Isometric Spiracular Scaling in Scarab Beetles: Implications for Diffusive and Advective Oxygen Transport

Julian M. Wagner1, C. Jaco Klok1, Meghan E. Duell1, John J. Socha2, Guohua Cao2, Hao Gong2, Jon F. Harrison1

*1School of Life Sciences, Arizona State University, Tempe, AZ 85287-4501, USA*

*2Department of Biomedical Engineering and Mechanics, Virginia Tech, VA 24061, USA*

Short title: Spiracle scaling of beetles

Corresponding author: Jon Harrison, School of Life Sciences, Arizona State University, Tempe, AZ 85287-4501 USA, +1 (480)-965-9459; j.harrison@asu.edu

**Abstract**

The scaling of respiratory structures has been hypothesized to be a major driving factor in the evolution of many aspects of animal physiology. Here we provide the first assessment of the scaling of the spiracles of insects, using ten scarab beetle species differing 180x in mass, including some of the most massive extant insects. Using micro-CT, we measured the cross-sectional area and depth of all eight spiracles. Areas, depths, diffusive and advective capacities of the spiracles scaled isometrically. Because diffusive capacities scale with lower slopes than metabolic rates, the largest beetles measured require 10-fold higher PO2 gradients across the spiracles to sustain metabolism by diffusion compared to the smallest species. Large beetles can exchange sufficient oxygen for resting metabolism by diffusion across the spiracles, but likely no beetles can do so during flight. In contrast, spiracular advective capacities scale more steeply than metabolic rates, so larger beetles should require less pressure to drive sufficient ventilation to meet oxygen demands, assuming similar extraction efficiencies. These data illustrate a general principle of gas exchangers: isometric scaling of respiratory transport structures diminish the role of diffusion but enhance advective capacities as animals increase in size.

**Keywords**

Spiracle, scaling, Scarabaeidae, tracheal system, body size, oxygen transport

**MAIN TEXT**

**Introduction**

As animal species evolve different sizes, many aspects of their physiology and morphology scale disproportionately with one another (allometrically) with consequences for animal behavior, life history, evolution, and diversity (1-3). A driver of this disproportionality lies in the nonlinear scaling of geometry: doubling the radius of a sphere gives quadruple the surface area and octuple the volume; in a similar way, scaling up a small body plan gives drastically altered ratios of surface area, volume, and body length. Since the challenges associated with changes in body size have a geometric origin, they are ubiquitous. As a result, understanding the mechanisms animals use to overcome the effects of changes in geometric proportions remains a pervasive, important, and challenging biological problem. Three related aspects of animal function modulated by allometry are scaling of animal metabolic rates (4, 5), limits on the maximal body sizes of specific taxa (6, 7), and gas exchange strategies (8). For gas exchange, volume of tissue and hence potential gas exchange needs of animals scale with the cube of length (like the sphere), while surface areas tend to scale with the square of length. This leads to a decline in surface area to volume ratio with size. As a consequence, when animals evolve larger sizes, they may need to adapt the proportions of their respiratory structures or increase the use of advection (bulk flow) to avoid facing limitations on their size or metabolic rates.

Limitations on the capacity of larger animals to support oxygen delivery to tissues have been proposed to drive the hypometric scaling of metabolic rates with size, as well as the hypometric scaling of many physiological (e.g. heart and ventilation rates) and behavioral/ecological traits (e.g. territory size, dispersal distance) (1-4, 9). However, competing theories suggest that other factors, such as heat dissipation constraints, nutrient uptake constraints, or performance-safety trade-offs drive the hypometric scaling of metabolic rates and correlated variables, and that evolutionary adaptations of respiratory systems to size allow animals to match oxygen supply to need regardless of body size (10-13). One important step in resolving this controversy is determining how respiratory structures and mechanisms scale. The vast majority of prior studies of the scaling of gas exchange structures have focused on vertebrates, especially mammals, and we have relatively limited information on the scaling of gas exchange structures in invertebrates, despite the fact that most animal species are invertebrates (5, 14). The scaling of the insect respiratory system is of particular interest as aspects of tracheal system structure have been reported to scale hypermetrically, in contrast to the isometric or hypometric scaling of respiratory structures in vertebrates, supporting the hypothesis that possession of a tracheal respiratory system limits insect body size (6, 15-18). Here we report the first study of the scaling of the spiracles of insects, the first step in oxygen delivery from air to tissues in this most biodiverse clade of terrestrial animals.

Gas exchange usually occurs in a series of steps, often a sequence of alternating diffusive and advective processes. The capacity for a respiratory surface to conduct oxygen (diffusive conductance, Gdiff) can be described using Fick’s law, that is

(1) .

K is the Krogh’s diffusion constant for oxygen in the barrier. The diffusive oxygen exchange across the surface (Jdiff, mol sec-1) is given by

(2) Jdiff = Gdiff \* ΔPO2.

ΔPO2 is the partial pressure gradient for oxygen across the exchanger. When gas exchange relies on diffusion across a barrier, either or ΔPO2 must increase to match the increased oxygen demand inherent in a larger body size (a larger relative tissue volume), or oxygen supply will limit metabolic rate. Increases in may be accomplished by either a decrease in diffuser thickness or increase in area. The ΔPO2 from air to mitochondria can be no greater than atmospheric PO2 (approximately 21 kpa at sea level), so this sets an upper limit on the ability of large animals to utilize increases in ΔPO2 to overcome a that does not increase in proportion to oxygen consumption rate.

