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Chapter 20
Multigenerational Effects of Rearing Atmospheric Oxygen Level on the Tracheal Dimensions and Diffusing Capacities of Pupal and Adult *Drosophila melanogaster*

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Abstract Insects are small relative to vertebrates, and were larger in the Paleozoic when atmospheric oxygen levels were higher. The safety margin for oxygen delivery does not increase in larger insects, due to an increased mass-specific investment in the tracheal system and a greater use of convection in larger insects. Prior studies have shown that the dimensions and number of tracheal system branches varies inversely with rearing oxygen in embryonic and larval insects. Here we tested whether rearing in 10, 21, or 40 kPa atmospheric oxygen atmospheres for 5–7 generations affected the tracheal dimensions and diffusing capacities of pupal and adult *Drosophila*. Abdominal tracheae and pupal snorkel tracheae showed weak responses to oxygen, while leg tracheae showed strong, but imperfect compensatory changes. The diffusing capacity of leg tracheae appears closely matched to predicted oxygen transport needs by diffusion, perhaps explaining the consistent and significant responses of these tracheae to rearing oxygen. The reduced investment in tracheal structure in insects reared in higher oxygen levels may be important for conserving tissue PO_2 and may provide an important mechanism for insects to invest only the space and materials necessary into respiratory structure.

Keywords Insect • Evolution • Tracheal system • Gigantism

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20.1 Introduction

The correlation between apparent late-Paleozoic hyperoxia and gigantic insect fossils has galvanized interest in the hypothesis that possession of a blind-ended tracheal system limits insect body size, and that historical variation in atmospheric oxygen partial pressure (aPO_2) has controlled the maximal body size of insects. Such evolutionary questions are inherently challenging to address since we cannot yet rear Paleozoic insects in various oxygen levels, and even if this becomes possible through a "Jurassic Park" miracle of molecular genetics, we will be unable to directly test ecological hypotheses for insect gigantism (such as the idea that insect giants were made possible by the lack of large flying vertebrates) without a time machine and some alternative universes at our disposal. Nonetheless, if the physiological characteristics of extant insects, including their developmental and evolutionary responses to aPO_2, do not suggest plausible mechanisms by which hyperoxia might have facilitated gigantism, this hypothesis would be greatly weakened. In addition, there are many reasons for investigating the responses of modern insects to aPO_2, including biomedical interest in the remarkable hypoxia-tolerance of insects, and the need to understand the effects of environmental variation on the ecology and physiology of this important group of animals.

20.2 Hypotheses and Approaches for Testing Links Between aPO_2 and Insect Size

The first hypothesis for why hyperoxia might have allowed evolution of giant insects revolved around the concept that hyperoxia would allow maintenance of an adequate PO_2 for tissues deeper into a larger insect. This argument rests on the concept that calculations suggest that diffusive gas exchange is possible in most insects [25]. All insects tested to date (we have tested insects up to 20 g) can recover from complete short-duration anoxia. Since most insects appear to lack appreciable anaerobic capacity and are therefore completely paralyzed by anoxia, it appears that all insects can achieve the minimal oxygen delivery necessary to jump-start life by diffusion. However, most insects that have been examined utilize some type of convection, including tiny *Drosophila* [27, 38, 39]. Large active insects such as grasshoppers and scarab beetles are highly dependent on convective gas exchange [9, 33]. Second, a simple diffusion-limited system would predict that larger insects would tend to have smaller safety margins for oxygen delivery (higher critical PO_2); this does not appear to be the case [10]. Thus, even though diffusion is clearly important in the blind-ended tracheoles, and perhaps in the distal regions of the tracheal system in general, simple diffusive models do not seem to explain links between aPO_2 and insect size.

Oxygen can affect many aspects of animal performance and development, and thus there are many possible avenues by which aPO_2 might affect the evolution of insect size [11]. At the individual level, on an acute timescale, higher aPO_2 might enhance maximal locomotory capacity, improving survival and fecundity. In support of this idea, hyperoxia sometimes increases locomotory performance of dragonflies and grasshoppers [13, 21]. Reduced ventilation in response to higher aPO_2 would reduce water loss rates. Over a longer, developmental timescale, higher internal PO_2 might increase body size via direct effects on cell size or proliferation [15, 35], or allow insects to grow for a longer period within an instar, since oxygen becomes more limiting as insects grow without molting [7, 8]. Most insects are smaller (and have smaller cells) when reared in hypoxia, but to date there is little evidence for hyperoxia increasing body size developmentally [12, 23].

