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RESEARCH ARTICLE

Body size is not critical for critical P_{O_2} in scarabaeid and tenebrionid beetles

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SUMMARY

Constraints on oxygen delivery potentially limit animal body size. Because diffusion rates are highly distance dependent, and because tracheal length increases with size, gas exchange was traditionally thought to be more difficult for larger insects. As yet the effect of body size on critical oxygen partial pressure (P_{crit}) has not been measured for any clade of insect species for which there are interspecific data on tracheal scaling. We addressed this deficiency by measuring P_{crit} over a 4150-fold mass range (ratio of largest to smallest species mean) of two families of Coleoptera (Tenebrionidae and Scarabaeidae). We exposed adult beetles to progressively lower oxygen levels and measured their ability to maintain CO_2 release rates. Absolute metabolic rates increased hypometrically with beetle body mass (M) at both normoxic ($M^{0.748}$) and hypoxic ($M^{0.846}$) conditions. P_{crit}, however, was independent of body size. Maximum overall conductances for oxygen from air to mitochondria ($G_{O_2,max}$) matched metabolic rates as insects became larger, likely enabling the similar P_{crit} values observed in large and small beetles. These data suggest that current atmospheric oxygen levels do not limit body size of insects because of limitations on gas exchange. However, increasing relative investment in the tracheal system in larger insects may produce trade-offs or meet spatial limits that constrain insect size.

Key words: Coleoptera, allometry, insect, oxygen delivery, respiration, trachea, scaling, metabolism.

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INTRODUCTION

Oxygen delivery is important for most animals, especially insects, which are highly aerobic. Insects transport oxygen *via* an air-filled tracheal respiratory system, with gas exchange occurring by a mix of convection and diffusion (Harrison, 2009). The tracheal system acts as a ventilation system (with spiracles acting as air input valves, air sacs as bellows and tracheae as a tubular air duct system) to bring oxygen to the tracheoles, the blind-ending tubules that act as the diffusive exchange surfaces of the tracheal system. The tracheoles act analogously to vertebrate capillaries in that they supply oxygen to the cells of the body. The shapes and dimensions of the tracheal system are subject to plasticity and selection when oxygen limitation occurs, and respiratory system morphology [e.g. tracheal diameters (Henry and Harrison, 2004; Loudon, 1988); tracheolar branching (Jarecki et al., 1999)] shows compensation for changing oxygen availability.

Oxygen limitation can occur during low oxygen supply [e.g. in a hypoxic environment (Chown and Holter, 2000)] and/or high oxygen demand [e.g. during flight (Harrison and Roberts, 2000)]. Body size strongly affects oxygen demand, and metabolic rate generally increases with increasing body size. Many other anatomical, physiological and ecological characteristics of animals have also been shown to be directly related to body size (Brown and West, 2000; Brown et al., 2004; Calder, 1984; Lindstedt and Calder, 1981; McMahon and Bonner, 1983; Peters, 1983; Schmidt-Nielsen, 1984; Taylor et al., 1981), and the variation in an animal trait over a range of body sizes can be described by the allometric equation $y=aM^b$, where y is the trait, a is a normalization constant, M is body mass and b is the scaling exponent.

We might expect that traits associated with oxygen delivery would also be affected by (and affect) body size. On the one hand, oxygen diffusion becomes more difficult over longer distances, but on the other hand, tissue-level oxygen demand (i.e. mass-specific metabolic rate) is higher for smaller animals. Indeed, many anatomical and physiological aspects of respiratory systems exhibit isometric and allometric patterns with respect to body mass (M). In vertebrates, lung volume scales with body mass as M^1 (Tenney and Remmers, 1963), lung diffusing capacity scales as M^1 (Gehr et al., 1981) and breathing frequency scales as $M^{-0.25}$ (Worthington et al., 1991). Respiratory parameters of some invertebrates have also been shown to scale with animal body size and, importantly, to have functional consequences. For example, insect tracheal volume scales intraspecifically [*Schistocerca americana*; Orthoptera (Lease et al., 2006)] and interspecifically [Tenebrionidae (four species); Coleoptera (Kaiser et al., 2007)] with body mass as $M^{1.3}$, explaining enhanced respiratory capacity (Greenlee and Harrison, 2004a) and increased convective ventilation [$M^{1.3}$ (Greenlee et al., 2009)] of older insects (*S. americana*). Tracheal volume scales intra-instar (i.e. within an intermolt period) as $M^{-2.4}$ in *S. americana* (Lease et al., 2006) because within-instar tissue accumulation is constrained by a relatively rigid/fixed exoskeleton, as hypothesized by Greenlee and Harrison (Greenlee and Harrison, 2004b) and demonstrated recently (Kirkton et al., 2011; Callier and Nijhout, 2011). This >75% reduction in respiratory volume (Lease et al., 2006) explains the decreased aerobic capacity of late-instar insects [*S. americana*;

Orthoptera (Kirkton et al., 2005; Kirkton et al., 2011)] and can also lead to oxygen limitation that triggers the molting process itself [*Manduca sexta*; Lepidoptera (Callier and Nijhout, 2011)]. Additionally, insect maximal tracheal system conductance scales interspecifically as $M^{0.7}$ [Orthoptera, 23 species from five families (Greenlee et al., 2007)]. When considered in conjunction with the hypermetric scaling of orthopteran tracheal volume and convection – $M^{1.3}$ (Lease et al., 2006; Greenlee et al., 2009) – these data support the idea that the matching of metabolic function to tissue needs requires more respiratory system volume and convection in larger insects.

One way to test the respiratory capacity of an organism is to determine the animal's critical oxygen partial pressure (critical P_{O_2}, or P_{crit}). This is the lowest P_{O_2} that an animal can tolerate without compromising its metabolic capacity (Greenlee and Harrison, 2004a; Greenlee et al., 2007; Harrison et al., 2006; Kam and Lillywhite, 1994; Yeager and Ultsch, 1989). Insects are quite tolerant of hypoxic and anoxic conditions compared with vertebrates (Schmitz and Harrison, 2004), and generally have low P_{crit} values [1–5 kPa (Greenlee and Harrison, 2004a; Klok et al., 2010)]. Insect P_{crit} can be affected by respiratory system type (Schmitz and Harrison, 2004), temperature (Woods and Hill, 2004), ontogeny (Greenlee and Harrison, 2004b) and activity (reviewed by Harrison et al., 2006; Schmitz and Harrison, 2004). The pattern for how the P_{crit} for CO_2 release rate is related to body size seems to vary among insects. P_{crit} decreased with increasing body mass across developmental stages of the grasshopper *S. americana* (Greenlee and Harrison, 2004a), and appears to decrease with advanced life stage across developmental stages of the blowfly *Phormia regina* (Diptera) (Keister and Buck, 1964); however, it was constant across instars during the development of the caterpillar *M. sexta* (Greenlee and Harrison, 2005). In the only prior interspecific comparison, P_{crit} was found to be independent of body mass for grasshoppers (Greenlee et al., 2007). We are aware of no data to date that show how P_{crit} varies with size among adults of a single species of insects, although in amphibians P_{crit} was found to increase with increasing body size in *Siren lacertina* (Ultsch, 1974) and to not be significantly correlated with body size in the frog *Rana muscosa* (Bradford, 1983).