The scaling of surface area, barrier thickness and ΔPO2 for gas exchangers across species of animals varies with clade and developmental stage. In adult vertebrates, the scaling of the passive diffusing capacity of the lung scales hypometrically, but matches the scaling of metabolic rates (5). However, the scaling of respiratory morphology differs in endotherms and ectotherms (5), as barrier thickness is constant with size in ectotherms, but increasing with size in endotherms (so endotherms must scale surface area of the lung more steeply than endotherms in order to match the scaling of Gdiff to the scaling of metabolic rate. Bird eggs, which rely on diffusion through pores for oxygen, have a different strategy. Larger bird eggs have relatively thicker shells (scaling with mass0.45), increasing barrier thickness with size, likely to mitigate a higher likelihood of mechanical damage (19). Pore area increases proportionally with shell thickness, so Gdiff per pore is relatively constant across egg size, and larger eggs have a higher density of pores (20). The scaling of the Gdiff of the shell overall matches the scaling of metabolic rate, with both scaling hypometrically (19, 20). Pycnogonids (sea spiders) show yet another pattern for the diffusing capacity of their respiratory structures (their legs). Unlike either bird eggs or vertebrate lung membranes, pycnogonid barrier thickness scaled isometrically (7). As for bird eggs, there is an increase in the area-specific diffusing capacity of the leg cuticle of pycnogonids, although the morphological basis remains unclear (7). However, unlike adult vertebrates or bird eggs, increases in diffusive conductance of the respiratory exchanger do not match increases in metabolic rates with size, requiring an increased ΔPO2 across their diffusers which may limit the maximal size of this taxa (7).

Advective steps in gas exchange can occur using either air or fluid media and represent a second broad strategy for delivering gases to tissues. The morphological capacity for a structure to transport a fluid by advection can be described from Poiseulle’s law,

(3) .

Given this, the advective transport of oxygen through the structure is given by

(4) Gadv \* [O2].

[O2] is the concentration of O2 in the fluid. Some examples in mammals illustrate how morphology scales for structures relying on advection. In mammals, the radius of the aorta scales with mass0.375, and the length of the aorta scales with mass0.25 , suggesting that Gadv of the aorta scales with mass1.25 (4 \* 0.375 – 0.25) (21). The tracheal-bronchial system is the advective structure for air transport in vertebrates; radius scales with mass0.39 while lengths scale with mass0.27, suggesting that Gadv for mammalian tracheal systems scale with mass1.29 (22).

The design of the insect tracheal system is fundamentally different from either the vertebrate respiratory system or that of skin-breathing aquatic invertebrates; it remains unclear how scaling of the components of this system match diffusive versus advective purposes. In insects, spiracles provide (usually) gated opening to an air-filled branching system that ramifies through the insect, with oxygen transported in the gas phase (assuming air-filled tracheae) to the most distal surface of the tracheoles, with diffusion then occurring from tracheole to mitochondria (23). Since Krogh’s demonstration that diffusion should suffice for oxygen transport in a relatively large Lepidopteran larvae, diffusion has been considered to be an important mechanism of gas exchange in insects (24, 25). However, most insects supplement diffusion with advection, especially when active (23, 26, 27). The spiracles are potentially an important step in insect gas exchange, since they are relatively small (difficult to see by eye in most insects) and yet must sustain all gas flux. It appears that spiracles are not excessively over-designed, since sealing of just one thoracic spiracle reduces flight metabolic rate in Drosophila (28). At present it is not clear whether spiracles should be designed to match Gdiff, Gadv or some other physiological capacity to metabolic rate. To shed light on this question, here we use micro-computed tomography (micro-CT) (29) to provide the first interspecific examination of the scaling of spiracles, using ten species of scarab beetles spanning two orders of magnitude in mass, including some of the most massive extant species.

**Methods**

*Acquisition of raw micro-CT images*

Seventeen individuals of ten species (1-2 individuals per species) of scarab beetles (Fig. 1a) with a size range from 0.097 to 18 grams were obtained via breeders from online sources. The species examined included the following: *Goliathus goliathus*, *Coelorrhina hornimani*, *Dicronorrhina derbyana*, *Mecynorrhina torquata*, *Eudicella euthalia*, *Protaetia orientalis*, *Popilia japonica*, *Trypoxylus dichotomus*, *Dynastes hercules*,and *Cyclocephalis borealis*. Most species had both male and females represented. Most species of beetles were scanned using a SkyScan1172 micro-CT scanner equipped with a Hammamatsu 1.3 MP camera and Hammamatsu SkyScan Control software at Virginia Tech. To maintain tracheal structure in their natural configuration, we used a minimal preparation of fresh samples (30). All beetles were killed using ethyl acetate fumes, stored at 4°C, and scanned within three days. They were warmed back to room temperature to avoid motion artifacts from fluid flow, placed in x-ray translucent polyimide tubing (Kapton, Dupont), and centered on a brass stage with putty. Power was set at 10W, voltage was adjusted for optimum brightness and contrast (70-96 kV), with currents between 104-141 μA. Beetles were scanned with 0.4° rotation steps for 180° with frame averaging. A flat-field correction was applied to all scans to account for subtract aberrations. All images had 1024x1280 pixel resolution, yielding a scaling of 12-98 μm/pixel that was independent of beetle size. Small beetles could be captured in a single scan, but larger beetles were scanned in segments along their longitudinal axis by varying their position relative to the beam.