Large body sizes likely evolve over many generations in response to natural or sexual selection. Thus a key question is whether there are mechanisms by which higher oxygen might facilitate evolution of larger insect size. *Drosophila* evolves larger body sizes in hyperoxia [23]. The mechanism for this effect is unclear; and experiments are needed to determine whether hyperoxia preferentially enhances survival or fecundity of larger *Drosophila*. Hyperoxia does not interact synergistically with selection for large body size; animals evolve the same larger sizes in 21 and 40 kPa aPO_2 [22]. However, hypoxia clearly limits body size in flies during artificial selection for large body mass [22].

Studies of laboratory evolution using model organisms like *Drosophila* are useful for demonstrating the potential for body size evolution in response to aPO_2, but can be criticized on the grounds that patterns of selection in artificial laboratory environments may differ from natural systems. In contrast, comparative studies are potentially useful for capturing macro-evolutionary patterns such as body size differences among clades. To determine whether atmospheric hyperoxia might explain Paleozoic insect gigantism, we need evidence for proximate mechanisms by which aPO_2 might influence the fitness of different-sized insects.

20.2.1 Tracheal System Structure: Scaling and Response to aPO_2

Respiratory structure and function appear to scale differently in vertebrates. An intensive series of studies from many labs over many years have examined respiratory structure allometry and function in vertebrates [6, 17, 18, 42, 43]. In both mammals and other vertebrates, convective ventilation, cardiac output, and tissue oxygen diffusion capacity scale in parallel with metabolic rate across body size, with larger animals having lower mass-specific values (mass scaling exponents in the range of 0.75). Respiratory organ sizes and volumes (lungs, tidal volume, heart, stroke volume) tend to scale isometrically, while frequencies (breathing frequency, heart rate) tend to scale with $mass^{-0.25}$ [2, 37].

In insects, metabolic rate scales with body mass to the 0.6–0.85 in most interspecific studies, so the metabolic scaling pattern is very similar to that observed in vertebrates [4]. However, in contrast to vertebrates, the limited data available suggest that larger insects have a greater mass-specific investment in respiratory structures, and have greater mass-specific ventilation. The first evidence for this trend was presented by Miller in a comparative study of large beetles' respiratory mechanisms [33]. He showed that mass-specific ventilation during flight increases with size in large beetles, leading him to suggest that "*the capacity of the thoracic pump may therefore impose a limitation on the size of flying beetles*". We documented tracheal hypermetry in the leg muscle of grasshoppers (tibia extensor) using histology and electron microscopy [14]. Using an inert gas technique, we showed that whole-body mass-specific tracheal volumes scaled with mass$^{1.3}$ (significantly greater than the isometrically predicted value of 1) in this same species [26]. We then conducted a comparative study of tracheal investment in tenebrionid beetles, using synchrotron X-ray phase contrast imaging. We developed a point-counting method for estimating tracheal system volumes from two orthogonally directed, digitized X-ray images to calculate tracheal volumes [20]. Tracheal volumes scaled with mass$^{1.29}$, significantly above the isometrically predicted value of 1. The percent body volume occupied by tracheae increased from 0.5 % in the smallest beetle (2 mg *Tribolium castaneum*) to 5 % in the largest beetle measured (2 g *Eleodes obscura*). We have recently completed a synchrotron imaging study of tracheal dimensions and function in developing grasshoppers, and shown that tracheal hypermetry occurs in all segments, and that convection scales hypermetrically for inactive animals, again with a scaling exponent of 1.3 [9]. This pattern of increased mass-specific investment in tracheal structure is a surprising finding, especially when contrasted with the patterns documented for vertebrates.

20.2.2 Potential Trade-Offs Associated with Tracheal Hypermetry in Large Insects

> Trends in compensation by differential increase of area-dependent structures cannot be continued indefinitely without producing structural absurdities.—S.J. Gould. Biol. Rev. 1986.

Hypermetric scaling of a structure imposes costs and possibly constraints on larger animals. For example, larger vertebrates must have more upright postures, proportionally larger and thicker skeletons and reduced agility [1]. Tracheal hypermetry may similarly constrain insect size via negative selection effects due to associated trade-offs such as decreased body density, increased respiratory system costs, displacement of other tissues, and exhaustion of internal space available for tracheae.

