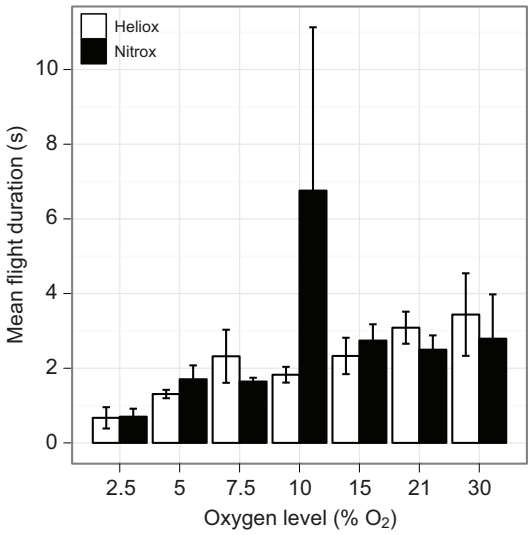

Fig. 4. Mean flight duration versus oxygen level in dragonflies. White bars represent species mean flight times in hypodense air and black bars represent flight times in normodense air. Lower oxygen levels reduced flight bout durations, but durations were not significantly affected by air density (Table 1). Data are means ± s.e.m.

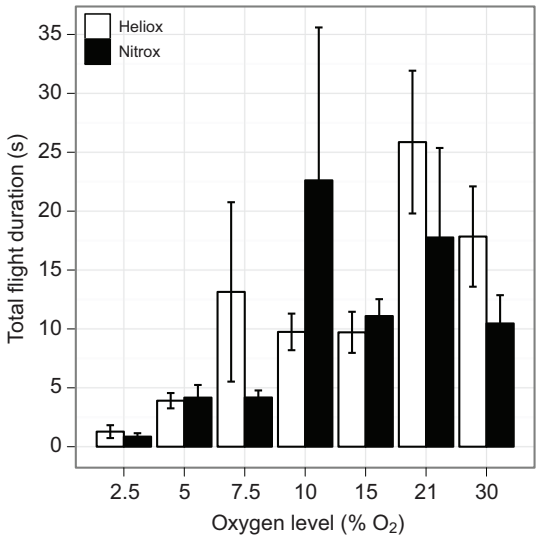

Fig. 5. Total flight duration versus oxygen level in dragonflies. White bars represent the total time spent in flight in hypodense air and black bars represent flight times in normodense air. Hypoxia reduced total flight duration, but there was not a significant effect of air density (Table 1). Data are means ± s.e.m.

any animal species. In contrast to predictions based on the hypothesis that larger insects are increasingly oxygen limited, but consistent with prior results using resting animals, the oxygen sensitivity of flight metabolic rate and behavior were independent of mass. However, regardless of size, the flight behavior but not the metabolic rate of dragonflies was highly sensitive to changes in atmospheric oxygen content. This strategy of reducing flight duration and the number of flight attempts in response to hypoxia may allow flight metabolic rates to remain sufficiently high to support limited flight in all but the most hypoxic environments.

Reliability of the flight CO_2 emission rates and sample size issues

The reluctance of most dragonfly species to fly for a long duration in a respirometry chamber provides a major technical challenge, and has limited prior attempts to measure dragonfly flight metabolism. Flight durations were quite short in this study (averaging 2.9±0.36 s) because most of the dragonfly species (though able to hover) were not willing to maintain longer flights in the chamber. Also, our methods required integrating CO_2 peaks that extended temporally well beyond the flight periods because the relatively large chambers required to permit flight required relatively long equilibration times (Fig. 9). One study measured much longer flight durations for one dragonfly species (Harrison and Lighton, 1998); however, that target species was specifically chosen for its long flight durations in a respirometer. The short flight durations and relatively long chamber equilibration times raises the question of whether the oxygen sensitivity of flight and CO_2 emission rates can be measured accurately.