*Dynastes hercules* were too large to be scanned with the same instrument, so these beetles were imaged using an in-house-built bench-top micro-focus x-ray computed tomography (micro-CT) platform (see (31, 32) for details). The x-ray tube (Oxford Instrument, Inc.) was operated at 70 kV and tube power was fixed at 20 W. Images were collected with an X-ray flat-panel detector (model C7921, Hamamatsu, Inc.) operated at 1x1 binning mode, with a detector element size of 50 x 50 µm. The axial scanning field-of-view (FOV) was 37.2 mm in diameter. In each scan, images were collected at 0.5° intervals as the beetle was rotated through 360°, resulting in a total of 720 x-ray projections per scan. Because the specimen was larger than the field of view, multiple scans were conducted consecutively along the animal’s anterior-posterior axis to image the entire body. The axial slice images were reconstructed using the standard filtered back-projection (FBP) reconstruction algorithm, with an image matrix of 1008 x 1012 px and an isotropic pixel size of 36.8 x 36.8 µm.

*Image Reconstruction and Measurements*

Raw micro-CT images were imported into NRecon reconstruction software from SkyScan. Ring artifact and beam hardening corrections were applied where necessary, and contrast was optimized using the software’s interactive histogram feature. For large beetles that required multiple scans, reconstructions were set to align and fuse automatically. Slices generated in NRecon were imported into Avizo 9 for 3D reconstructions.

Spiracles were identified by the characteristic slit-like shape of the opening, and the bellows-shaped air sac behind it (Figure 1b, 1c, 1d). Spiracle locations were confirmed by dissection on representative specimens. Measurements were taken for one of the paired six abdominal and two thoracic spiracles for each beetle (Figure 1b,1c). A few scans had small aberrant regions (e.g. blurriness) due to challenges in scanning, so measured spiracles varied between the symmetric right and left side of an animal based on which region of the scan was best resolved. Diameters of the spiracular opening were measured at the widest point of opening to the outside air in the transverse and sagittal planes; area of the opening was then calculated assuming an elliptical shape with the lengths of the semi-major/minor axes being the diameters described above (Figure 1d). The depth of the spiracle was measured from the outer opening to the interior valve connecting the spiracle to the tracheal trunk (Figure 1d).

*Calculations and Statistical Analyses*

We measured the scaling relationships for each spiracle separately, using log-log plots. As dependent variables in these regressions, we tested log10 transformed spiracular depth, area, area/depth (as an index of the diffusive capacity of the spiracle, see Eq.1), and area2/depth (as an index of the resistance of the spiracle to advective flow, see Eq. 3 below).

We used two statistical approaches to assess the role of the phylogenetic relatedness of the animals in scaling patterns: a phylogentic generalized linear model (pGLS) and a generative Bayesian model. We ran and plotted pGLS reults in R (33-39). The goal of pGLS is to account for non-independence of data points due to phylogenetics in construction of the linear model, and requires a phylogeny of study species(40). We spliced together such a phylogeny from multiple published scarab phylogenies. The branch positions for beetle subfamilies (Dynastinae, Rutelinae and Cetoniinae) were determined using Huntet al.(41*)*. The branches within Dynastinae were placed in the tree using work from Rowland and Miller (42), and the branches of Cetoniinae determined with two trees, one from Mico et al. and the other Holm (43, 44). Four of the genera in this study were present in the tree for Coleoptera constructed by Bocak et al (45), which indicated the same branch places as our spliced tree, providing some positive confirmation for this tree structure. Branch lengths of one were used in the phylogenetic tree because actual branch lengths are not known. Similar to pGLS, we built a Bayesian model assuming that the data were generated by a multivariate normal distribution with covariance matrix given by the amount of shared ancestry between species (amount of shared branch length). See supplemental methods for the details of the model, selection of priors, and python code.

Analyses indicated that the parameter characterizing the degree of phylogenetic signal in our data (λ) was non-identifiable (supplemental figures 1,2); this means that our data does not inform this parameter and it could take on any value from zero (no phylogenetic signal) to one (strong phylogenetic signal) with similar probability. Hence, we opted to omit the use of phylogenetic covariance from our models since 1) the total non-identifiability made selecting a single λ via maximum likelihood for the frequentist pGLS dubious and 2) including it added no explanatory value to our Bayesian regression (parameter samples for λ were essentially straight from the prior). We instead used nonparametric bootstrapping (10,000 bootstrap replicates with ordinary least squares regression slopes/intercepts/residual standard deviation as the summary statistics) to obtain confidence intervals for our slope and intercept values. Additionally, we performed a Bayesian linear regression. Our model was a normal likelihood with mean given by a line with slope and intercept parameters. To obtain parameter estimates, we sampled using the Stan implementation of Hamiltonian Monte Carlo (cmdstanpy). See supplemental methods for the details of the model, selection of priors, and python code.

We defined isometric scaling as scaling as follows:

mass0.67 for areas

mass0.33 for area/depth

mass1 for area2/depth,

according to basic principles of geometry (assuming mass is proportional to volume). We observed whether the 95% confidence interval given by bootstrapping/parameter samples for the slope of our measures of spiracle morphological overlapped the isometric prediction. To produce any *p* values, we calculated the number of bootstrap replicates with test statistic at least as extreme as a particular value of interest, e.g. slope compared to isometry.

