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Plastic and evolved responses of larval tracheae and mass to varying atmospheric oxygen content in *Drosophila melanogaster*

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Summary

Structural changes in the tracheal system during development have the potential to allow insects to compensate for varying oxygen availability. Despite possible compensation, oxygen level during development may also affect insect body size. We investigated how atmospheric oxygen level affects the dimensions of the main dorsal tracheae (DT) and masses of larval *Drosophila melanogaster* (Meigen) reared for up to six generations in 10%, 21% or 40% O_2 at 25°C. Wandering-stage third-instar larvae were weighed every other generation, and the dimensions of the DT were measured. Hypoxia produced significantly lighter larvae after one generation of exposure, while hyperoxia did not affect larval mass. Atmospheric oxygen content did not significantly change the diameters of the anterior portions of the main tracheae; however, the posterior diameters were strongly affected. During the first generation of exposure, tracheal diameters were inversely proportional to rearing oxygen levels, demonstrating that developmental plasticity in DT diameters can partially (8–15%) compensate for variation in atmospheric oxygen level. After multiple generations in differing atmospheres and two further generations in 21% O_2, larvae had tracheal diameters inversely related to their historical oxygen exposure, suggesting that atmospheric oxygen can produce heritable changes in insect tracheal morphology.

Key words: *Drosophila melanogaster*, tracheae, hypoxia, hyperoxia, selection, hypertrophy.

Introduction

Oxygen plays a pivotal role in the development of circulatory and respiratory organs from animals of diverse taxa. For example, vertebrates and invertebrates adapt to hypoxic environments by altering gill morphology [salamanders (Bond, 1960), fish (Chapman et al., 2002) and crustaceans (Astall et al., 1997)], and metabolic demands influence angiogenesis during development in a variety of vertebrates [birds (Snyder, 1987), rats (Snyder et al., 1992) and humans (Ashton et al., 1954; Knighton et al., 1981)]. In insects, gaseous oxygen is delivered to tissues *via* a system of highly branched, interconnected tubules, and evidence suggests that morphological changes in the tracheal system may be an important mechanism by which insects adjust to changes in atmospheric oxygen level. Terminal tracheal branching is increased by hypoxia and decreased by hyperoxia in *Drosophila melanogaster* (Meigen) (Jarecki et al., 1999). Similarly, rearing under hypoxic conditions increases tracheal diameters in *Tenebrio molitor* larvae (mealworms; Locke, 1958; Loudon, 1989). Since higher tissue oxygen levels increase oxidative stress (Sohal and Weindruch, 1996), modulation of tracheal diffusing capacity may help allow maintenance of a tissue oxygen partial pressure (P_{O_2}) sufficient to support aerobic metabolism without the accumulation of detrimental oxygen radicals.

In addition to affecting the tracheal system, rearing oxygen level may also affect insect body size. There is now considerable interest in the possibility that increased atmospheric oxygen level might correlate positively with maximal body size, across historical periods and present-day environments (Graham et al., 1995; Dudley, 1998; Chapelle and Peck, 1999). In support of this hypothesis, adult fruit fly body size is increased by hyperoxia and decreased by rearing in hypoxia (Frazier et al., 2001). Such correlation could be driven by developmental plasticity of body size (as in Frazier et al., 2001) or by evolutionary changes in insect size in response to changes in atmospheric oxygen. Interestingly, because changes in tracheal diffusing capacity could at least partially compensate for change in atmospheric P_{O_2}, plastic or evolved changes in tracheal dimensions seem likely to reduce effects of atmospheric P_{O_2} on body size.

In the present study, we test for both a developmentally plastic response and a heritable response to atmospheric oxygen (10, 21 and 40% oxygen) for dimensions of the main dorsal tracheae (DT) and larval masses of the fruit fly,