Increasing animal volume per unit mass could affect many performance aspects, including increasing drag, lever arms for locomotion, or niche space required. Based on tracheal system scaling in grasshoppers, a 1 kg grasshopper would have a 3.7 l volume (<30 % of the density of a 10 mg hopper), greatly increasing the mass-specific need for nutrient investment in the exoskeleton and

tracheal system and likely the susceptibility to breakage [9]. Tracheal system hypermetry could also lead to displacement of other tissues, reducing the animals' performance relative to those with a similar size. Percentage body volume occupied by nonrespiratory tissues falls exponentially with mass, from 92 % in 10 mg grasshoppers to 59 % in 10 g animals, and extrapolated to 27 % in theoretical 1 kg grasshoppers [9]. Potentially, larger grasshoppers' survival or reproduction could decrease relative to smaller individuals due to reduced locomotory, digestive, or reproductive capacities associated with such decreases in relative functional tissue content.

Increasing tracheal hypermetry could also directly limit maximal insect size by filling all available space within key body regions that cannot be expanded for biomechanical reasons. In interspecific comparisons of beetles, the most dramatic example of hypermetry occurred at the connection between legs and body [20]. Across four species of beetles, the leg orifice cross-sectional area scaled with $mass^{0.77}$, but the orifice-penetrating tracheae scaled with $mass^{1.02}$, occupying increasingly larger exoskeleton fractions in larger animals. Extrapolating these trends of tracheal hypermetry to the largest extant beetle, the leg would be 90 % full of tracheae, suggesting a spatial limitation [20]. Studies of the scaling of respiratory and nonrespiratory structures in the largest extant insects are needed to test these extrapolations.

20.2.3 Developmental and Evolutionary Responses of the Insect Tracheal System to aPO_2

The need for tracheal hypermetry in larger insects provides a plausible mechanism by which possession of a tracheal respiratory system might limit insect size, and also has implications for the intriguing question of Paleozoic insect gigantism. The morphology of animal respiratory systems often responds both developmentally and evolutionarily to compensate for changes in aPO_2 [34, 41]. Insects compensate for hypoxia by developmentally increasing the diameters of major tracheae and tracheole numbers; the converse occurs in hyperoxia [16, 19]. As in vertebrates, changes in tracheal dimensions with changing aPO_2 are controlled by HIF (hypoxia-inducible factor) pathway responses. Hypoxia increases the expression of fibroblast growth factor receptors on the tracheae, in response to stabilization of HIF by hypoxia, leading to enhanced tracheal growth [3]. *Drosophila melanogaster* larvae evolve larger tracheae when reared for multiple generations in hypoxia, and smaller tracheae when reared in hyperoxia [16], suggesting that tracheal investment has significant costs (materials, energy, or space) that result in selection against excess tracheal structure. Thus, the higher aPO_2 in the late Paleozoic (*c*. 31 kPa) may have reduced tracheal system investment, and reduced the negative fitness consequences of excessive tracheal investment in giant insects, providing a plausible mechanism by which elevation in atmospheric oxygen level could enable insect gigantism.

Several important questions remain about the pattern of compensatory responses of the insect tracheal system to aPO_2. One is whether the effects transfer across developmental stage. The prior studies that have quantitatively documented compensatory effects of rearing aPO_2 on tracheal dimensions have all utilized embryos or larvae [16, 19, 30], and the tracheal system of the one adult insect studied to date (the grasshopper, *Schistocerca americana*) did not change with aPO_2 [44]. Locke did report that hypoxic rearing of larval *Rhodnius prolixus* resulted in adults with increased and more "robust" wing tracheae; however, metrics of such changes were not provided [29]. Secondly, there is variation in reported responses of tracheal morphology to aPO_2 [10], and the source of this variation is unclear. One possibility is that the magnitude of responses of tracheal dimensions might vary spatially within the insect. In larval *D. melanogaster* there were detectable changes in the diameter of the posterior portion of the dorsal longitudinal tracheae (near the spiracle) but not in the anterior section [16]. Certain tissues, such as the leg muscles and antennal cells, are served by particularly long tracheae, which might be more challenged by decreasing aPO_2, leading to a greater effect on tracheal morphology. Tracheae through which substantial convection occurs might have less need to change dimensions in response to aPO_2; this may explain the lack of response of the grasshopper transverse tracheae to aPO_2 [10].