The diffusive capacity of a spiracle (Gdiff, nmol sec-1 kPa-1) at 25°C was calculated as:

with D (the diffusivity constant for O2 in air) = 0.178 cm2 sec-1 (46) and β (the capacitance coefficient for oxygen in air) = 404 nmol cm-3 kPa-1 (47). For a diffusive system,

1. Oxygen consumption rate (nmol s-1) = ∆ PO2 \* Gdiff,

with ∆PO2 = the partial pressure gradient for oxygen across the spiracle (kPa). Advective capacity (Gadv, m3 s-1•kPa-1) was calculated as:

assuming a dynamic viscosity of air of 1.86 \* 10-8 kPa sec (46). To calculate total diffusive or advective capacity per beetle, the diffusive/advective capacity for all eight spiracles was summed and doubled (to obtain the total for both sides of the animal).

To calculate the ∆PO2 across the spiracles needed to supply beetle metabolic demand by diffusion, the metabolic rate for a quiescent beetle at a body temperature of 25°C of a given mass was estimated from (48) with the following equation: log10(metabolic rate (μW)) = 3.2 + 0.75•log10(mass (g)) and the assumption of 20.7 kJ/L for O2. For O2 at 25°C, 24.5 mol/L was also assumed. Based on (49), flight metabolic rate of small insects is in the range of 8x resting, whereas it is on the order of 32x resting metabolic rate in large insects. Most scarab beetles are endothermic during flight, so flight metabolic rates of these warm beetles could double this value to 64x with a 10°C increase in thoracic temperature if the Q10 is 2. Even this calculation may be a conservative estimate of hovering metabolic rate, as oxygen consumption rose to 90x higher than those of quiescent, 25°C fig beetles in one study (50), and maximal flight metabolic rates may be 1.25-2x higher than during hovering (51-54). Therefore, we estimated maximal aerobic metabolic rate during flight as 90x those of quiescent beetles. The required PO2 gradient across the spiracles to support gas exchange by diffusion at rest and during flight was calculated by rearranging equation 2 and performing unit conversions as follows:

**Results**

All spiracles scaled isometrically for their area, depth, area/depth, and area2/depth (figure 2, supplemental figure 3-7). Some example regressions with confidence intervals for the slopes are shown in figure 2, illustrating scaling isometry, the larger size of the mesothoracic spiracle, and the tight size distribution of the more anterior spiracles as compared to the posterior. The mesothoracic spiracle was much larger than any of the other spiracles consistent with the general trend of increasing spiracular area closer to the anterior of the animal (supplemental figure 3). The area of the mesothoracic spiracles was approximately four times larger than both the metathoracic spiracles, and abdominal spiracles 1-3, and abdominal spiracles 4-6 were approximately half the size of the more anterior abdominal spiracles (supplemental figure 3). Not only were anterior spiracles larger than posterior, but also had much lower variability around the trend line within the species assayed in this study (figure 2abc). In comparison, the depth of the spiracles showed much less variability in tightness of the distribution around the scaling trend lines (figure 2def).

As with individual spiracles, the combined diffusing capacity of all the spiracles scaled isometrically (Fig. 3a). However, because this slope was less than the scaling exponent for metabolic rate (slope of 0.75 shown as the repeated light grey background lines), the required pO2 gradient across spiracles necessary to supply oxygen consumption by diffusion increased an order of magnitude from the small to the large scarabs (Fig. 3b). The advective capacity increased with a positive slope that was greater than the scaling exponent for metabolic rate (slope of 0.75, light grey lines) (Fig. 3c).

**Discussion**

Spiracles scaled isometrically. Isometric scaling of diffusive capacities means that diffusion becomes increasingly less able to meet oxygen demands in larger beetles, with the required gradient for oxygen transport by diffusion through the spiracles increasing by an order of magnitude over two orders of magnitude in body mass. Conversely, our data demonstrate that the advective capacities of the spiracles scale more positively than do metabolic rates. This result strongly suggests that, if insects switch from diffusive to advective gas transport, there is no physical constraint associated with spiracular gas exchange that limits insect size and metabolic rates.

It is important to note that isometry does not occur universally for tracheal structures, or consistently across clades. Comparing tenebrionid beetles inter-specifically, the leg tracheae scale hypermetrically, but the head tracheae scale isometrically (6). Within a bumblebee species, one spiracle scales isometrically (16). In the leg of growing locust (*Schistocerca americana*), the diffusing capacity of the large longitudinal tracheae of the leg scales hypometrically (17), while in a growing caterpillar (*Manduca sexta*), diameters of most tracheae scale isometrically (55). Why different scaling patterns are observed in these different cases is unclear; more in-depth analysis of the required gas transport and the mechanism of transport are needed to evaluate the scaling of individual tracheal system structures.

Diffusive capacities of the spiracles scaled with mass0.39, well below the scaling slope for oxygen consumption rate (approximately 0.75); thus, diffusion across the spiracles becomes more challenging for larger insects. The required O2 gradient across the spiracles to supply the metabolic demand by diffusion increases by approximately an order of magnitude from our smallest to largest beetles, but the size effect on the required PO2 gradient is less important than the effect of activity. For quiescent beetles, the PO2 gradients across the spiracles necessary for diffusion are low (0.05-0.46 kPa depending on size). However, during endothermic flight, the required PO2 gradient across the spiracles increases from 5 to 41 kPa which is impossible to achieve, as air has only 21 kPa PO2. Because the distance required for oxygen diffusion from air to the flight muscle is much greater than the distance through the spiracle, these data illustrate the impossibility of diffusion sustaining oxygen consumption during flight in these beetles, regardless of size. With metabolic rates scaling with mass0.75 and spiracular depth with mass0.33, spiracular area would need to scale with mass1.08 (0.75 + 0.33) to conserve the required PO2 gradient to support diffusion across all insect sizes.