Here we investigate P_{crit} and maximal overall conductance from air to mitochondria ($G_{O_2,max}$) in the only group of insects for which there are currently interspecific data on tracheal scaling – Coleoptera (Kaiser et al., 2007). We test the general hypothesis that the respiratory capacities of larger beetles will match their increased needs for oxygen delivery. More specifically, we predict that like many vertebrates and invertebrates, larger coleopterans will have increased absolute metabolic rates, and that metabolic rate will scale hypometrically with body mass (scaling with a slope of less than 1 on a log–log plot). Second, we predict that $G_{O_2,max}$ will scale with a slope similar to that of metabolic rate, and that P_{crit} will be similar for large and small animals. Alternatively, if oxygen delivery becomes more difficult as animals increase in size, $G_{O_2,max}$ will increase less strongly with size than metabolic rates, and P_{crit} will be higher for larger animals.

Finally, we predict that evolutionary history and life history traits may alter these relationships. In this study, the scarabaeid species were all flying species whereas the tenebrionid species were mostly non-flying species. We therefore expect these two families to exhibit different resting metabolic rates (Reinhold, 1999; Chown et al., 2007; Riveros and Enquist, 2011), and potentially different gas exchange requirements. Specifically, we predict that the higher metabolic capacities of Scarabaeidae associated with flight (Niven and Scharlemann, 2005) and the possession of air sacs (Harrison, 2009; Kaiser et al., 2007) might enable lower P_{crit} values and higher $G_{O_2,max}$. To test these predictions, we investigate the effect of body size on normoxic and hypoxic CO_2 release rates, P_{crit} and $G_{O_2,max}$ for 14 species of the order Coleoptera from two families, Tenebrionidae and Scarabaeidae.

MATERIALS AND METHODS

Animals

Adult beetles were obtained by collection in the field (Mojave Desert, CA; Sonoran Desert, AZ; Chihuahuan Desert, AZ; Magdelena and San Mateo Mountains, NM; Chiricahua and Superstition Mountains, AZ) and by purchase from biological supply companies (Hatari Invertebrates, AZ, USA; Carolina Biological Supply, Burlington, NC, USA). This sample included 96 individuals (14 species) from two families of Coleoptera: Tenebrionidae and Scarabaeidae (Table 1).

The tenebrionid species were analyzed in June 2006, and the scarabaeid species in September 2006. Tenebrionidae were collected and housed under laboratory conditions up to several months prior to experimental analyses. Because of shorter adult lifespans compared with the Tenebrionidae, the Scarabaeidae were collected or purchased within 1–2 weeks of experimental analysis. Food (apple, cereal or dung) was removed from animals >1 h before experimental analyses to minimize specific dynamic action. However, it should be recognized that neither metabolic rate nor the fuel utilized are likely in steady-state conditions within our experiments. Water was given *ad libitum* to animals until experimental analysis, but was not present during the experiment itself; thus animals were slowly, but only slightly, dehydrating.

Animals were weighed on a Mettler MX5 micro-balance (±0.001 mg; Mettler Toledo, Columbus, OH, USA). Mass was determined for each animal before and after hypoxic exposure to account for water loss during the experiment, and the mean of these two masses is reported. Fresh body mass of all animals ranged from 1.32 to 5444.85 mg (species means from 1.7 to 4444 mg; Table 1).

Flow-through respirometry

Animals were placed in a 60, 10 or 1 ml syringe modified into a flow-through respirometry chamber using CO_2-impermeable plastic tubing. We adjusted syringe size to animal body size (Table 2) to minimize variation of chamber time constants. The tips of the syringes were packed with glass wool to prevent animal escape. Syringes were enclosed to reduce stimulation of phototactic movement, and were maintained at 25±1°C using a water bath or temperature cabinet (PTC-1 Peltier cabinet, Sable Systems, Las Vegas, NV, USA). Locomotor activity and loco-static behavior were not recorded during the experiment.

We equilibrated each beetle in its chamber to experimental conditions for more than 10 min, with tubings open to room air, followed by ventilation with normoxic air for 15 to 20 min, which flushed CO_2 buildup from the animal chamber. After equilibration, chambers were connected to the analyzers and perfused with sample gas continually while the oxygen content of sample gas was changed. Each beetle was exposed to nine or 10 different partial pressures of atmospheric oxygen (21, 10, 5, 4, 3, 2, 1, 0.5 and 0 kPa O_2 plus 7.5 kPa O_2 for Scarabaeidae). Animals were exposed to each P_{O_2} in descending order for 15 min. This prevented metabolic rate increases caused by prior hypoxic exposure (Greenlee and Harrison, 1998). After exposure to anoxia, sample gas was restored to normoxia to determine whether animals survived and whether normoxic metabolic rates were recovered. Each experimental run lasted for 5 to 8 h.

Table 1. Mean CO_2 release rates and mass at each P_{O_2}, shown as species means (±s.e.m.)

		CO_2 produced ($\mu mol CO_2 h^{-1}$)											
	Mass (mg)	0kPa	0.25kPa	0.50kPa	0.75kPa	1kPa	2kPa	3kPa	4kPa	5kPa	7.5kPa	10kPa	21kPa
Tenebrionidae:													
Tribolium castaneum	1.70±0.04	0.02±0.00		0.04±0.00		0.04±0.00	0.06±0.00	0.06±0.00	0.07±0.01	0.07±0.00		0.07±0.01	0.08±0.01
Tenebrio molitor	117.47±7.01	0.96±0.09	1.01±0.04	1.64±0.21	2.11±0.23	1.81±0.13	3.01±0.28	3.61±0.45	3.83±0.43	4.49±0.47		5.20±0.55	5.31±0.65
Eleodes obsoletus porcatus	293.62±27.25	1.01	1.77	1.45±0.13	2.04±0.21	1.56±0.41	2.44±0.35	2.40±0.39	3.73±0.78	4.12±0.85		4.08±0.76	2.86±0.46
Cryptoglossa muricata	494.19±37.18	1.47±0.22		2.11±0.27	2.79±0.60	2.65±0.39	3.59±0.49	3.71±0.54	3.60±0.59	3.76±0.46		3.16±0.51	2.42±0.38
Eleodes longicollis	709.66	2.19		3.49		4.81	7.73	7.45	7.22	8.63		4.54	4.35
Eleodes armatus	872.70±203.20	3.34	4.74		4.34±0.78	5.25±1.01	6.35±1.56	8.04±2.18	8.58±2.15	6.33±1.18		5.99±1.44	6.36±1.84
Eleodes obscurus	1409.29±38.42	6.11±0.64	8.37±0.90		9.37±0.99	10.60±1.62	13.12±1.12	15.05±1.36	18.12±2.28	21.64±3.79		27.40±6.12	28.29±7.22
Scarabaeidae:													
Aphodius sp. 1	5.01±0.69		0.08±0.03	0.10±0.02		0.10±0.03	0.22±0.05	0.24±0.08	0.23±0.07	0.28±0.10	0.25±0.09	0.30±0.08	0.18±0.06
Aphodius sp. 2	6.14		0.03	0.06		0.13	0.32	0.43	0.48	0.53	0.46	0.32	0.37
Aphodius sp. 3	28.16		0.87	1.15		2.28	4.02	4.40	5.08	5.08	5.25	4.90	4.87
Tomarus gibbosus	309.21±25.34		2.41±0.25	3.02±0.07		4.90±0.95	4.43±1.44	4.16±1.32	3.89±0.69	4.06±0.99	3.92±1.03	5.69±1.95	2.94±0.35
Cotinis mutabilis	915.24±50.03		7.21±0.56	8.57±0.33		9.09±1.15	10.33±1.43	10.40±0.95	10.67±0.99	13.33±1.05	15.44±2.58	21.23±3.81	19.02±2.16
Xyloryctes jamaicensis	1252.61±144.58		9.41±4.35		14.84±5.09	22.33±5.51	41.31±18.12	40.04±17.73	44.52±29.60	44.97±31.80	42.18±29.58	39.29±25.77	36.33±22.87
Dynastes granti	4444.00±187.15		21.98±3.99	22.40±4.39		53.82±12.78	103.21±24.16	102.22±30.00	94.36±18.99	98.01±21.93	76.15±13.53	75.35±16.25	71.08±15.81

No standard errors are shown for species where there was no repetition (as species replicate).