Unlike vertebrates, insects rely almost exclusively on aerobic metabolism to fuel flight (Beenakkers et al., 1985), so it is unlikely that flights of several seconds were sustained by non-aerobic catabolism. One possible explanation for the low sensitivity of flight to hypoxia would be if oxygen stores within the body serve as significant reservoirs of oxygen during these flights. There are no reported measurements of tracheal volumes for dragonflies. We examined several X-rays of different species of dragonflies at Argonne National Laboratory; for those few observations, we visually estimated that tracheal volumes approximated 30% of body volume. To estimate the duration of flight possible using internal oxygen stores, we estimated that tracheal oxygen level would be minimally 1% below air values, and that flight must stop when tracheal oxygen levels drop to 2%, as flight behavior was minimal at 2.5% (Figs 3–5). Using our average CO_2 emission rates of 907 μmol h^{-1}, and assuming a respiratory quotient of 1, we estimated that when dragonflies are flying in 21, 10 and 5% air, the tracheal oxygen stores could maximally support 7.4, 2.8 and 0.8 s of flight, respectively. These durations are relatively long compared with the 2 s flight bouts observed in many cases; thus it is plausible that use of internal oxygen stores may have blunted the oxygen sensitivity of the CO_2 emission rate, perhaps explaining the ability of dragonflies to sustain similar metabolic rates in atmospheres with as low as 5 kPa O_2. However, we found no relationship between flight duration and flight CO_2 emission rate within individuals, even comparing flights lasting only 1–2 s with flights lasting over 20 s (Fig. 1), suggesting that most of our flights did represent steady-state conditions, even though they were of quite short durations. In addition, as the estimated 0.8 s of flight is less than we observed in 5% oxygen, the use of oxygen from internal stores cannot be completely responsible for the observed flight under these conditions.

Also supporting the reliability of our measured CO_2 emission rates, our estimated metabolic rates (range 0.05–0.38 W) were in the range of previously reported values. Polcyn reported oxygen consumption rates for four of the same species we used (*Anax junius*, *Aeshna multicolor*, *Libellula saturata* and *Tramea lacerata*); these metabolic rates ranged from 0.24 to 0.43 W (Polcyn, 1994). A flight metabolic rate of 0.12 W was reported for the dragonfly *Erythemis simplicicollis* (Harrison and Lighton, 1998), falling in the range of our values. Using heat loss models, the metabolic rate for the dragonfly *Sympetrum sanguineum* was 0.04 W (Wakeling and Ellington, 1997), while *A. junius* had a rate of 0.37 W (May, 1995), bracketing our range of estimates. In sum, these analyses suggest that our flow-through respirometry system was able to accurately assess the in-flight CO_2 emission rates of these dragonflies.

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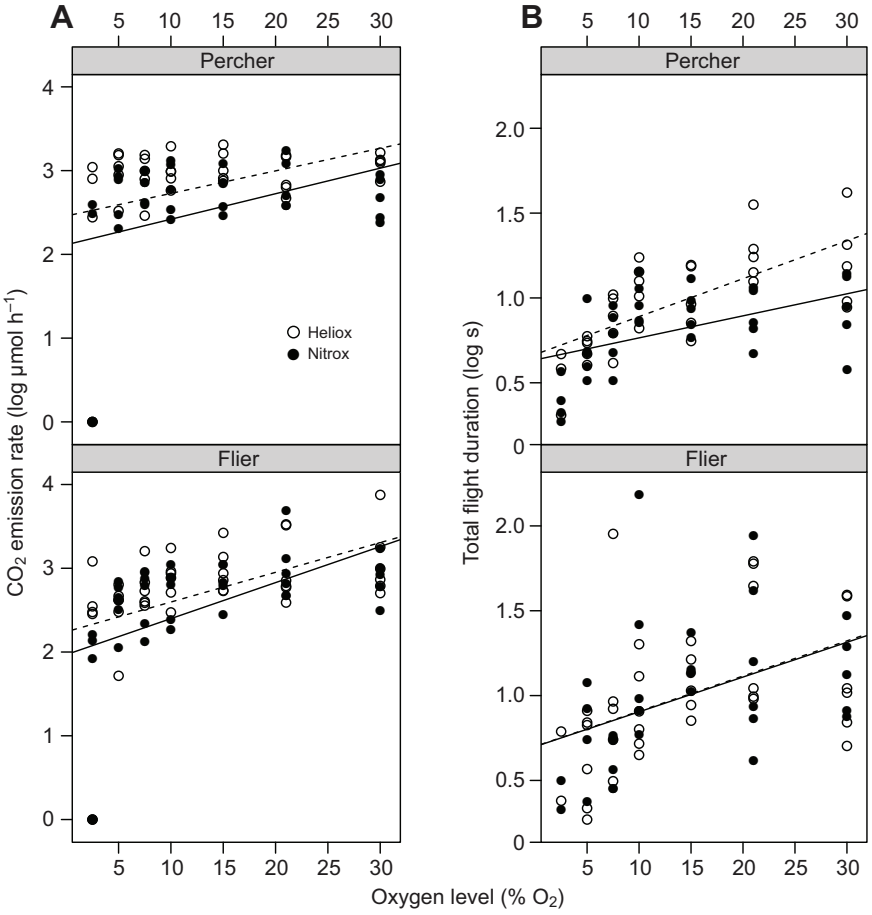

Fig. 6. Carbon dioxide emission and total flight duration versus oxygen level in dragonflies. Both CO_2 emission rates (A) and total flight durations (B) increased with higher oxygen levels. Perchers and fliers did not differ significantly in regression slopes or intercepts. Solid lines indicate nitrox regressions; dashed lines indicate heliox regressions.