By contrast, the advective capacity scales with mass1.1, much higher than the slope for the metabolic rate, so oxygen delivery via advection becomes easier for larger animals even as diffusion becomes impossible. This effect might reflect a shift toward increasing reliance on advection as scarab beetles increase in size; alternatively, this increasing advective capacity relative to metabolic rate may allow larger beetles to require lower pressures to drive advection through the spiracles in larger beetles, assuming no scaling of extraction efficiency.

We also note that we observe much tighter distributions in the scaling pattern for the area of the large anterior spiracles as compared to the smaller posterior ones. This may suggest that the large anterior spiracles are more constrained in their morphology, since they presumably provide the gas exchange needed for metabolically demanding tissues like the flight muscle and brain.

*Caveats*: Our finding of isometric scaling of spiracles should be taken critically. Insect spiracles are morphologically complex structures. We analyzed air transport capacities treating the spiracle as a cylinder, which could over or underestimate capacity depending on factors like valve position and the complex shape of the spiracular atrium. The assessment of spiracles of living insects could offer insights not possible with static CT scans. For example, insects might control the shape of the bellows-like atrium and valves in a concerted way to promote air flow. As yet, we know little about how the tracheal system structure and function might scale differently in different species. As an example of a fairly dramatic difference in tracheal system function across beetle clades, some Cerambycid beetles use draft inward ventilation through the mesothoracic spiracle during flight, whereas most scarab beetles autoventilate the thorax using wing movements (56, 57). Dung beetle species vary between exhaling nearly all to none of their air out the mesothoracic spiracles, with species from more arid environments exhibiting more expiration via the mesothoracic spiracle (58). The phylogenetic, life history, and environmental influences on tracheal system structure, function, and scaling seem likely to be a ripe area for future research.

Our finding of isometric scaling of insect spiracles would appear to differ from reports for tracheae of mammals, in which radius scales with mass0.39 and lengths with mass0.27 (59). However, confidence limits from our study included these scaling slopes. Tenney and Bartlett’s study (59) had greater power, as it examined 43 species ranging over 5 orders of magnitude in body mass. However, it worth noting that they did not consider error in their slope estimates, test for statistical differences in slopes between the radii and lengths, or consider phylogeny, so the conclusion that mammalian tracheae scale non-isometrically (and differently from insect spiracles) could benefit from rigorous comparative analysis.

*Conclusions*: Insect spiracles scale isometrically in beetles, which means that diffusive capacities increase much less than metabolic rates, while advective capacities increase more rapidly than metabolic rates. These are general principles of gas exchange that should apply to respiratory structures of any animal clade exhibiting isometric scaling. Nonetheless, resting oxygen consumption rates of the largest beetles can likely be supplied by diffusion, allowing even very large insects to recover from drowning or other forms of anoxia. In contrast, endothermic flight, and likely strong terrestrial locomotion require a strong ventilatory system, especially in larger insects.

**Authors' contributions**

JW helped with study design, collected spiracle measurements from the micro-CT data, carried out statistical analyses, and drafted the manuscript and figures. JFH helped with study design, coordinated the study, and drafting the manuscript. CJK and JJS collected the micro-CT data; additionally, Dynastes data was collected in collaboration with HG and GC. MED helped develop methods for analysis of micro-CT data, and helped with phylogenetic correction analysis. JJS also helped draft the manuscript. All authors gave approval for publication.

**Funding**

This research was supported in part by funds from the School of Life Sciences Undergraduate Research (SOLUR) Program through the School of Life Sciences at Arizona State University, Tempe Campus, and by NSF IOS 1122157 and 1558052.

**References**

1. Bonner JT. Why size matters: from bacteria to blue whales. Princeton, N.J.: Princeton University Press; 2006.

2. West GB. Scale: The Universal Laws of Growth, Innovation, Sustainability, and the Pace of Life in Organisms, Cities, Economics, and Companies. New York: Penguin Press; 2017.

3. Sibly RM, Brown JH, Kodric-Brown A. Metabolic Ecology: A Scaling Approach: Wiley-Blackwell; 2012.

4. West GB, Brown JH, Enquist BJ. A general model for the origin of allometric scaling laws in biology. Science. 1997;274:122-6.

5. Gillooly JF, Gomez JP, Mavrodiev EV, Rong Y, McLamore ES. Body mass scaling of passive oxygen diffusion in endotherms and ectotherms. Proceedings of the National Academy of Sciences. 2016;113(19):5340-5.

6. Kaiser A, Klok CJ, Socha JJ, Lee W-K, Quinlan MC, Harrison JF. Increase in tracheal investment with beetle size supports hypothesis of oxygen limitation on insect gigantism. Proceedings of the National Academy of Sciences. 2007;104(32):13198-203.

7. Lane SJ, Shishido CM, Moran AL, Tobalske BW, Arango CP, Woods HA. Upper limits to body size imposed by respiratory–structural trade-offs in Antarctic pycnogonids. Proceedings of the Royal Society B: Biological Sciences. 2017;284(1865).

8. Perry SF, Lambertz M, Schmitz A. Respiratory Biology of Animals. Evolutionary and Functional Morphology. Oxford, U.K.: Oxford Press; 2019.

9. Banavar JR, Moses ME, Brown JH, Damuth J, Rinaldo A, Sibly RM, et al. A general basis for quarter-power scaling in animals. Proceedings of the National Academy of Sciences. 2010;107(36):15816-20.