In this study, we examine the effect of multigenerational rearing in hypoxic (10 kPa), normoxic (21 kPa) and hyperoxic (40 kPa) aPO_2 on the morphology and diffusing capacities of adult and pupal tracheae of *D. melanogaster*. The flies used in this study were from the same lines as a prior study that demonstrated that changes in these aPO_2 environments result in changes in body size [23]. Within the adults, we compared the effect of aPO_2 on the abdominal and femoral tracheae of both males and females.

20.3 Material and Methods

20.3.1 Drosophila *Rearing, Laboratory Natural Selection Protocols, and Atmospheric Oxygen Control*

Wild type *Drosophila melanogaster* cultures (Oregon R strain) were acquired from Carolina Biological Supplies (www.carolina.com) and were reared as previously described [23]. We reared three replicate lines for seven generations in either hypoxia (10 kPa aPO_2) or normoxia (21 kPa aPO_2) or hyperoxia (40 kPa aPO_2) in a laboratory natural selection experiment to investigate the effects of multigenerational rearing on adult body size [23]. Adult flies, taken from one of the three replicate populations during generations 6–7 were used for this experiment.

20.3.2 X-ray Synchrotron Imaging and Image Analyses of Tracheae

Adult flies from generation 6 were shipped from our laboratory to the Advanced Photon Source (APS) at Argonne National Laboratory (ANL) for X-ray examination. Due to rapid pupal developmental rates in *D. melanogaster* mature third instar larvae from generation 7 were allowed to pupate at Argonne to permit imaging of flies during the pupal stage. The fly cultures were packed in gas impermeable containers, sealed and perfused with their rearing aPO_2 level prior to shipping. Prior tests done in the ASU lab showed that the perfused containers could maintain a steady PO_2 for at least 4 days. Upon arrival at the APS laboratories we restored the experimental aPO_2 by perfusing the 10 and 40 kPa containers with air from prepared gas cylinders, so flies were maintained in their treatment aPO_2 until they were imaged.

Flies were imaged at the APS 32-ID beam line using X-ray imaging methods as previously described [20, 40]. Selected flies were killed in an ethyl-acetate bottle and groups of ten individuals per sex were mounted, heads facing up, on narrow strips of Kapton (X-ray transparent polyimide film, DuPont) taking care to position the wings backward so as not to obscure the body, and the legs were straightened ventrally to provide the best view of the leg tracheae. The Kapton strips were then clamped onto an electronic manipulation stage (horizontal, vertical, rotation) to orientate the specimens for examination in the X-ray beam. A 90° side view orientation provided the best view of both leg and abdominal tracheae. Puparia, emerging within a 24 h window, were mounted in rows on small sturdy Kapton sheets (2 cm × 2.5 cm) and positioned in a dorsoventral position in the beam. The X-rays created projection images of the adult flies and puparia on a scintillation screen and the images were then reflected with a prism into a CCD Sensicam recording system for the capturing of high quality still images.

In the adult flies we focused on the metathoracic femoral tracheae and the dorsal tracheal branches coming off the sixth abdominal spiracles of female and male flies (see Fig. 20.1 and also [31] for detailed tracheal layouts). For the pupal-stage flies, we focused our attention on the major thoracic puparium tracheal branches that connect the developing pupa inside the puparium casing with the outside atmosphere for gas exchange (Fig. 20.2). Very early in the pupal stage, within the first few hours after pupation, these tracheal trunks are still connected to the tracheal system of the recently pupated larval tissues, but as the new pupae starts to develop, the tracheal trunks detach from the new pupa while the new pupa starts to develop a tracheal and spiracular configuration similar to that of an adult fly. The two tracheal branches form two ‘snorkels’ that connect the developing pupa to the outside atmosphere.

X-ray images of the flies and puparia were captured in tiff format and converted into jpg format. The converted and resized jpg files were imported in Matlab and a custom programmed Matlab M-file was used to determine the average tracheal diameters for each trachea; the tracheae was manually traced and the average diameter was calculated from the area and length of the polygon and the length of the