Inducing maximal flight power output by using hypodense air

As found by previous researchers, hypodense air increases the metabolic rate of flying insects (Roberts et al., 2004), probably because of the increase in power production required to generate lift in hypodense air (Dudley, 1995). Orchid bees increase power output by 45% when hovering in heliox (Dudley, 1995). In carpenter bees, flight in heliox increased power output by 10% and metabolic rate by 33% compared with flight in nitrox. In our study, the average increase in CO_2 emission rate was only approximately 10% higher in heliox than nitrox (Fig. 2), suggesting that lowering the air density may not affect the power requirement for flight as much in dragonflies as bees. The lower wing beat frequencies, smaller stroke amplitudes and reduced wing-loading of dragonflies relative to bees could cause them to be less affected by lowering air density.

Oxygen sensitivity of flight metabolism and behavior

A striking result, similar to a prior study of tethered flight in grasshoppers (Rascón and Harrison, 2005), is that flight behavior was much more sensitive to hypoxia than flight metabolic rate. Total flight durations declined 2- to 6-fold as oxygen level declined (Figs 5, 7), with a large part of this effect due to the number of bouts attempted (Fig. 3). These data suggest that hypoxia suppresses flight motivation. Reduced locomotor performance by humans in hypoxia is also believed to be partially explained by hypoxic suppression of central nervous system drive (Amann et al., 2006; Noakes, 2009).

In contrast to the relatively strong sensitivity of flight behavior to hypoxia, the CO_2 emission rates of flying dragonflies tended to be quite insensitive to oxygen levels over a broad range (Fig. 2, Fig. 6A), with significant declines only observed in 2.5% oxygen (Fig. 2). Thus dragonflies seem able to achieve adequate oxygen delivery to their flight muscles to support flight muscle behavior and metabolism in relatively extreme hypoxia, at least during short flights. Dragonflies seem significantly less sensitive to hypoxia than bees, which showed decreased wing-beat frequency and CO_2 emission rate at 7.5% oxygen and below (Joos et al., 1997), perhaps because of their lower wing-loading.

The conclusion that flight metabolic rate of dragonflies is relatively insensitive to atmospheric oxygen level differs from prior findings for the dragonfly *E. simplicicollis* (Harrison and Lighton, 1998). For this species, it was found that hyperoxia stimulated CO_2 emission rates during flight by approximately 10%, and moderate hypoxia (10% O_2) significantly decreased flight metabolic rate by 18%. One obvious explanation for the difference in results between these two studies is the relatively low power of this comparative study to detect changes in CO_2 emission rate in response to oxygen. The *E. simplicicollis* study measured 25 individuals at each oxygen level (Harrison and Lighton, 1998); in the present study, we used species means for 11 species as our data set. In addition to the smaller samples sizes, variation is expected to be greater across rather than within species, leading to a much greater power to detect significant, within-species changes in flight parameters with oxygen. In contrast, both studies support the general observation that atmospheric oxygen can be dropped fourfold with relatively minor changes in flight metabolic rate. It would be very interesting to explore the mechanisms by which dragonflies and other insects are able to maintain gas exchange rates despite strong drops in the oxygen content of the atmosphere. Although it has been reported that autoventilation due to wing movement predominates in producing gas exchange in dragonflies (Weis-Fogh, 1967), our