10. Glazier DS. Metabolic scaling in complex living systems. Systems. 2014;2(4):451-540.

11. Harrison JF. Do performance-safety trade-offs cause hypometric metabolic scaling in animals? Trends Ecol Evol. 2017;32(9):653-64.

12. Harrison JF. Approaches for testing hypotheses for the hypometric scaling of aerobic metabolic rate in animals. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2018;315(5):R879-R94.

13. White CR, Kearney MR. Metabolic scaling in animals: methods, empirical results, and theoretical explanations. Comprehensive Physiology. 2014;4:231-56.

14. Peters RH. The Ecological Implications of Body Size. Cambridge: Cambridge University Press; 1983.

15. Harrison JF, Kaiser A, VandenBrooks JM. Atmospheric oxygen level and the evolution of insect body size. Proceedings of the Royal Society B-Biological Sciences. 2010;277(1690):1937-46.

16. Vogt JF, Dillon ME. Allometric scaling of tracheal morphology among bumblebee sisters (Apidae: *Bombus* ): compensation for oxygen limitation at large body sizes? Physiol Biochem Zool. 2013;86.

17. Harrison JF, Lafreniere JJ, Greenlee KJ. Ontogeny of tracheal dimensions and gas exchange capacities in the grasshopper, Schistocerca americana. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology. 2005;141(4):372-80.

18. Greenlee KJ, Henry JR, Kirkton SD, Westneat MW, Fezzaa K, Lee WK, et al. Synchrotron imaging of the grasshopper tracheal system: morphological components of tracheal hypermetry and the effect of age and stage on abdominal air sac volumes and convection. American Journal of Physiology: Comparative, Regulatory and Integrative Physiology. 2009;297:1343-50.

19. Ar A, Rahn H. **Pores in avian eggshells: gas conductance, gas exchange and embryonic growth rate**. Respir Physiol. 1985;61(1):1-20.

20. Toien O, Paganelli CV, Rahn H, Johnson RR. Diffusive resistance of avian eggshell pores. Respir Physiol. 1988;74:345-54.

21. Holt JP, Rhode EA, Holt WW, Kines H. Geometric similarity of aorta, venae cavae, and certain of their branches in mammals. Am J Physiol. 1981;241:100-4.

22. Stahl WR. Scaling of respiratory variables in mammals. J Appl Physiol. 1967;22(3):453-60.

23. Harrison JF, Wasserthal LT, Chapman RF. Gaseous exchange. In: Simpson SJ, Douglas AE, editors. The Insects: Structure and Function. 5th ed. New York: Cambridge University Press; 2013. p. 501-45.

24. Krogh A. Studien über Tracheenrespiration. II. über Gasdiffusion in den Tracheen. Pflügers Archiv. 1920;179:95-112.

25. Hetz SK, Bradley TJ. Insects breathe discontinuously to avoid oxygen toxicity. Nature. 2005;433(7025):516-9.

26. Socha JJ, Forster TD, Greenlee KJ. Issues of convection in insect respiration: Insights from synchotron X-ray imaging and beyond. Respir Physiol Neurobiol. 2010;173S:S65-S73.

27. Wasserthal LT, Cloetens P, Fink RH, Wasserthal LK. X-ray computed tomography study of the flight-adapted tracheal system in the blowfly *Calliphora vicina*, analysing the ventilation mechanism and flow-directing valves. J Exp Biol. 2018;221:1-12.

28. Heymann N, Lehmann F-O. The significance of spiracle conductance and spatial arrangement for flight muscle function and aerodynamic performance in flying *Drosophila*. J Exp Biol. 2006;209(9):1662-77.

29. Iwan D, Kamiński MJ, Raś M. The Last Breath: A μCT-based method for investigating the tracheal system in Hexapoda. Arthropod Struct Dev. 2015;44(3):218-27.

30. Socha JJ, DeCarlo F. Use of synchrotron tomography to image naturalistic anatomy in insects. SPIE 2008;2008(Developments in X-Ray Tomography VI: San Diego, CA, USA):70780A-7.

31. Sharma KS, Gong H, Ghasemalizadeh O, Yu H, Wang G, Cao G. Interior micro-CT with an offset detector. Medical Physics. 2014;41(6):061915.

32. Gong H LJ, Zhou O, & Cao G Medical Imaging 2015: Physics of Medical Imaging, (International Society for Optics and Photonics), p 94124N, editor Implementation of interior micro-CT on a carbon nanotube dynamic micro-CT scanner for lower radiation dose. SPIE Medical Imaging; 2015.

33. Garnier S. Paxkage ‘viridis’ 2016 [Available from: <https://cran.microsoft.com/snapshot/2016-08-05/web/packages/viridis/viridis.pdf>.

34. Orme D. The caper package: comparative analysis of phylogenetics and evolution in R 2018 [Available from: <https://cran.r-project.org/web/packages/caper/vignettes/caper.pdf>.

35. Pinheiro J, Bates D, DebRoy Sa, Sarkar D, Team RC. Linear and Nonlinear Mixed Effects Models 2021 [Available from: {<https://CRAN.R-project.org/package=nlme>}.

36. Harmon LJ, Weir JT, Brock CD, Glor RE, Challenger W. GEIGER: investigating evolutionary radiations. Bioinformatics. 2008;24.

37. Revell LJ. phytools: an R package for phylogenetic comparative biology (and other things). Methods Ecol Evol. 2012;3.