Gas mixtures were created by diluting dry, CO_2-free air with N_2. The ratio of air to N_2 flow was controlled with a Tylan RO-28 mass flow controller and two Tylan FC-280 mass flow meters (Tylan General, San Diego, CA, USA). Air and N_2 were mixed in a 1 liter glass container with a 10 cm electrical fan, and pumped from the mixing container using a standard aquarium pump (up to 1.5 l min^{-1}). The air–N_2 mixture was split into three lines, each consisting of an Ascarite-packed 60 ml syringe, to scrub the air of residual CO_2, an animal respirometry chamber (syringe) and a CO_2 analyzer (two LiCor Li-6252s and one Li-6262; LiCor, Lincoln, NE, USA). Flow rates (25–100 ml min^{-1}) for the three lines were controlled by Brooks mass flow controllers and mass flow meters (Brooks Instruments, Hatfield, PA, USA); resolution of these flow meters is approximately 1% of the flow rate. The flow rates were adjusted to animal and chamber size (Table 2). One of the lines was equipped with an Oxzilla (tenebrionids) or a FoxBox (scarabaeids) O_2 analyzer (both Sable Systems) upstream of the animal chamber to measure/survey the set P_{O_2}. Baseline CO_2 was measured before and after each experiment for each line with animal chambers excluded from the air flow, and baseline O_2 was measured continuously throughout each experiment.

During the scarabaeid experiments, 0.1 ml of water was injected approximately once per hour into the Ascarite scrubbing columns to minimize desiccation of animals during data collection. Increased humidity in desiccation-prone species (such as non-desert-adapted scarabaeids) reduces stress and therefore reduces the risk of stress-induced activity or metabolic rate artifacts (Hadley and Quinlan, 1993; Williams et al., 1998). This water addition elevated the humidity marginally (7% relative humidity at 25°C; to offset the desiccation potential of dry air), but the quantity of water added to the air was small enough to be considered negligible in terms of oxygen dilution. Oxygen was measured downstream of the humidifying columns.

Expedata software (Sable Systems) was used to record CO_2 (±0.2 p.p.m.), temperature and O_2 (±0.1%) throughout experimental runs at a 1 s sampling rate (Fig. 1). At the completion of each experiment, a drift correction was performed (using Expedata software and baseline air) to adjust for any (linear) CO_2 drift that may have occurred over the course of the multi-hour experiment.

The LiCor analyzers were calibrated by flowing CO_2 standard gases (measured ±0.1 p.p.m.) through the entire system as set up for the animal measurements, at the identical flow rates (and therefore chamber pressures) as when the animal measurements were performed. Ambient temperatures were also constant (±1°C) throughout the experiments. We used multiple calibration gases to confirm that the recorded output was linearly related to CO_2 concentration. Overall resolution of the CO_2 release rates was better than ±3% of the measured rates (LiCor resolution of 1%, adjusted for flow rates and CO_2 concentrations of the smallest beetles), and equilibration times of combined animal chambers and tubing (~2.82 ml; <40 cm of Bev-A-Line tubing, 3 mm inner diameter) are presented in Table 2.

Determination of P_{crit} and $G_{O_2,max}$

Mean CO_2 release rate was determined for each animal, at each P_{O_2}, averaged over a ~10 min period. If CO_2 release appeared cyclic or discontinuous, care was taken to ensure that the sample selection encompassed one or more complete cycles of CO_2 burst release. Individual P_{crit} values for CO_2 release rate were then determined by statistically identifying the P_{O_2} at which CO_2 release rate dropped. Because of a high variability in CO_2 release, we slightly modified Greenlee and Harrison's method of calculating P_{crit}

Table 2. Summary of sample size, flight capacity, body size, experimental parameters and critical P_{O_2} values derived in this study

Species	N (CO_2: P_{crit})	Type of locomotion	Mass (mg) Mean	Min.	Max.	s.e.m.	Chamber size (ml)	Flow rate (ml min^{-1})	95% Equilibrium time (min)	P_{crit} (kPa) Mean	Min.	Max.	s.e.m.
Tenebrionidae													
Tribolium castaneum	20:18	Volant	1.70	1.32	1.95	0.04	0.5	25.0	0.30	2.30	0.38	10.02	0.73
Tenebrio molitor	15:15	Volant	117.47	77.24	173.39	7.01	9.0	43.5–50.0	0.62	2.98	0.85	4.96	0.40
Eleodes obsoletus porcatus	5:5	Non-flying	293.62	199.67	341.38	27.25	9.0	43.5–50.0	0.62	2.87	0.86	3.91	0.56
Cryptoglossa muricata	12:11	Non-flying	494.19	333.17	715.54	37.18	9.0	25.0–50.0	1.09	1.17	0.09	2.00	0.20
Eleodes longicollis	1:1	Non-flying	709.66	n.a.	n.a.	n.a.	9.0	25.0	1.09	1.93	n.a.	n.a.	n.a.
Eleodes armatus	5:5	Non-flying	872.70	525.20	1632.65	203.20	9.0	43.5–50.0	0.62	1.57	0.74	3.10	0.44
Eleodes obscurus	6:6	Non-flying	1409.29	1291.15	1503.90	38.42	9.0	43.5–50.0	0.62	2.28	0.20	4.93	0.87
Scarabidae													
Aphodius sp. 1	6:5	Volant	5.01	2.85	7.65	0.69	0.5	25.0	0.30	0.91	0.25	2.12	0.34
Aphodius sp. 2	1:1	Volant	6.14	n.a.	n.a.	n.a.	0.5	25.0	0.30	2.11	n.a.	n.a.	n.a.
Aphodius sp. 3	1:1	Volant	28.16	n.a.	n.a.	n.a.	0.5	25.0	0.30	3.01	n.a.	n.a.	n.a.
Tomarus gibbosus	5:2	Volant	309.21	277.38	370.31	25.34	9.0	50.0	0.54	0.73	0.45	1.01	0.28
Cotinis mutabilis	6:6	Volant	915.24	819.02	1187.96	50.03	25.0	50.0	1.28	0.52	0.22	1.02	0.11
Xyloryctes jamaicensis	3:3	Volant	1252.61	1001.73	1588.41	144.58	25.0	50.0	1.28	1.33	1.33	1.33	0.00
Dynastes granti	10:8	Volant	4444.00	3788.30	5444.85	187.15	50.0	75.0	1.62	0.92	0.43	1.02	0.07

(Greenlee and Harrison, 2004a), which utilizes the confidence intervals (CI) of CO_2 release. P_{crit} values presented in this paper represent the P_{O_2} where: (1) mean CO_2 release rate at P_{crit} drops below the lower 95% CI of CO_2 release rate at the previous, higher P_{O_2} and (2) mean CO_2 release rate at any subsequent, more hypoxic P_{O_2} did not rise above the mean CO_2 release rate at the P_{crit}.