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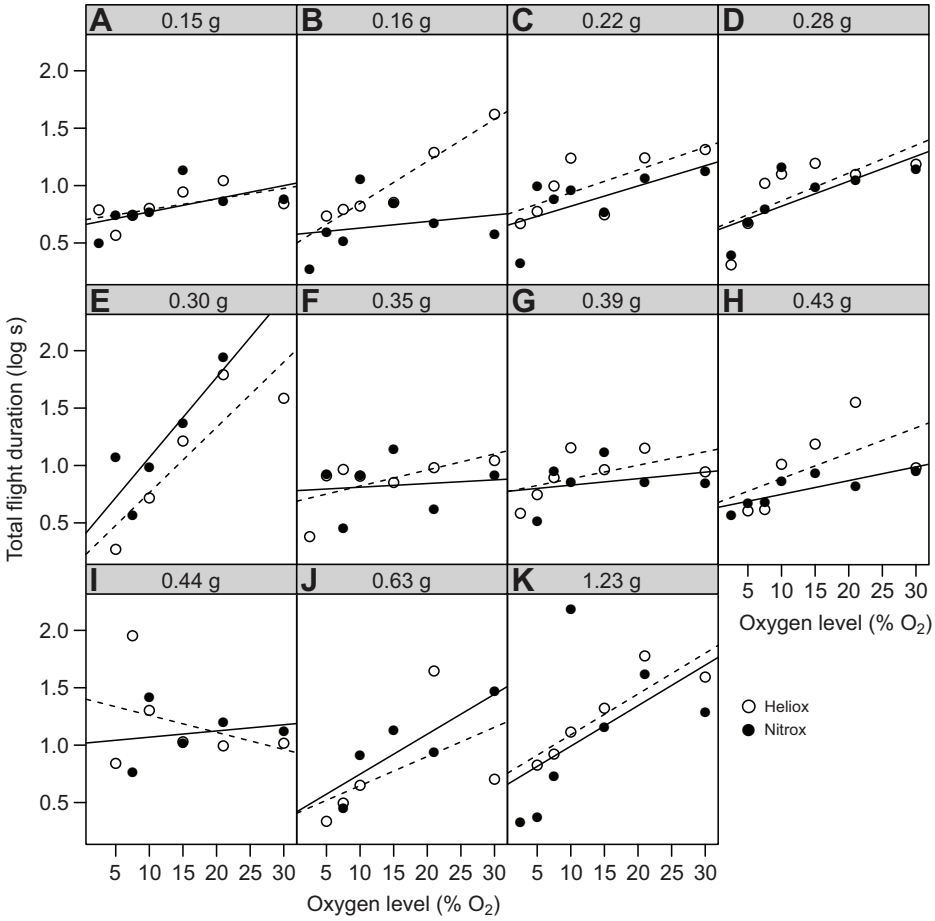

Fig. 7. Representative graphs showing the linear regressions performed within each species for total flight duration per trial versus oxygen for dragonfly species of different masses. (A) *Pantala flavescens*, (B) *Pachydiplax longipennis*, (C) *Macrodiplax balteata*, (D) *Libellula luctuosa*, (E) *Pantala hymenaea*, (F) *Tramea onusta*, (G) *Libellula comanche*, (H) *Libellula saturata*, (I) *Tramea lacerata*, (J) *Aeshna multicolor* and (K) *Anax junius*. The resultant slope from each graph was recorded and used to test whether there was an interaction between each dependent variable and mass. There was no significant effect of air density (Table 1) or interaction between air density and oxygen level. Solid lines indicate nitrox regressions; dashed lines indicate heliox regressions.

personal observations of dragonflies using X-ray synchrotron imaging suggest that dragonflies use abdominal pumping during flight. In honeybees, increases in evaporative water loss during flight in hypoxia suggest that convection is stimulated by hypoxia (Joos et al., 1997); perhaps this is also true in dragonflies.

Effects of body size on the oxygen sensitivity of CO_2 emission rates and flight behavior

The CO_2 emission rates, number of flight bouts, mean flight durations and total flight durations of large and small dragonflies responded similarly to atmospheric oxygen level; there was no significant interaction between body mass and oxygen on metabolic rate (Table 1, Fig. 8). These results suggest that large and small flying dragonflies have similar safety margins for oxygen delivery, which is similar to what has been observed with resting (Greenlee and Harrison, 2004; Greenlee et al., 2007) and hopping grasshoppers (Kirkton et al., 2005), resting beetles (Lease et al., 2012) and feeding caterpillars (Greenlee and Harrison, 2005). The finding that oxygen sensitivity is independent of size during flight is particularly important because the oxygen demand of insects is highest during flight.

Oxygen as a constraint on insect body size

Our study contributes to the increasing body of evidence that large extant insects have oxygen sensitivities similar to those of smaller insects, and thus are not particularly physiologically or behaviorally constrained by atmospheric oxygen. These data suggest that the giant insects of the Paleozoic (and the fact that extant insects are smaller than vertebrates) cannot be explained by a simple, direct stimulation of aerobic metabolism in tracheal systems by higher atmospheric oxygen levels. However, several studies suggest that atmospheric oxygen level is negatively correlated with size and number of tracheae (Henry and Harrison, 2004; Jarecki et al., 1999; Loudon, 1989), and other studies indicate that larger insects invest proportionally more of their bodies to the tracheal system (Greenlee et al., 2009; Lease et al., 2006; Kaiser et al., 2007). Thus, insect body size might be limited by trade-offs associated with the need for insects to devote more resources to the respiratory system when oxygen levels are lower (Harrison et al., 2010).