D. melanogaster. The simplicity of the system, ease of visualization (the tracheae of larvae are visible through the cuticle with a dissecting microscope) and short generation time make larval fruit flies an ideal model for the study of oxygen effects on tracheal morphology. We hypothesize that, like mealworms, dimensions of the DT are regulated during development in order to compensate for oxygen availability, and such compensation will be heritable and maintained after sustained, multiple-generation exposure to hypoxic or hyperoxic atmospheres. We further propose that oxygen can influence the evolution of body size in fruit flies and can act as a limiting factor if flies are artificially selected for large body size. In one experiment, flies were reared for multiple generations in different atmospheric oxygen levels, with females being randomly mated. In an artificial selection experiment designed to test whether oxygen is limiting, only the largest females were allowed to mate and found the next generation. In these experiments, flies were reared for 5–6 generations in the test oxygen levels and then subsequently reared for two generations in common normoxic conditions. This two-generation common-garden design at least partially controls for plasticity and parental effects and allows us to test for heritable responses of larval mass and DT diameter to atmospheric oxygen level. In this paper, we report the effect of two trials of these experiments on the masses and diameters of the DT of third-instar larvae.

During the 1st, 2nd and early 3rd instars, *D. melanogaster* possess only two functional spiracles, located at the posterior end of the animal, that open into two DT (Manning and Krasnow, 1993). While late 3rd instars use two anterior spiracles in addition to the aforementioned posterior spiracles (Manning and Krasnow, 1993), diameters along the DT may be more affected by P_{O_2} depending on proximity to the posterior spiracles if oxygen affects tracheal dimensions during the earlier stages of development. Unlike many other insects, the spiracles of fruit fly larvae lack valves and do not close except by fine hydrophobic hairs that fold over the spiracular openings when the animal becomes submerged in liquid medium (Manning and Krasnow, 1993). Since the posterior spiracles are continuously open, and the diameters of the spiracles are similar to the diameters of the DT, major resistance to gas exchange must occur in the tracheae and tracheoles. DT diameters tend to decrease posterior-to-anterior by segment in larval flies (Beitel and Krasnow, 2000). Because the posterior sections of the DT must supply oxygen to an entire larva, while the anterior sections of the DT need only supply the cranial regions of a larva, it seems possible that the posterior regions of the DT may be more sensitive to atmospheric oxygen level.

Gas exchange is likely to be primarily diffusive in larval *D. melanogaster* (Krogh, 1920) since these insects are small (<2 mg) and lack air sacs (Manning and Krasnow, 1993). In the present study, we calculate tracheal diffusing capacities of the DT to gain insight into the degree to which changes in diameters of the DT compensate for changes in atmospheric oxygen. Using larval metabolic rates (Berrigan and Pepin, 1995), we also estimate the drop in P_{O_2} from the spiracles to the anterior termini of the DT.

Materials and methods

Experimental design

To test for developmental plasticity, we compared larval body mass and tracheal diameters of *D. melanogaster* reared from egg to the third larval stage in 10, 21 or 40% oxygen. Two trials of this test of developmental plasticity were performed. These evolutionary experiments are part of a larger experiment examining the effect of atmospheric oxygen level on adult body mass in fruit flies.

To test for possible evolved responses, *D. melanogaster* were reared in three different oxygen levels (10, 21 and 40% oxygen) over five to six generations in two trials each of two types of experiments (Fig. 1). In the oxygen selection experiment (OS), females were randomly mated with males; this study is a ‘laboratory natural selection experiment’ (Gibbs, 1999) in which oxygen may serve as a selective agent affecting larval body or tracheal sizes. In the artificial selection experiment (AS), the females with the largest body masses were chosen to mate and found the next generation. After the flies were reared under the OS or AS conditions for 5–6 generations, flies were reared for an additional two generations (generations 7–8) in normoxic (21% O_2) conditions. We tested for significant evolutionary