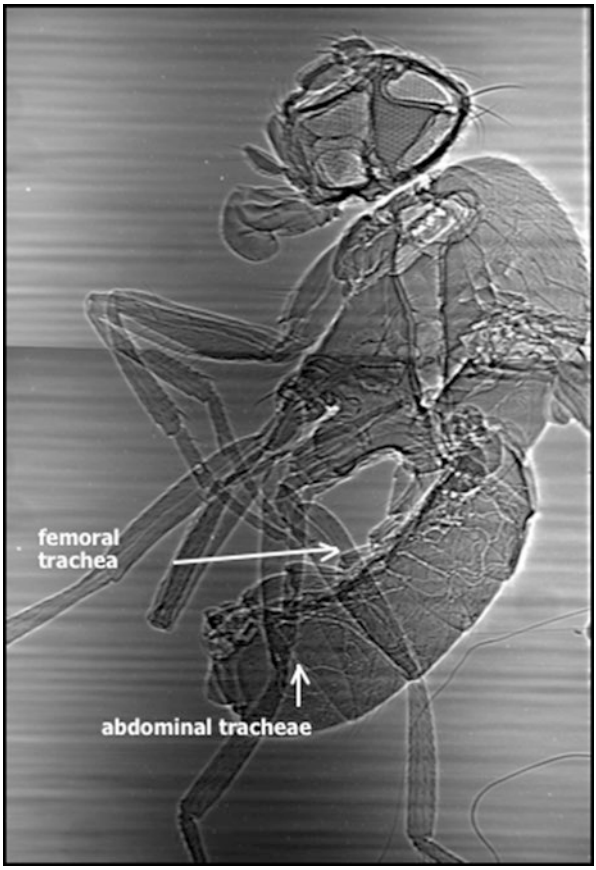

Fig. 20.1 An X-ray image of a male *Drosophila melanogaster* fly reared for six generations at 10 kPa aPO_2. The *arrows* indicate the sixth abdominal and meta-thoracic femoral tracheae measured for this study

tracheae. The lengths of the meta-thoracic femora of the flies and of the puparial thoracic snorkels were additionally measured with Able Image Analysis software (www.mulabs.com).

20.3.3 Tracheal Oxygen Diffusing Capacities

Calculation of oxygen diffusing capacity of an individual trachea requires measurement of both length and cross-sectional area. X-ray images of the adult femoral and puparium snorkel tracheae were well defined and we could get reliable diameter and length measures for those tubes for each adult or pupal individual. However, this was more difficult to do for the adult abdominal tracheae, which curved and branched irregularly. In this case diameters of the adult abdominal tracheae were measured along the best defined lengths available in the X-ray images for the various flies and a mean value of all these lengths (94 μm) was used to calculate a

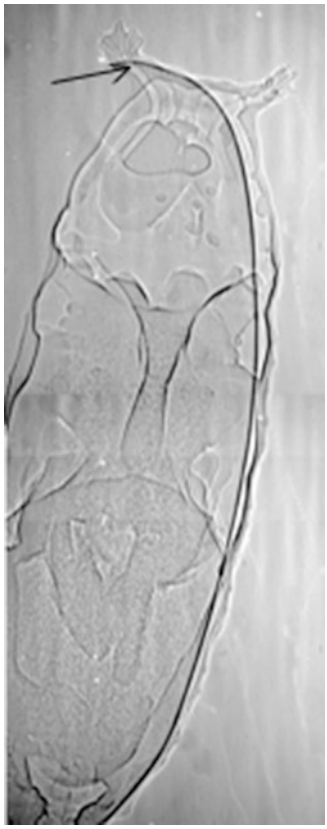

Fig. 20.2 An X-ray image of *Drosophila melanogaster* pupae reared for seven generations at 10 kPa aPO_2. The *arrows* indicate the "snorkel tracheae" whose diameters were measured in this study

standardized estimate of abdominal tracheal oxygen diffusing capacities across all individuals. Oxygen diffusing capacities (GT in nmol kPa^{-1} s^{-1}) along the lengths of tracheae were calculated as:

$$GT = \pi r^2 \times DO_2 \times \beta gO_2 L^{-1} \tag{20.1}$$

where r is the mean tracheal radius, L is the tracheal length, DO_2 is the oxygen diffusion coefficient at 25 °C (0.178 cm^2 s^{-1}, [28]), and βgO_2 is the capacitance coefficient of oxygen in air (4.04×10^5 nmol l^{-1} kPa^{-1}, [36]).

20.3.4 Statistical Analyses

Differences in tracheal diameters and diffusing capacities between the various fly groups were determined either with one way analyses of variance (ANOVA) or with analyses of covariance (ANCOVA). For the femoral tracheae we used ANCOVA, femur length as a covariate since oxygen affected femur size (see below). Male and

female flies were analyzed separately due to significant sexual dimorphic differences in body size. Linear regression analyses of body size indicators on experimental aPO_2 were done to indicate positive, negative or neutral trends in body sizes. All analyses conducted with Statistica 8 (www.StatSoft.com, Tulsa OK).