For approximately 10 individuals, we could not identify a P_{crit} with these criteria; in these cases, visual inspection of the original CO_2 release trace was used to identify the P_{crit}. Mean P_{crit} for each species was calculated by averaging the P_{crit} values for individuals. To calculate O_2 consumption from CO_2 release, a respiratory quotient (RQ) of 0.8 was used (Lighton, 1988). By dividing O_2 consumption at the P_{crit} by the P_{crit}, we were able to estimate maximum conductance ($\mu mol\,O_2\,h^{-1}\,kPa^{-1}$) for oxygen from the atmosphere to the oxygen-consuming tissues of the beetles ($G_{O_2,max}$) [first defined for an insect model by Kestler (Kestler, 1985)], based on the assumptions that spiracles are completely open, tracheolar fluid is removed and ventilation is maximized at P_{crit} (Greenlee and Harrison, 2004a). Under these conditions, it is plausible that the major resistance to gas exchange is in the liquid phase (from tracheoles to mitochondria), given the much higher capacitance and diffusion rates of oxygen in air compared with water (Kestler, 1985).

Statistical analyses

Statistical analyses were conducted to determine whether there was a body size and/or phylogenetic effect on CO_2 release rate at normoxia, P_{crit} for CO_2 release rate, CO_2 release rate at anoxia and $G_{O_2,max}$. For this, we performed ordinary least squares (OLS) and standardized/reduced major axis (SMA) regression analyses of mean species values across all species examined, with family as a categorical value. We additionally conducted an independent contrast analysis in Mesquite (Maddison and Maddison, 2006) using the PDAP module (MESQUITE V1.12) and in R using the 'ape' package (Paradis et al., 2004) to make phylogenetic corrections. The topology of the phylogenetic tree was constructed using previously published work (Doyen and Tschinkel, 1982; Kaiser et al., 2007; Mestrovic et al., 2006; Smith et al., 2006) and consultation with Dr Kelly Miller (Museum of Southwestern Biology, University of New Mexico, Albuquerque, NM, USA) and Dr Clarke Scholtz (Department of Zoology and Entomology, University of Pretoria, Gauteng, South Africa). To test for the influence of phylogenetic effects, we performed OLS and SMA line-fits on independent contrasts using normalized branch lengths. To control the influence of phylogenetic relationships, we repeated the analysis with branch lengths set equal to one and haphazardly altered positions of individual species within the phylogenetic tree. We additionally tested for potential within-species size effects by analyzing the regression functions of P_{crit} on size within the species for which we had sufficient specimens.

RESULTS

Scaling of normoxic CO_2 release rates

CO_2 release during normoxia (21 kPa O_2) was independent of phylogenetic relationship (t=–0.39, P>0.5), but was significantly correlated with body mass (t=6.36, P<0.005). OLS regression analysis revealed that absolute metabolic rate (MR) scaled with body mass (M) for Tenebrionidae ($MR=0.06M^{0.73\pm0.14}$; r^2=0.84, P=0.004), Scarabaeidae ($MR=0.09M^{0.78\pm0.13}$; r^2=0.88, P=0.002) and all

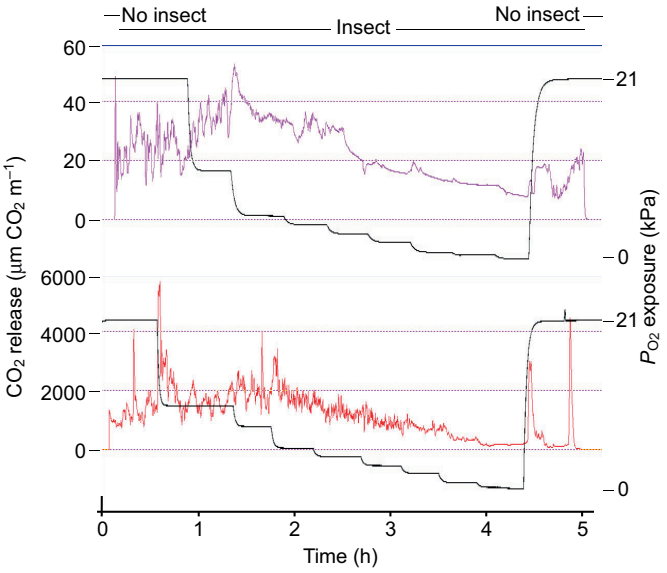

Fig. 1. Sample traces of CO_2 release rates during progressive decrease in oxygen partial pressure (P_{O_2}) exposure for two individual beetles (top: *Eleodes obsoletus porcatus*; bottom: *Dynastes granti*) at 25°C. P_{O_2} ranged from 21 to 0 kPa O_2 for each animal, and CO_2 release rates varied with body size and species (see Table 1 for typical values). Flow rates varied from 25 to 100 ml min^{-1}, and CO_2 (±1 p.p.m.) and O_2 (±0.1%) were recorded throughout, and depend on ambient temperature and pressure changes and on vapor pressure after humidification.

Coleoptera (MR=$0.08M^{0.75\pm0.10}$; r^2=0.83, P<0.00; Fig. 3, Tables 1, 3). None of these slopes were statistically distinguishable from $M^{0.75}$. Because the scaling relationship was not significantly different between tenebrionid and scarabaeid beetles, we analyzed the influence of phylogenetic relationships for all species using a phylogenetic tree that included both families (Fig. 2). SMA regression analysis on phylogenetically independent contrasts (PICs) (MR=$0.12M^{1.04}$; 95% CI=0.73–1.48) was not significantly different from OLS regression analysis (MR=$0.08M^{0.75}$; Fig. 3, Table 3). Mass dependence of normoxic CO_2 release was higher (i.e. CO_2 release scaled steeper with body mass) in scarabaeid beetles (MR=$M^{1.05\pm0.22}$; r^2=0.82) than tenebrionid beetles (MR=$M^{0.89\pm0.20}$; r^2=0.80) when phylogenetically corrected at the level of family (OLS on PIC, forced through zero), though this difference was not statistically significant. Please note that animal activity was not measured during this experiment and our data for normoxic CO_2 release are therefore not standard resting measurements of metabolic rate.

Effects of hypoxia on CO_2 release rate patterns

The general tendency across individuals (not shown), species (Fig. 4, Table 1) and families (Fig. 5) of Coleoptera was for mean CO_2 release to decrease as P_{O_2} decreased. However, the pattern of decreased CO_2 release rates varied considerably between individuals, species and families. In some species, moderate hypoxia stimulated elevated CO_2 release rates, perhaps because of stimulation of escape behavior (e.g. *Cryptoglossa muricata* and *Eleodes armatus*; Fig. 4A). For individuals, the reaction to hypoxia was even more variable (Fig. 1), with frequent cases of increases and decreases in CO_2 release rate within the same animal at sequentially decreasing P_{O_2}. This individual variation was more pronounced in some species than others, as illustrated by the size of the standard error bars in Fig. 4 (e.g. *Tribolium castaneum versus Dynastes granti*).