Other hypotheses unrelated to oxygen may also explain why insects are frequently small relative to other terrestrial animals, and have smaller maximum sizes now relative to some fossilized insects from the Paleozoic. The wall thickness of an insect's exoskeleton may need to increase faster than its diameter, imposing a limit before the cuticle buckles (Price, 1997). Low temperatures during the late Carboniferous (Royer et al., 2004) could have also contributed to the evolution of larger species of insects in the past (Kingsolver and Huey, 2008). From an ecological standpoint, niche competition and predation may have also led to the reduction of insect sizes as larger, more successful vertebrates evolved (Damuth, 1981; Blackburn and Gaston, 1994; Clapham and Karr, 2012). A major challenge for evolutionary biologists is to find approaches to distinguish these alternative hypotheses.

MATERIALS AND METHODS

Animals and study sites

Eleven species of dragonflies (Table 2) were collected from the Soda Springs Desert Studies Center at Zzyzx, CA, USA, which is located at the western

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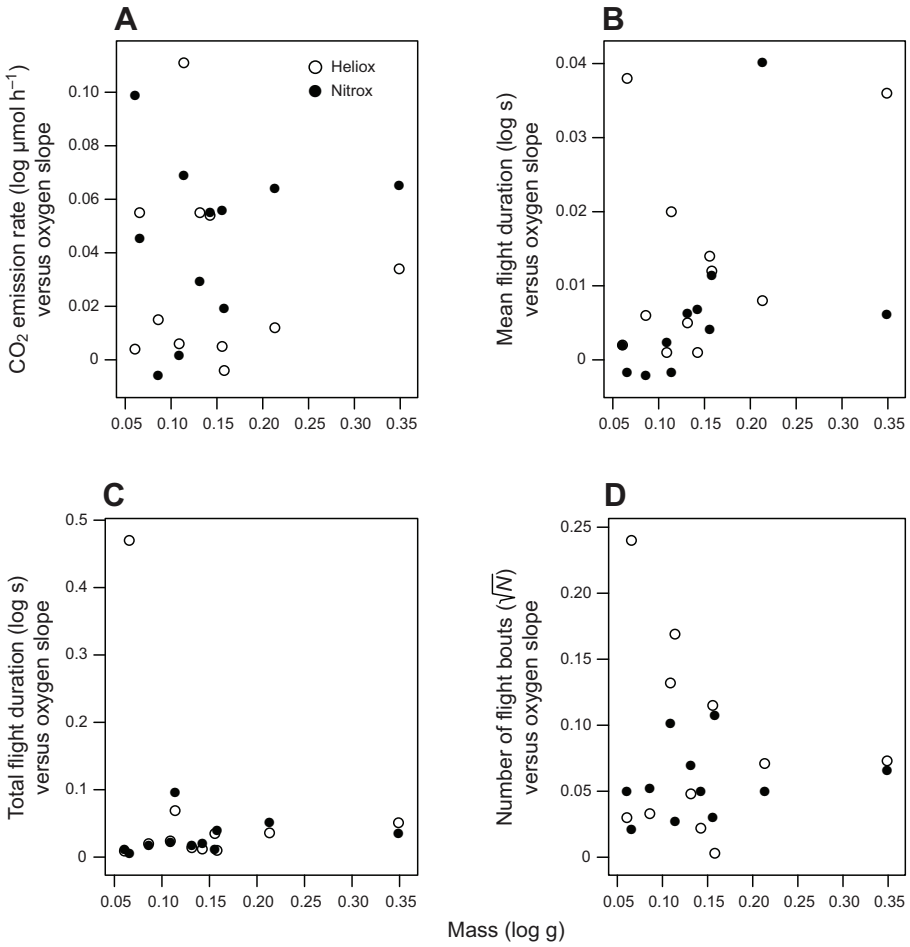

Fig. 8. The oxygen sensitivity of CO_2 emission rates and flight behaviors of larger dragonflies were not significantly related to body mass in either normobaric or hypobaric atmospheres. (A) CO_2 emission rate. (B) Mean flight duration. (C) Total flight duration. (D) Number of flight bouts. Phylogenetically uncorrected data are shown, with oxygen sensitivity (slope of a linear regression fit of the parameter on atmospheric O_2) plotted versus species mass. All fits of the oxygen sensitivity versus mass regressions were poor (R^2<0.1) and slopes did not differ from zero.

end of the Mojave National Preserve (35°08′35″N, 116°06′15″W). A man-made, spring-fed pond (Lake Tuendae) supports at least 13 species of dragonflies (Polcyn, 1994). As different dragonfly species emerge as adults during different summer months, we conducted three collecting trips – one in July 2004, one in May 2005 and a final trip in August 2005.