20.4 Results

20.4.1 Effect of Multigenerational Rearing at Different aPO_2 on Fly Size

After 5–7 generations' rearing at differential aPO_2 the adult body masses and femur lengths were positively related to atmospheric aPO_2 (Table 20.1; see [23] for comprehensive discussion of aPO_2 effects on adult sizes).

20.4.2 Tracheal Dimensions in Adult Flies and Pupae

The aPO_2 differentially affected adult abdominal, adult femoral, and pupal snorkel tracheae (Table 20.2). The diameters of the abdominal tracheae of female flies were statistically unaffected by aPO_2. In males, there was a significant effect of aPO_2, with a significant decrease in diameter for flies reared in 40 kPa aPO_2, but no effect of hypoxic rearing (Table 20.2). In contrast, the diameters of the femoral tracheae were affected by aPO_2 in both males and females, with diameters increasing in hypoxia and decreasing in hyperoxia (Table 20.2).

In the puparia, the thoracic tracheal trunk lengths showed no relationship with aPO_2 ($F_{(1, 29)} = 0.68$, $p = 0.52$—Overall mean length—481.1 ± 33.8 μm) and were therefore not used as a covariate to correct for the effects of body size. Rearing significantly affected diameters of the snorkel tracheae (Table 20.2). Puparia reared in 40 kPa had significantly narrower tracheae than those reared in either 10 or 21 kPa (Table 20.2).

Table 20.1 Linear regression analyses for the effect of oxygen level on body size parameters for flies reared for 5–7 generations at 10, 21, or 40 kPa aPO_2

Intercept ± SE	Slope ± SE	*F*	df	*p*	R^2
Meta-femur length (μm) on body mass (mg)—♀ and ♂ combined					
484.5 ± 38.71	131.7 ± 39.66	11.02	1, 4	<0.03	0.73
Body mass on aPO_2 at generation 6					
♀ 0.950 ± 0.012	0.008 ± 0.0005	269.38	1, 268	<0.0001	0.50
♂ 0.636 ± 0.010	0.006 ± 0.0004	260.38	1, 178	<0.0001	0.59
Meta-femur length (μm) on aPO_2 at generations 5–7					
♀ 597.9 ± 3.91	1.36 ± 0.15	88.06	1, 115	<0.0001	0.434
♂ 543.6 ± 5.34	1.98 ± 0.20	97.62	1, 118	<0.0001	0.453

Table 20.2 Summary statistics of adult and puparium tracheal dimensions (in μm) after 5–7 generations of laboratory natural selection at variable atmospheric oxygen concentrations

aPO_2	Mean ± SE	Minimum	Maximum	*n*
Oxygen selection group—30♀ + 20♂ per line (3) per generation				
Abdominal tracheal diameters (μm)				
10 kPa ♀	17.05 ± 0.46^{A}	14.38	18.97	10
21 kPa ♀	17.51 ± 0.70^{A}	14.08	20.47	10
40 kPa ♀	16.23 ± 0.42^{A}	14.71	18.86	10
10 kPa ♂	15.47 ± 0.28^{A}	14.78	17.61	10
21 kPa ♂	15.82 ± 0.57^{A}	13.65	18.80	10
40 kPa ♂	13.88 ± 0.27^{B}	13.09	15.61	10
ANCOVA ♀: $F_{(2, 26)} = 1.35$, $p = 0.276$				
ANCOVA ♂: $F_{(2, 26)} = 5.42$, $p = 0.01$				
Femoral tracheal diameters (μm)				
10 kPa ♀	14.17 ± 0.33^{A}	12.79	16.35	10
21 kPa ♀	13.38 ± 0.40^{AB}	11.65	15.40	10
40 kPa ♀	11.88 ± 0.37^{B}	10.35	13.59	10
10 kPa ♂	13.73 ± 0.42^{A}	12.08	15.83	10
21 kPa ♂	13.00 ± 0.23^{B}	11.99	14.25	10
40 kPa ♂	11.20 ± 0.25^{C}	9.51	12.10	10
ANCOVA ♀: $F_{(2, 26)} = 6.20$, $p = 0.0063$				
ANCOVA ♂: $F_{(2, 25)} = 11.34$, $p = 0.0003$				
Puparium tracheal diameters (μm)				
10 kPa ♂	148.37 ± 5.67^{A}	126.22	190.46	11
21 kPa ♂	149.49 ± 4.55^{A}	127.08	175.65	10
40 kPa ♂	130.14 ± 3.79^{B}	104.93	145.54	11
ANCOVA: $F_{(2, 26)} = 5.30$, $p < 0.011$				