There were also some differences between Coleopteran families with respect to CO_2 release patterns. Scarabaeid species had significantly higher CO_2 release rates than tenebrionid species at every P_{O_2} (ANCOVA with CO_2 release rate as a dependent variable, mass as a covariate, and family and P_{O_2} as categorical predictors, P=0.01; Fig. 5). Tenebrionid species tended to decrease mean CO_2 release rates upon hypoxic exposure (Fig. 5), though the P_{O_2} triggering the drop varied between species (e.g. *Eleodes obscurus* dropped at ~5 kPa O_2, while *T. castaneum* had a slight drop at 5 kPa O_2, but then a more prominent drop at ~2 kPa O_2; Fig. 4A). Scarabaeid species, in contrast (Fig. 5), showed a tendency to increase average CO_2 release rates upon initial exposure to hypoxic conditions (e.g. *D. granti* at ~5 kPa O_2, *Aphodius* sp. 1 at ~10 kPa O_2; Fig. 4B), followed by a decrease in average CO_2 release rate upon exposure to a lower P_{O_2} (~2 kPa O_2 for both species; Fig. 4B). The

Table 3. Comparison of the allometric scaling of mean CO_2 release rates at normoxia (21 kPa O_2) and anoxia (0 kPa O_2), critical P_{O_2} (P_{crit}) and maximum overall conductances from air to mitochondria ($G_{O_2,max}$) regressed on mean fresh mass (log CO_2 release *versus* log mass) of beetles using ordinary least squares (OLS) and standardized major axis (SMA) regression analyses

Regression type	Treatment	Slope	Intercept	MSE offset	r^2	P
OLS	CO_2 release at normoxia	0.748±0.097	–1.111±0.239	0.077	0.833	<0.001
	CO_2 release at anoxia	0.846±0.069	–1.816±0.169	0.015	0.927	<0.001
	P_{crit} for CO_2 release	n.s.	n.s.		0.100	0.265
	$G_{O_2,max}$	0.780±0.077	–1.269±0.190	0.054	0.895	<0.001
OLS on phylogenetic independent contrasts forced through 0	CO_2 release at normoxia	0.961±0.143	0		0.791	<0.001
	CO_2 release at anoxia	0.938±0.103	0		0.874	<0.001
	P_{crit} for CO_2 release	n.s.	n.s.		0.001	0.907
	$G_{O_2,max}$	0.883±0.089	0		0.892	<0.001
SMA on phylogenetic independent contrasts	CO_2 release at normoxia	1.039±0.158	–0.934±0.063	0.117	0.706	<0.001
	CO_2 release at anoxia	1.010±0.123	–0.978±0.046	0.105	0.825	<0.001
	P_{crit} for CO_2 release	n.s.	n.s.		0.021	0.640
	$G_{O_2,max}$	0.942±0.108	–0.966±0.039	0.108	0.850	<0.001

Regressions were conducted on species means for CO_2 release rates, which were calculated from average CO_2 release rates for each individual at each P_{O_2}, and on species means of P_{crit} and $G_{O_2,max}$, which were calculated from individual P_{crit} and $G_{O_2,max}$ values. Metabolic scaling equation (MSE) offsets indicate scaling intercepts in μmol CO_2 h^{-1} (i.e. not logged), and reflect CO_2 release rates, P_{crit} and $G_{O_2,max}$ of a 1 mg insect.
Slope and intercept values are means ± s.e.m.
n.s., not significant.

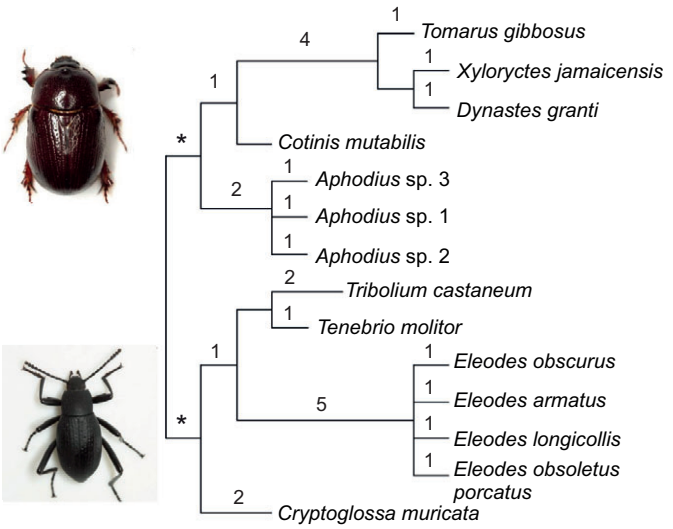

Fig. 2. Phylogenetic tree used in the independent contrast analysis. Asterisks indicate uncertainty in branch lengths at the base of each Coleopteran family. The analyses presented in this paper were conducted with *=1, although *=50 was also tested (data not shown) with no change in significance. Images used with permission from Mike Quinn, TexasEnto.net.

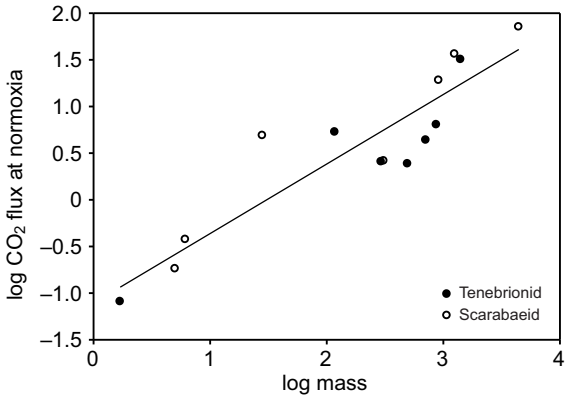

Fig. 3. Mean CO_2 release rates (μmol CO_2 h^{-1}; averaged across all individuals tested) at normoxia as a function of mean fresh mass (mg; log–log plot) for tenebrionid (filled circles) and scarabaeid species (open circles) examined in this study. The slope is shown as ordinary least squares regression analysis on species means.

effect of P_{O_2} on CO_2 release rates was significant for both families (P=0.03), although the difference in release patterns between the families was not significant (ANCOVA with CO_2 release rate as a dependent variable, mass as a covariate, and family and P_{O_2} as categorical predictors, P>0.5 for family × P_{O_2}).

Effect of anoxia on the CO_2 release rate scaling

The effect of body mass on CO_2 release rates during normoxia (21 kPa O_2) and anoxia (0 kPa O_2) was compared using OLS on species means (i.e. phylogenetically uncorrected), OLS on PIC, and SMA on PIC (Table 3). During both normoxia and anoxia, CO_2 release rate scaled with body size, but to different degrees depending on the oxygen exposure and the type of statistical analysis. Scaling coefficients again tended to be slightly higher when phylogenetically corrected than when uncorrected (e.g. b=0.94 *versus* 0.85 for anoxic conditions; Table 3), although these differences were not statistically significant. Scaling coefficients for CO_2 release were higher in normoxic conditions compared with anoxic conditions when phylogenetically corrected, although in contrast slopes were higher in anoxic conditions compared with normoxic conditions when analyzed without phylogenetic correction (Table 3). However, regardless of analysis type, the scaling intercept for CO_2 release was lower for anoxic than normoxic conditions, and this pattern was consistent across all Coleoptera (Table 3) and within each family (data not shown). The effect of P_{O_2} on CO_2 release rate was significant (ANCOVA with CO_2 release rate as a dependent variable, mass as a covariate and P_{O_2} as a predictor, $F_{11,127}$=2.39, P=0.01).