Oxygen effects on flight metabolic rates and performance were measured within 5 min of collecting the animal, at an outdoor respirometry setup located next to Lake Tuendae. The wet body mass (±0.001 g) of all captured animals was measured using an analytical balance (Mettler AE100; Mettler Toledo, Columbus, OH, USA).

Roughly half of the species captured tended to patrol the lake continuously throughout the day and were categorized as ‘fliers’, whereas the remaining species, categorized as ‘perchers’, preferred to sit on vegetation and only flew when actively hunting or attempting to mate (Heinrich and Casey, 1978). Activity levels, coloration and the amount of wing wear indicated that all animals were mature adults.

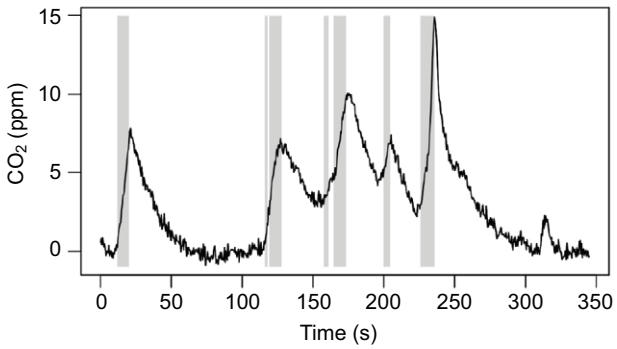

Fig. 9. Representative trace of the CO_2 content of excurrent air sampled from the flight chamber. This particular example shows the CO_2 produced by the darner, *Anax junius*, in 30% nitrox. Each flight bout duration is represented by the width of the light gray bars.

Experimental design

The power of interspecific comparative analyses depends on the phylogeny of the sampled species, number of species used, and, for questions of mass effects, on the range of species masses (Harvey and Pagel, 1991). Because many measurements were made on individuals (flight behavior and metabolic rate in 14 different gas mixes plus some additional controls), several hours were required to complete measurements for a single individual. In addition, some species were relatively rare and difficult to collect. Thus we decided to focus on obtaining the maximum number of species and clades, and our study had a relatively low number of individuals per species (Table 2). In general, we collapsed individual data to species means; unless otherwise stated, reported values are averages across individuals of that species.

Individual dragonflies were captured with a net or by hand, and transferred to the clear plastic flight chamber, which was also a flow-through respirometry system. A 4 l chamber was used, which allowed unimpeded free flight, which generally produces higher metabolic rates than tethered flight (Kammer and Heinrich, 1978), although some species did occasionally fly into the chamber walls (see supplementary material Movie 1 for typical flight behavior in the chamber). After allowing 5 min for the dragonfly to equilibrate to the experimental atmosphere, a video recording of the flight chamber was initiated, and, if necessary, flight was induced by gently shaking the flight chamber. CO_2 released during flight activity was recorded (Fig. 9). If animals did not fly, the chamber was shaken relatively continuously for at least 2 min. If the animals never achieved lift during those 2 min, they were not recorded as flying. Flight

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Table 2. Mean masses, CO_2 emission rates and behavior characteristics for each species stimulated for ~3 min to fly in 21% oxygen balanced with nitrogen

Species	Category	Number and sex of animals	Mass (g)	CO_2 emission rate (μmol h^{-1})	Mean flight duration per bout (s)	Total flight time (s)	Number of flight bouts
Aeshna multicolor	F	1 male	0.6338	350.29	4.54	13.61	3
Anax junius	F	1 male	1.2329	1015.44	4.77	38.13	8
Libellula comanche	P	1 male	0.3882	1206.54	2.74	10.95	4
Libellula luctuosa	P	2 males, 1 female	0.2847±0.06 (0.1558–0.3597)	348.93±127.32 (128.66–569.71)	1.36±0.12 (1.12–1.50)	11.49±4.50 (2.9–18.08)	9±3
Libellula saturata	P	3 males	0.4311±0.03 (0.3767–0.4521)	1210.80±273.15 (719.71–1663.60)	4.23±1.82 (1.75–7.78)	34.49±24.62 (5.24–85.54)	6±2
Macrodiplax balteata	P	1 male	0.2189	582.10	2.21	11.03	5
Pachydiplax longipennis	P	3 males, 1 female	0.1631±0.02 (0.1105–0.2020)	475.96±115.72 (209.04–765.91)	2.75±1.86 (0.89–4.61)	11.9±6.54 (5.36–18.44)	5±1
Pantala flavescens	F	3 males	0.1496±0.03 (0.0913–0.1970)	474.91±207.87 (207.07–884.19)	1.40±0.18 (1.25–1.75)	10.05 ±3.41 (6.26–16.85)	8±3
Pantala hymenaea	F	1 male	0.2997	2909.53	2.57	25.67	10
Tramea lacerata	F	1 male	0.4387	742.13	2.95	8.86	3
Tramea onusta	F	2 males	0.3534±0.003 (0.3508–0.3559)	660.64±290.64 (370.00–951.27)	1.90±0.31 (1.59–2.21)	8.58±4.16 (4.42–12.73)	5±3