38. Paradis E, Claude J, Strimmer K. APE: Analyses of Phylogenetics and Evolution in R language. Bioinformatics. 2004;20(2):289-90.

39. R-Core-Team. R: A language and environment for statistical computing Vienna, Austria: R Foundation for Statistical Computing; 2016 [Available from: <http://www.R-project.org/>.

40. R. P. Freckleton, P. H. Harvey, M. Pagel. Phylogenetic Analysis and Comparative Data: A Test and Review of Evidence. The American Naturalist. 2002;160(6):712-26.

41. Hunt T, Bergsten J, Levkanicova Z, Papadopoulou A, John OS, Wild R, et al. A comprehensive phylogeny of beetles reveals the evolutionary origins of a superradiation. Science. 2007;318(5858):1913-6.

42. Rowland JM, Miller KB. Phylogeny and systematics of the giant rhinoceros beetles (Scarabaeidae: Dynastini). Insecta Mundi 2012;0258-0263:1-15.

43. Holm E. On the genera of African Cetoniinae: Anisorrhina Westwood 1842, Melinesthes Kraatz 1880 and Inhambane Péringuey 1907. Tropical Zoology. 1993;6(1):165-77.

44. Micó E, Morón MA, Šípek P, Galante E. Larval morphology enhances phylogenetic reconstruction in Cetoniidae (Coleoptera: Scarabaeoidea) and allows the interpretation of the evolution of larval feeding habits. Syst Entomol. 2008;33(1):128-44.

45. Bocak L, Barton C, Crampton-Platt A, Chesters D, Ahrens D, Vogler AP. Building the Coleoptera tree-of-life for >8000 species: composition of public DNA data and fit with Linnaean classification. Syst Entomol. 2014;39(1):97-110.

46. Lide DR, editor. CRC Handbook of Chemistry and Physics. 72 ed. Boca Raton: CRC Press; 1991.

47. Piiper J, Dejours P, Haab P, Rahn H. Concepts and basic quantities in gas exchange physiology. Respir Physiol. 1971;13:292-304.

48. Chown SL, Marais E, Terblanche JS, Klok CJ, Lighton JRB, Blackburn TM. Scaling of insect metabolic rate is inconsistent with the nutrient supply network model. Funct Ecol. 2007;21:282-90.

49. Niven JE, Scharlemann JPW. Do insect metabolic rates at rest and during flight scale with body mass? Biol Lett. 2005;1:346-9.

50. Chappell MA. Thermoregulation and energetics of the green fig beetle *Cotinus texana* during flight and foraging behaviour. Physiol Zool. 1984;57:581-9.

51. Roberts SP, Harrison JF, Dudley R. Allometry of kinematics and energetics in carpenter bees (*Xylocopa* varipuncta) hovering in variable-density gases. J Exp Biol. 2004;207(6):993-1004.

52. Heinrich B. Thermoregulation in bumblebees II. Energetics of warm-up and free flight. Journal of Comparative Physiology. 1975;96:155-66.

53. Wolf TJ, Schmid-Hempel P, Ellington CP, Stevenson RD. Physiological correlates of foraging efforts in honey-bees: oxygen consumption and nectar load. Funct Ecol. 1989;3:417-24.

54. Lehmann FO, Dickinson MH. The changes in power requirements and muscle efficiency during elevated force production in the fruit fly Drosophila melanogaster. J Exp Biol. 1997;200(7):1133-43.

55. Lundquist TA, Kittilson JD, Ahsan R, Greenlee KJ. The effect of within-instar development on tracheal diameter and hypoxia-inducible factors α and β in the tobacco hornworm, Manduca sexta. J Insect Physiol. 2018;106:199-208.

56. Miller PL. The supply of oxygen to the active flight muscles of some large beetles. J Exp Biol. 1966;45:285-304.

57. Amos WB, Miller PL. The supply of oxygen to the active flight muscles of Petrognathus gigas (F.) (Cerambycidae). Entomologist. 1965;98:88-94.

58. Duncan FD, Byrne MJ. The role of the mesothoracic spiracles in respiration in flighted and flightless dung beetles. J Exp Biol. 2005;208(5):907-14.

59. Tenney SM, Bartlett D. Comparative quantitative morphology of the mammalian lung: Trachea. Respir Physiol. 1967;3(2):130-5.

**Figure Legends**

Figure 1. Scarab beetles include large bodied individuals and have eight spiracles. (a) Phylogenetic tree for the scarab beetles used in this study showing size distribution among clades (branch lengths are meaningless). (b) Location of the eight spiracles in the scarab body. (c) 3D reconstruction of the tracheal trunks in the thorax, legs and abdomen of *Dicronorrhina derbyana*; spiracles are shown in white. The larger images of spiracles show the size of the opening (dark in color) compared to the mushroom-shaped (white) atrium behind and the differences in spiracle shape. (d) Transverse x-ray slice through the third abdominal spiracle with diameter, α, and depth, β, measures illustrated.

Figure 2. Isometric scaling of scarab beetle spiracles. Spiracle area scales isometrically (A, B), with much tighter distribution about the isometric model for the large anterior spiracles (e.g. the mesothoracic spiracle in seen in A) as compared to the smaller posterior spiracles that may be less critical for gas exchange to metabolically demanding tissues. C shows estimates for the variability for regression models for the various spiracles (S mesothoracic, T metathoracic, 1-6 abdominal), calculated as the standard deviation divided by 10regression intercept, which represents the spiracle area for a 1g beetle. Black diamond and line show the median and 2.5th-97th residual standard deviation divided by 10regression intercept calculated on non-parametric bootstrap samples. The white diamond and grey interval represent the median and 3rd-97th highest posterior density interval for the standard deviation divided by 10regression intercept calculated from parameter samples from the Bayesian regression. We see a trend towards much higher variability in posterior spiracle area as compared to anterior. In contrast to spiracle area, spiracle depth shows similar variability in all spiracles (D-F) regardless of position.