Effects of family and mass on P_{crit}

The mean P_{crit} for CO_2 release rate for each beetle species examined in this study was ≤3 kPa O_2 (Fig. 4, Table 2), and across all species was 1.76 kPa O_2. Some species had a relatively higher P_{crit} than others (e.g. *Eleodes obsoletus porcatus versus Tomarus gibbosus*; Fig. 4A,B). The mean P_{crit} for scarabaeid beetles (1.36 kPa) was lower than that for tenebrionid beetles (2.16 kPa), but this difference was not statistically significant (ANOVA with P_{crit} as dependent variable and family as predictor, $F_{1,12}$=3.601, P=0.082). This lack of significance may be attributable to low statistical power because of the small sample size (N=7 for each family). However, the variance in P_{crit} values did differ significantly between the two coleopteran families examined. Specifically, tenebrionid beetles showed a wider range of P_{crit} values than did scarabaeid beetles (two-sample F-test for variance, P=0.01; also see Fig. 6, top graphs *versus* bottom).

P_{crit} was independent of body size both across and within beetle families (Fig. 7). There was no significant effect of beetle body size on P_{crit} regardless of whether data were phylogenetically corrected and analyzed at the level of coleopteran order (SMA on PIC; P>0.5) or family (SMA on PIC; tenebrionid P>0.5, scarabaeid P>0.5), or whether they were analyzed across coleopteran order prior to phylogenetic correction (OLS; P>0.5). In addition, when P_{crit} was examined intraspecifically, it again appears to be independent of body size. No scaling relationship between body size and P_{crit} occurred within any one taxon (Fig. 6).

Effects of family and mass on $G_{O_2,max}$

$G_{O_2,max}$ was independent of phylogenetic relationship (t=–1.62, P>0.5), but was significantly correlated with body mass (t=12.82, P<0.005). As beetle body size increased, $G_{O_2,max}$ increased hypometrically, with specific exponents and intercepts again dependent on the method used to fit the line (e.g. OLS, $G_{O_2,max}=0.05M^{0.78}$ *versus* SMA on PIC, $G_{O_2,max}=0.01M^{0.94}$; 95% CI=0.73–1.21; Table 3).

The mass dependence of $G_{O_2,max}$ was similar for scarabaeid and tenebrionid beetles regardless of whether analyzed as species averages (OLS: tenebrionid, $G_{O_2,max}=0.03M^{0.79}$, r^2=0.95; scarabaeid, $G_{O_2,max}=0.08M^{0.81}$, r^2=0.98) or phylogenetically corrected at the level of family (OLS on PIC, forced through zero: tenebrionid, $G_{O_2,max}=M^{0.88}$, r^2=0.90; scarabaeid, $G_{O_2,max}=M^{0.90}$, r^2=0.92). The slopes of both of these relationships overlapped $M^{0.75}$ (for tenebrionid $G_{O_2,max}$, 95% CI=0.34–1.59; for scarabaeid $G_{O_2,max}$, 95% CI=0.63–1.25).

DISCUSSION

In this study we examined P_{crit} in conjunction with insect body size, and thus simultaneously assessed the net outcome of several supply

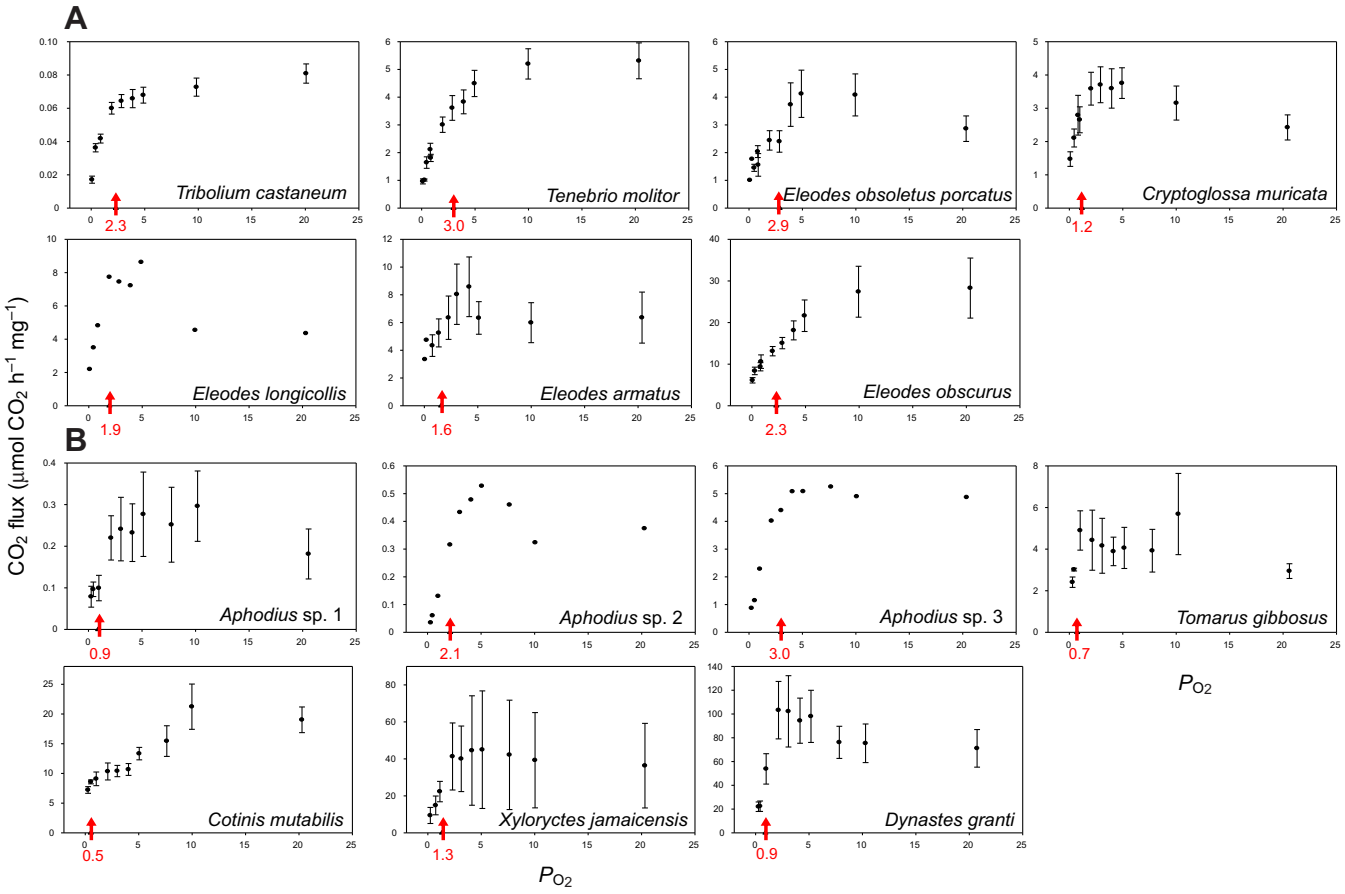

Fig. 4. Mean CO_2 release rates (averaged across all individuals tested) *versus* P_{O_2} for each species of (A) tenebrionid and (B) scarabaeid. Bars indicate standard errors; data points with no error bars indicate species with no replication. Mean critical P_{O_2} values are indicated with a red arrow. The species are ordered based on body mass.

and demand trade-offs that might affect insect oxygen limitation: increased oxygen demand (due to increased body size), decreased mass specific oxygen demand (due to increased body size), potentially increased oxygen supply (due to increased convective capacity from larger tracheal volumes) and potentially decreased oxygen supply (due to decreased diffusive capacity over longer tracheal lengths). Our data show that the capacity of the insect respiratory system matches oxygen needs across a large range of masses. Metabolic rates and tracheal conductances scaled similarly with size, and P_{crit} was size-independent, whether examined within species, across species within families or across the two tested beetle families.