If multiple animals from the same species were tested, s.e.m. values and ranges are included. F, flier; P, percher.

performance and CO_2 emission rates were measured in test gases of 2.5, 5, 7.5, 10, 15, 21 and 30% oxygen, with the balance gas being either nitrogen (nitrox) or helium (heliox). Each animal was flown in all of the gas mixtures; whether the individual was first flown in nitrox or heliox mixes was alternated through the study. The order in which the animal was exposed to the differing oxygen levels was randomly determined. A subset of animals was flown in normoxia before switching to each new test gas to test for degradation in performance over time. Although a *t*-test comparing these animals' CO_2 emissions during their first and last flights in 21% normoxia showed that there was a significant difference (t=–4.153, d.f.=39.481, P=0.0002), metabolic rates were not reduced. Instead, the mean CO_2 emission rate was higher for the final flight; thus there was no evidence for degradation of flight performance of individuals over the trial.

Video analysis of behavior

The video camera's angle of view encompassed the entire flight chamber (see supplementary material Movie 1); a flight bout was defined as a continuous period of time when the wings were active and animals were off the chamber floor. Flight bout durations were measured using frame-by-frame analysis of video taken by a digital video recorder (ZR series; Canon, Melville, NY, USA). Video images were shot at a standard rate of 30 frames s^{-1}; thus, measured flight durations 0.03 s could be detected. Total flight duration during the 3 min test bout was calculated as the sum of the duration of all flight bouts in that test oxygen.

Respirometry

The flow rate of air through the flight chamber (constant at 16.2±0.1 l min^{-1}) and the oxygen concentration of the mixture were regulated by mass flow controllers and meters (Omega, Stamford, CT, USA). The flow rate was chosen to reduce washout effects and improve temporal resolution (95% equilibration time was less than 1 min) while keeping flows low enough so that the CO_2 content of the excurrent air could be accurately determined. Our oxygen analyzer was not sufficiently precise to measure oxygen consumption rates during flight; the oxygen readings were instead used to confirm the gas mixes. Excurrent air from the chamber was dumped into a manifold from which the air was subsampled at a rate of 500 ml min^{-1}. The subsampled air was first dried (magnesium perchlorate) and then pulled sequentially through a CO_2 analyzer (LI-6252; Li-Cor, Lincoln, NE, USA), Ascarite II (for CO_2 removal) and then an O_2 analyzer (FOXBOX, Sable Systems, Las Vegas, NV, USA) by a pump (R-1; AMETEK, Pittsburg, PA, USA). The output of both analyzers was digitized and recorded using Sable Systems DATACAN. The metabolic rates during flight were calculated by integrating the area under each CO_2 emission peak (Fig. 9) that corresponded to a burst or closely timed burst of flight, and dividing by the time spent in flight as determined using the video recording of behavior.

The flight chamber was housed in a temperature-controlled environment to reduce the effects of temperature on metabolic rate. The temperature was maintained at 31.6±0.1°C by monitoring the temperature within a 0.76×0.76×0.91 m wood-framed, Plexiglas chamber and adjusting the output from an attached air conditioner accordingly. The barometric pressure at Zzyzx during the course of the experiment was 101.07±0.04 kPa (286 m elevation).

Statistical analyses

We tested for general effects of oxygen and air density on our dependent parameters (flight CO_2 emission rate, number of flight bouts, flight bout duration, total flight duration) with general linear models using oxygen, air density and body mass as independent factors. For CO_2 emission rates and number of flight bouts, all species and individuals were tested at all oxygen levels and air densities. For flight bout durations and total flight time, many animals did not fly in 2.5% oxygen, so the sample sizes were reduced for these variables. We first tested for three-way interaction terms, and then two-way interaction terms. If these higher-level terms of the model were insignificant, they were dropped from the model, which was then rerun. To avoid problems with oversampling, all measurements of our dependent parameters were first collapsed into an individual mean and then all measurements within a species were further collapsed into a species mean.

To assess the oxygen responsiveness of dragonflies, we plotted the dependent variables versus oxygen for each species and calculated the linear slope. We then tested whether these slopes (oxygen responsiveness) were statistically related to body mass using linear regression.