Superscripts denote significant differences in tracheal diameters. Meta-thoracic femur lengths were used as covariates correcting for body size differences. The puparium tracheal lengths showed no significant differences (ANOVA: $F_{(2, 29)} = 0.676$, $p = 0.516$)

20.4.3 Tracheal Oxygen Diffusion Capacities

As for tracheal diameters, the responses of diffusing capacities to aPO_2 varied among the tracheae studied (Table 20.3). Diffusing capacities of the abdominal tracheae of females and the puparium snorkel tracheae were not affected by aPO_2 (Table 20.3). For males, the diffusing capacities of the abdominal tracheae were reduced by 14 % in hyperoxia, but were unaffected by hypoxic-rearing (Table 20.3). Femoral tracheal diffusing capacities were strongly affected by both hypoxia and hyperoxia (18–25 % compensatory change; Table 20.3).

Table 20.3 Summary statistics of tracheal diffusion capacities (in nmol kPa^{-1} s^{-1}) for representative adults and puparia after 5–7 generations of laboratory natural selection at variable atmospheric oxygen concentrations

aPO_2	Mean ± SE	Minimum	Maximum	*n*
Abdominal tracheal diffusion capacities (nmol kPa^{-1} s^{-1})				
10 kPa ♀	0.0178 ± 0.0009^{A}	0.0128	0.0225	10
21 kPa ♀	0.0187 ± 0.0015^{A}	0.0119	0.0254	10
40 kPa ♀	0.0160 ± 0.0008^{A}	0.0131	0.0215	10
ANCOVA ♀: $F_{(2, 26)} = 1.48$, $p = 0.246$				
10 kPa ♂	0.0145 ± 0.0006^{A}	0.0131	0.0189	10
21 kPa ♂	0.0152 ± 0.0011^{A}	0.0112	0.0212	10
40 kPa ♂	0.0116 ± 0.0005^{B}	0.0103	0.0146	10
ANCOVA ♂: $F_{(2, 26)} = 4.85$, $p < 0.016$				
Femoral tracheal diffusion capacities (nmol kPa^{-1} s^{-1})				
10 kPa ♀	0.0019 ± 0.0001^{A}	0.0014	0.0024	10
21 kPa ♀	0.0016 ± 0.0001^{A}	0.0012	0.0021	10
40 kPa ♀	0.0013 ± 0.0001^{B}	0.0010	0.0017	10
ANCOVA ♀: $F_{(2, 26)} = 5.67$, $p = 0.009$				
10 kPa ♂	0.0020 ± 0.0001^{A}	0.0015	0.0026	10
21 kPa ♂	0.0016 ± 0.0001^{B}	0.0014	0.0019	10
40 kPa ♂	0.0012 ± 0.0001^{C}	0.0008	0.0014	10
ANCOVA ♂: $F_{(2, 25)} = 8.54$, $p < 0.0015$				
Puparium snorkel tracheal diffusion capacities (nmol kPa^{-1} s^{-1})				
10 kPa ♀	0.29 ± 0.05^{A}	0.13	0.76	11
21 kPa ♀	0.31 ± 0.03^{A}	0.20	0.46	10
40 kPa ♀	0.24 ± 0.02^{A}	0.15	0.39	11
ANCOVA: $F_{(2, 29)} = 0.91$, $p = 0.414$				

Superscripts denote significant differences in tracheal diffusion capacities

20.5 Discussion

20.5.1 aPO_2 and Tracheal Dimensions

In general, our data support the general conclusion that the morphology of the insect tracheal system responds in a compensatory manner to aPO_2. However, responses varied among the various tracheae, and to some degree, among the sexes. Even after 5–7 generations of rearing, the magnitude of these compensatory changes in diffusive capacities were relatively small (Table 20.3), suggesting that either compensation is incomplete (perhaps explaining why aPO_2 affects body size), that the primary sites of compensation occur at other locations (e.g., the tracheoles) or that convection is important in at least some Drosophila tracheae.

At present it is unclear whether the changes in tracheal morphology observed here are due to developmental plasticity or evolution. Tracheal diameters of the major longitudinal tracheae of larvae do evolve in a compensatory manner in response to

aPO_2, showing 8–15 % compensatory changes in diameters and diffusing capacities in response to 6–7 generations of rearing in 10 or 40 kPa aPO_2. To test for evolution of adult tracheal dimensions in response to oxygen, it will be necessary to test animals reared for multiple generations in hypoxia or hyperoxia, and then returned for at least two generations to normoxia to control for developmental and parental effects.