Figure 3. Scaling of the spiracles is insufficient to conserve diffusive capacities but more than sufficient to conserve advective capacities in large beetles. (a) The log10 of total spiracular diffusive capacity per beetle (nmol s-1 kPa-1) scales hypermetrically, but with a slope significantly less than the 0.75 slope of metabolic demand (slope greater than 0.75 in 0 of 10,000 bootstrap replicates). Metabolic rate slope is shown in light grey. (b) The log10 PO2 gradient (kPa) required to diffusively supply the oxygen demand of beetles increases with beetle size. The upper band shows the range of PO2 gradients across the spiracles needed for diffusive oxygen supply in flying beetles, while the lower line shows the values for resting beetles at a body temperature of 25°C. The scatter points at the bottom of the flight band represent required PO2 gradients across the spiracles if metabolic rates during flight are eight times that of rest; the upper scatter points indicate required PO2 gradients across the spiracles if metabolic rates are ninety times resting values, likely if thorax temperature warms to 40°C and beetles exhibit maximal flight performance. (c) Hypermetric scaling of log10 summed advective capacity (m3 s-1•kPa-1) versus log10(body mass), showing a decrease in resistance to advective gas exchange in larger insects. The advective capacity scales significantly more steeply than the 0.75 slope of metabolic rate (slope higher than 0.75 in all but 93 out of 10,000 bootstrap replicates). Metabolic rate slope is shown in light grey. Equations of regression lines and R2 shown for each plot.

Supplemental Materials

Supplemental figure 1. Non-identifiability of phylogenetic signal parameter in pGLS regression model. Left-hand column shows both OLS regression and pGLS regression models plotted for a given spiracle morphology (log of area, depth, area/depth, or area2/depth) vs log of mass. The Right-hand column shows the log likelihood space for the parameter λ, which scales the off-diagonal covariance terms of the regression model thereby providing an indication of how strongly phylogeny influences the pattern of data distribution. A λ of one indicates strong phylogenetic signal whereas a value of zero indicates no sign of covariance in the data due to phylogeny. The solid red line indicates the maximum likelihood for the parameter used in the model on the left, the dashed red line indicates confidence interval estimates for the parameter value. Note that the log likelihood for the parameter is fairly flat across large section of the possible parameter space, and all confidence intervals on the parameter include zero (no phylogenetic signal). Together, these indicate that many values for λ are similarly likely, and that the strength of the phylogenetic signal is hence non-identifiable (our data does not strongly inform the parameter). Given the weakly-to-non-peaked likelihood distributions, selecting a particular value for λ to use in the model is a largely arbitrary choice, poorly supported by the data.

Supplementary figure 2. Non-identifiability of the phylogenetic signal parameter as indicated by Bayesian modeling. We constructed a generative model for our data (a multivariate normal distribution with covariance matrix given by the phylogenetic signal) which is analogous to the model assumed in pGLS regression. We included a parameter for λ that scaled the off-diagonal terms of the covariance matrix, analogous to pGLS, and sampled from our model for the various regressions of log spiracle morphology versus log mass. We then plotted the prior for the λ parameter (a beta distribution with shape parameters 1.4 and 1.4) against the samples from HMC sampling. This indicated that the samples for this parameter had no shrinkage: the posterior samples for the parameter matched the prior. This indicates that the data does not inform this parameter, a clear indication that the parameter is entirely non-identifiable given our data. Together, with supplemental figure 1, this indicates that the phylogenetic signal is not informative for our models and does not provide useful information or insight to our analysis.

Supplementary figure 3. Slopes, intercepts, standard deviations, and quasi-coefficient of variation with confidence intervals generated via non-parametric bootstrapping or Bayesian regression for all spiracles and area, depth, area/depth, or area2/depth vs mass. All spiracles scaled isometrically. The anterior spiracles are much larger than the posterior ones and hence provide much of the gas exchange capacity for the animal. They also exhibit a much tighter distribution for their area and gas exchange capacity than the posterior ones. The spiracle depth, however, did not show the same level of variation in dimension; all spiracular depths had similar degree of variation. This suggests there may be tighter selective regulation on the morphology of the large spiracles that likely supply the airflow needed for highly metabolically demanding tissue like the flight muscle, legs, and brain.

Supplementary figures 4-7. Regression plots for both Bayesian and non-parametric bootstrap analyses. . The top set of panels provide results for Bayesian linear regression; the light grey bands provide the 80th and 95th percentile for the posterior predictive distribution for the regression model, the dark grey interval shows the 95th percentile for regression line ranges given by the slope and intercept samples, the black line the 10th percentile for these regression lines. The bottom panels show regression results from non-parametric bootstrap replicates drawn from the data, with ordinary least squares regression as the summary statistic applied to the subsamples. The grey interval shows the 95th percentile for the regression lines generated by the bootstrap sampling. The repeated light grey lines in both sets of figures indicate the theoretical isometric slope value for the regression of a given morphological trait.

Supplementary table 1. Raw spiracle measurements.