Because observed differences in metabolic rate and behavior may be affected by phylogenetic relatedness in addition to physical size differences, phylogenetically independent contrasts were calculated for each of the independent and dependent variables used in this study using the ape package in R (Paradis, et al., 2004; Felsenstein, 1985).

To calculate the phylogenetically independent contrasts, a supertree (Fig. 10A) that included all of the species tested at Zzyzx was constructed by combining two other trees (Saux et al., 2003; Ware et al., 2007) using a strict supertree algorithm (Sanderson et al., 1998). A second tree was generated that assumes that all libellulid species were equally related to each other in a monophyletic clade, while keeping the aeshnids separated (Fig. 10B). A third tree was constructed using random branch lengths and relatedness (Fig. 10C).

All statistical analyses were carried out using R language (R Development Core Team, 2010; http://www.R-project.org/); graphs were generated using

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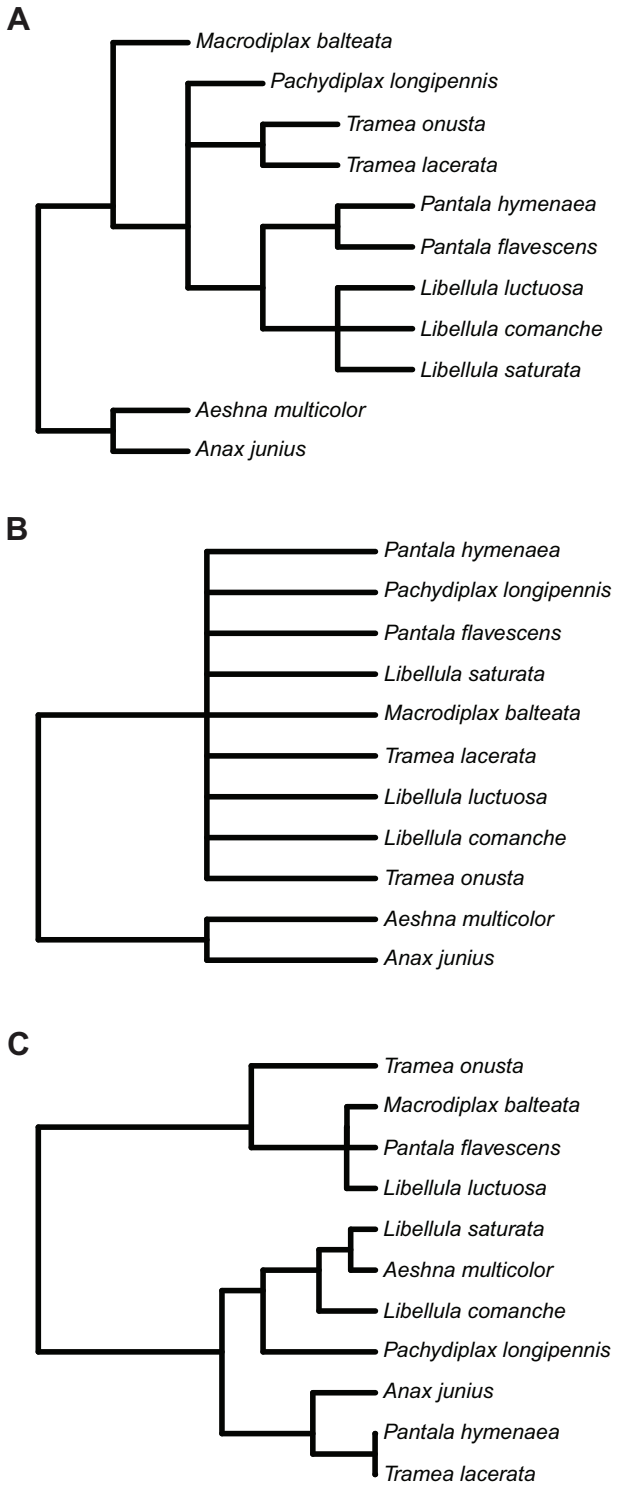

Fig. 10. The three phylogenetic trees used to calculate phylogenetically independent contrasts. (A) Supertree, (B) simple tree dividing dragonflies into two clades, and (C) random branch lengths and relatedness.

the ggplot2 and lattice packages (Wickham, 2009; Sarkar, 2008). Results were determined to be significantly different from the null hypothesis using an experimental type I error less than or equal to 5%. Analysis of covariance and linear regressions were used in the analysis of mass effects on metabolic rates and flight behaviors. Data were log transformed prior to running parametric tests. All values are shown as means ± s.e.m. unless otherwise noted.

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Competing interests
The authors declare no competing financial interests.

Author contributions
Both authors designed this project. J.R.H. collected data and performed all analyses. Both authors wrote the manuscript and approved the final version.

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