Why do different tracheae respond differently to aPO_2? One hypothesis is that a greater fraction of gas exchange through abdominal tracheae occurs via convection, while transport through the leg may be more diffusive-based. Responses of the fifth and sixth abdominal tracheae to aPO_2 were statistically identical, as were those of the mesothoracic and metathoracic femoral tracheae (data not shown for brevity), supporting the hypothesis that there are consistent differences in the response of abdominal and femoral tracheae to aPO_2. *Drosophila* exhibit abdominal pumping during flight, though it is of small amplitude [27]. *Drosophila* also exhibit ventilation due to proboscis-pumping during flight [27], and it is plausible that some of these pressure gradients carry through to the abdomen. In addition, the abdominal tracheae appeared flexible and were in contact with the digestive tract, and so it is plausible that convection in these tracheae occur due to digestive peristalsis. One can also imagine that convection might occur in the leg tracheae due to compression of the tracheae by contracting leg muscles. However, we did not observe any compressions of these leg tracheae when flies struggled, and the tracheae appear relatively straight and rigid, supporting the possibility that gas transport in the leg tracheae might be primarily diffusive. Are the diffusing capacities of the femoral tracheae sufficient to support adequate gas exchange by diffusion? Resting oxygen consumption rates of these flies averaged about 4 $\mu l\ h^{-1}$ [24] or about 0.05 nmol s^{-1}. While the metabolic rates of walking *Drosophila* have not been measured, we know that walking increases the metabolic rates of insects by 2–5 fold [5], thus the oxygen consumption of a walking fly is unlikely to be higher than 0.25 nmol s^{-1}. It seems reasonable to guess that half of that oxygen consumption would occur in the legs (brain, respiratory and cardiovascular functions will also increase), suggesting that 0.125 nmol s^{-1} might be divided among the six legs; thus oxygen consumption of a single metathoracic leg of *Drosophila* is unlikely to exceed 0.02 nmol s^{-1}. Dividing this predicted oxygen consumption by the tracheal conductance of the femoral tracheae yields a predicted PO_2 gradient down the tracheae for completely diffusive gas exchange of 10 kPa, 12.5 kPa and 15 kPa for the 10 kPa, 21 kPa, and 40 kPa to aPO_2 reared flies, respectively. These calculations suggest that diffusive gas exchange should be able to support aerobic metabolism in the legs of walking flies in normoxia, though perhaps not in 10 kPa aPO_2.

The snorkel tracheae of pupae did not show compensatory changes in diffusing capacities, though diameters were smaller for flies reared in hyperoxia. One plausible explanation for the lack of an effect of to aPO_2 on these tracheae is that they are so large that the PO_2 gradient down them is minimal. The maximal oxygen consumption rates of *Drosophila* pupae are reported to be 0.04 nmol s^{-1} [32]. Dividing half of this amount by the diffusing capacity of a single snorkel tracheae yields a predicted PO_2 gradient down these tracheae of only 0.06 kPa for the normoxic-reared flies. These calculations suggest that compensatory changes in the dimensions of these tracheae are not necessary to compensate for hypoxia.

Hyperoxia had a more consistent effect on tracheal morphology than hypoxia (Tables 20.2 and 20.3). Perhaps this occurs to help regulate internal PO_2, preventing oxidative damage due to excessive tissue PO_2. Alternatively this effect could occur because tracheae have a significant cost (in materials and/or space occupied).

20.5.2 Implications for Insect Gigantism

These results support prior studies showing a generally negative relationship between insect tracheal investment and aPO_2 [10], extending these results by showing that these morphological changes also occur in pupal and adult stages. This response provides a plausible mechanism for how Paleozoic hyperoxia might have facilitated larger body sizes in insects. A reduction in the need for tracheal investment might facilitate larger insects by allowing greater investment in nonrespiratory tissues [11]. The negative relationship between tracheal dimensions and oxygen might also facilitate insect gigantism by relieving spatial constraints. As noted above, larger insects invest proportionally more in their tracheal systems, leading to an increasing fraction of the body being occupied by tracheae. In the leg coxae, extrapolations suggest that the entire available space might be filled by tracheae in the largest extant insects [19]; with such a scenario, hyperoxia might allow insects to achieve larger sizes with the same-sized tracheae.

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