Normoxic metabolic rates of beetles increased hypometrically ($M^{0.75}$ OLS, but $M^{1.04}$ SMA on PIC) with respect to beetle body size in our study, and did so with interspecific exponents similar to those in the published literature for insects [$M^{0.82}$ OLS, but $M^{0.75}$ phylogenetic generalized least squares (Chown et al., 2007)] and for beetles [$M^{0.66}$ OLS, but $M^{0.73}$ SMA (Riveros and Enquist, 2011)] despite the hypermetric scaling of the dimensions of the insect respiratory system (Lease et al., 2006; Kaiser et al., 2007; Greenlee et al., 2009). The scarabaeid scaling of metabolic rate in our study ($M^{0.78}$) was higher than that reported in the scientific literature [$M^{0.54}$ (Riveros and Enquist, 2011)], and our tenebrionid scaling exponent ($M^{0.73}$) fell between previously reported values of $M^{0.68}$ (Riveros and Enquist, 2011), $M^{0.94}$ (Lighton and Fielden, 1995) and $M^{1.06}$ (Duncan et al., 2002) (all OLS). These slope similarities occurred despite the fact that the intention of the present study was to achieve a baseline CO_2 release rate against which to index metabolic decline during oxygen limitation respirometry, so our normoxic CO_2 release rate measurements were for a truncated period of time compared with those of studies focused on measurement of resting metabolic rates.

All beetles investigated in this study showed a general decrease in mean CO_2 release rate as P_{O_2} decreased, though the patterns of CO_2 release rates varied across species (Fig. 4). Most tenebrionid species showed a simple and steady decrease in mean CO_2 release rate as P_{O_2} decreased, whereas most scarabaeid species showed an increase in mean CO_2 release rate at mild hypoxia, followed by a decrease in mean CO_2 release rate at extreme hypoxia. This suggests a temporary increase in scarabaeid tracheal conductance (ventilation frequency and/or tidal volume) at mild hypoxia, which may ameliorate/delay the effects of hypoxia at the tissue level. Alternatively, this increase in CO_2 release rate could be an indication of hypoxia-induced activation of escape behavior, as seen in some larvae (Wingrove and O'Farrell, 1999). Dissimilar CO_2 release rate patterns for the families may also be due to an inherent lack of discontinuous gas exchange cycles in most North American Tenebrionidae (Lighton, 1998).

P_{crit} in this study is the critical transition point between aerobic metabolic function of inactive animals and oxygen-compromised metabolic function. The P_{crit} for CO_2 release rate of all coleopteran species examined in this study was $\leq$3 kPa, a value that supports

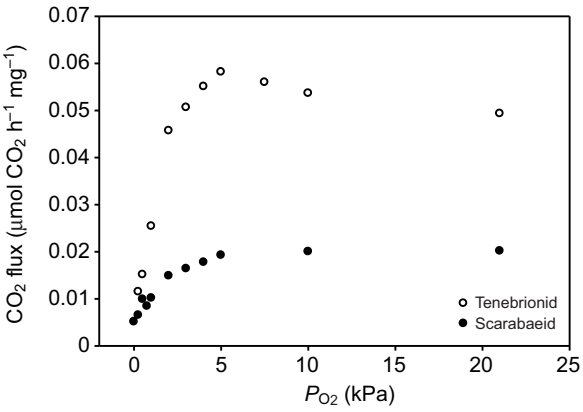

Fig. 5. Mass-corrected mean CO_2 release rates (averaged across species) plotted *versus* P_{O_2} for tenebrionid (filled circles) and scarabaeid (open circles) species. Scarabaeids had significantly higher CO_2 release rates than tenebrionids at every P_{O_2} (P<0.05), and P_{O_2} had a significant effect on CO_2 release rates across both families (P<0.05).

other evidence that insects tolerate much lower partial pressures of oxygen than vertebrates (reviewed in Greenlee and Harrison 2004a; Schmitz and Harrison, 2004). The mean P_{crit} of scarabaeid and tenebrionid species did not significantly differ, suggesting that gas exchange capacities are matched to metabolic rates similarly in these two families. However, the P_{crit} values of scarabaeid species were more constrained (i.e. occurred over a narrower range of values; P<0.05) than those of tenebrionids, suggesting that Scarabaeidae may have greater selective pressure on oxygen delivery capabilities than Tenebrionidae. This may be based on their life history, because some scarabaeid species live in burrows and/or decaying organic material, which often makes for hypoxic micro-environments (Holter, 1991; Holter, 1994), and other scarabaeid species may have high metabolic capacities associated with flight (Niven and Scharlemann, 2005).

Coleopteran P_{crit} did not vary with body size for inactive animals treated identically, suggesting that gas exchange capacities are well-matched to tissue needs across this wide range of beetle body sizes. The largest and smallest scarab species, *D. granti* (mean mass of 4.44 g) and *Aphodius* sp. 1 (mean mass of 5.01 mg), both had P_{crit} values of 0.9 kPa; the largest and smallest tenebrionid species (*E. obscura*, mean mass of 1.41 g, and *T. castaneum*, mean mass of 1.70 mg) both had P_{crit} values of 2.3 kPa (Table 2). Thus, our data support previous conclusions (Greenlee et al., 2007) that larger insect species are not more easily limited by oxygen delivery than smaller insects. However, the relationship between insect body size and oxygen delivery is a complex one. Proportional investment in the tracheal system is higher in larger insects (Lease et al., 2006; Kaiser et al., 2007), and insect developmental P_{O_2} has been shown to affect both adult body size (smaller at lower P_{O_2}) (e.g. Klok et al., 2009) and tracheal system investment (lower at higher P_{O_2}) (e.g. Henry and Harrison, 2004), which together offer clear evidence that insect body size is linked to insect oxygen delivery capacity (reviewed by Harrison et al., 2010). An additional consideration is that the animals in our study were measured mostly at rest or during low-level activity. Critical values may very well show different patterns with respect to body size when measured on animals completely at rest, or alternatively, during highly aerobic activities such as flight or running. Hypoxic limitations to activity may in fact be of greater ecological importance than hypoxic limitations at rest; for example, if P_{crit} during maximal activity were to positively scale with body size, gas exchange capacities may limit maximum body size in highly active insects.

$G_{O_2,max}$ scaled with mass in a manner similar to that of metabolic rate (Table 3); a similar result was found in an interspecific comparison of grasshoppers (Greenlee et al., 2007). This may have been achieved by the hypermetric scaling coefficients that exist for tracheal volume and air sac volume (reviewed earlier), and may enable the similar P_{crit} values seen in large and small beetles. Although $G_{O_2,max}$ may vary because of nutritional influences on RQ, $G_{O_2,max}$ was higher for the scarabaeid family than the tenebrionid family, consistent with the fact that scarabaeid species fly and have extensive air sacs, and thus likely have greater maximal oxygen needs and delivery capacities than tenebrionid species. The mass dependence of $G_{O_2,max}$, however, was similar for scarabaeid and for tenebrionid beetles. Assessment of maximal conductance during

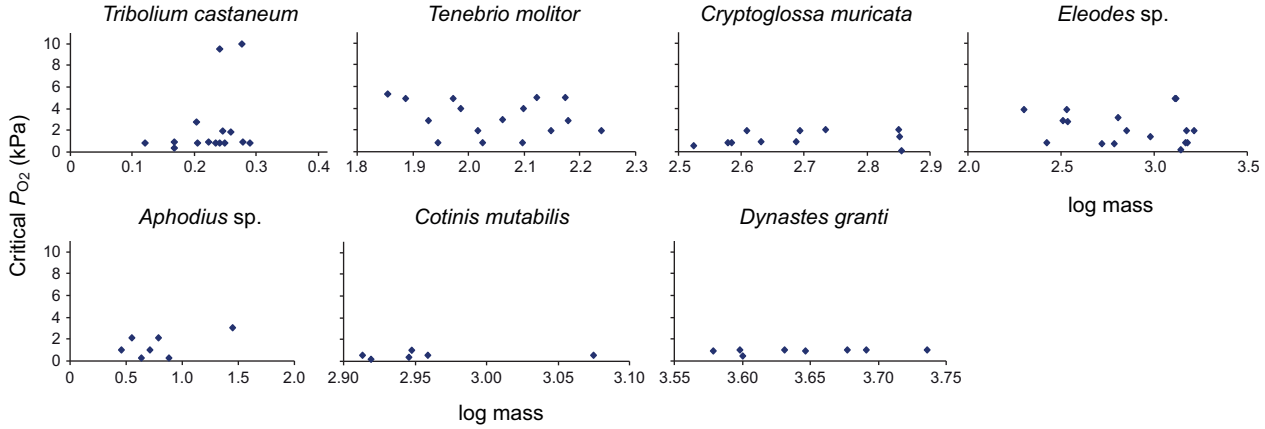

Fig. 6. Critical P_{O_2} (P_{crit}) for individual beetles within several species, plotted *versus* log fresh mass (mg). The top panel represents several Tenebrionidae species; the bottom panel represents several Scarabaeidae species. *Eleodes* and *Aphodius* species were lumped into one generic group each, as no other genera had multiple species represented in the study, and *Xyloryctes jamaicensis* and *Tomarus gibbosus* were excluded because they each contained three or fewer P_{crit} values. There was no tendency for P_{crit} to increase with size within any species (P>0.05), though the tenebrionid species show more variance in P_{crit} values than the scarabaeid species (P<0.05).

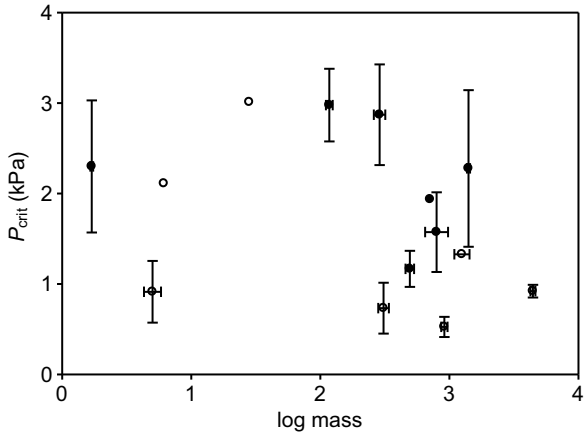

Fig. 7. Mean P_{crit} for each species (averaged across individuals within that species), plotted *versus* log mean mass (mg) of individuals in that species (filled circles, Tenebrionidae; open circles, Scarabaeidae). Bars indicate standard errors of species means, and data points with no error bars indicate species with no replication. There was no significant effect of body size on P_{crit} regardless of whether data were analyzed at the level of order (P>0.5) or family (Tenebrionidae, P>0.5; Scarabaeidae, P>0.5).

maximal tissue needs could further elucidate whether gas exchange capacity limits maximum body size of insects. $G_{O2,max}$ is likely higher during activity because of increased convection (and perhaps other changes), and conceivably the scope for overall conductance varies with size. Ideally, all of these tests would be conducted during maximal aerobic performance. However, this is technically challenging, and no scaling study has ever done this for any group of animals.

It should be noted that systematic variation in RQ, particularly interactions between body mass and P_{O_2}, might have affected our assessment of mass effects on P_{crit} and $G_{O2,max}$, as well as on the standard errors of these values. Oxygen consumption rates were calculated assuming a constant RQ of 0.8. However, if, for example, larger animals were more sensitive to hypoxia, then we might expect larger animals to increase RQ more significantly during hypoxia (i.e. to have a higher CO_2 emission rate relative to oxygen consumption). This could cause the P_{crit} value for CO_2 emission to be higher than the P_{crit} for oxygen consumption for larger species; such an effect could also bias the scaling exponent for $G_{O2,max}$ towards higher values. Oxygen consumption is much more technically challenging to measure over short time periods in small insects than CO_2 emission rates; thus, as yet, there are no data to suggest that RQ does vary systematically with mass and hypoxia in insects. However, eventually, it will be important to test this possibility to affirm the lack of a relationship between body mass and P_{crit} for aerobic metabolic rate in insects.

Conclusions

The interspecific scaling of P_{crit} has not previously been investigated for any group of insects for which we know how tracheal investment scales, and the intraspecific scaling of P_{crit} has not previously been reported for adults of any insect species. Here we investigated the effect of body size on normoxic and hypoxic CO_2 release rates and on P_{crit} for two families of beetles. As predicted, both tenebrionid and scarabaeid beetles exhibit mass-related metabolic scaling similar to hypometric equations reported in the literature. Coleopteran P_{crit}, in contrast, was not size-dependent. Although variance in P_{crit} was smaller for scarabaeid beetles than tenebrionid beetles, reflecting potential differences between these coleopteran families with respect to oxygen delivery constraints, P_{crit} was similar for large and small animals, regardless of family. The P_{crit} thus appears to be unaffected by body size for both tenebrionid and scarabaeid beetles.

However, morphological constraints on oxygen delivery capacity could limit maximum body size in insects (Kaiser et al., 2007) and other animals (Payne et al., 2010). Investment in structural support may also contribute to an upper size limit for vertebrates, because endoskeleton scales hypermetrically with body mass (Prange et al., 1979), although it probably does not generally do so for insects, because exoskeletal chitin increases isometrically with body mass (M^1) (Lease and Wolf, 2010). Two exceptions, interestingly, are Coleoptera and Orthoptera, where exoskeletal chitin scaling is hypermetric ($M^{1.1}$; statistically distinguishable from M^1 for Coleoptera) (Lease and Wolf, 2010). Regardless of the exoskeleton's potential role in limiting beetle maximal body size, there is a large and growing body of evidence that insect body size is constrained by tracheal oxygen delivery (Harrison et al., 2010). Our results, however, suggest that atmospheric oxygen level is not likely to limit maximal insect body size because of limitations on metabolic rate. Instead, it appears that the limit on insect body size may be trade-offs associated with the need for increased tracheal investment as insect size increases (Harrison et al., 2010; Kaiser et al., 2007; Lease et al., 2006) and/or that in some regions of the body (e.g. the legs) an increasing need for investment in the tracheal system may lead to space limitations (Harrison et al., 2010; Kaiser et al., 2007).

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