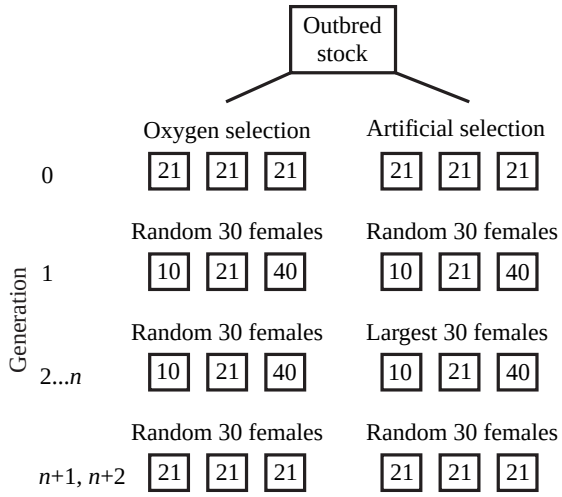

Fig. 1. Experimental design for the multi-generation experiments. In the oxygen selection experiment, 30 randomly chosen female flies were mated and used to found each generation. The same procedure was used to found the first generation and last two generations of the artificial selection experiment, whereas for generations two to *n*, only the heaviest 25% of the females were allowed to mate and found the next generation. For both experiments, flies were reared in 21% O_2 in generation 0 and the final two generations. Flies were reared in 10, 21 or 40% O_2 during all other generations. Two trials of both experiments were performed, each with a different relative humidity. Multiple main dorsal tracheae (DT) diameters were measured in the first trial; only posterior DT diameters were measured in the second trial. See text for details.

effects of rearing oxygen level on larval masses or tracheal diameters by comparing these values within generations 7–8. Investigation of the effect of OS and AS on adult body mass is continuing and these results will be reported elsewhere.

Study organism

To ensure that stock flies were variable and to increase the likelihood that experimental responses observed are general to *D. melanogaster*, rather than specific to a particular strain, our experiments were conducted with fly stocks recently collected from a diversity of locations. *Drosophila melanogaster* used in the first multi-generation trial were captured from the wild by Melanie Frazier in the state of Washington. Fruit flies used in the second multi-generation trial and first plasticity trial were purchased from Carolina Biological Supply Company (Burlington, NC, USA). *Drosophila melanogaster* used in the second plasticity trial were captured from a compost heap in Tempe, Arizona. All populations were maintained in the lab as outbred stocks for no more than five generations prior to starting the experiment.

Rearing conditions

Flies were reared in 237 ml plastic vials containing 50 ml of a diet composed of dextrose, agar, yeast, cornmeal, Tegosept antifungal agent (Genesee Scientific, San Diego, CA, USA) and water. Ascorbic acid (0.1 g per 50 ml media) was also added to the media while boiling to possibly reduce oxygen toxicity effects that the hyperoxic flies may experience (Fridovich, 1998; Sohal and Weindruch, 1996). The plastic vials were housed in 5 liter glass bottles that were all kept in a single temperature-controlled incubator set at 25°C (range of temperatures: 24.2–25.5°C) and lit using a fluorescent lamp set to a 14 h:10 h L:D cycle. At this air temperature, rearing oxygen level has strong effects on adult body size (Frazier et al., 2001).

Drosophila melanogaster were reared from egg to adult in hypoxic (10%; 9.7 kPa O_2), normoxic (21%; 20.4 kPa O_2) and hyperoxic (40%; 38.8 kPa O_2) conditions; the balance of the test gasses was N_2. For the first trial of the multi-generation experiments, airflow from each gas cylinder was kept near 100% relative humidity by bubbling air through a water reservoir. For the second multi-generation trial and both of the plasticity trials, relative humidity was kept near 65% by directing saturated 25°C air through a 19°C water bath to reduce condensation of water along the sides of the rearing bottles. Airflow rates through each glass chamber were set at 15 ml min^{-1}, as measured by a Cole-Parmer flowmeter (model N032-41; Vernon Hills, IL, USA). Excurrent air was monitored using an Ametek S-3A/I oxygen analyzer (Paoli, PA, USA) to confirm that oxygen levels within each chamber were very similar to air coming from the gas cylinders. Measurements using similar vials and flow rates indicate that air within 1 mm of the fly media had oxygen levels within 0.1% of the incurrent air (Frazier et al., 2001).

Virgin collection and mating

Rearing vials were monitored each morning for the presence of newly emerged flies. Because their age was unknown, these newly emerged adult flies were anesthetized using CO_2 and frozen. We collected flies no more than 8–10 h later, to ensure the virginity of collected females (Ashburner, 1989a). We anesthetized flies on a CO_2 flowbed and sorted them by sex. After a 24 h isolation period, we placed 30 females individually in 12×75 mm culture tubes containing two males and 1 ml of fly media. After 24 h, 15 pregnant females were placed in each plastic bottle and allowed to lay for two days; this method ensured that population density remained nearly constant for each generation.

Multi-generation experiment protocols

The protocols for the OS and AS experiments are shown in Fig. 1. At the beginning of each experiment, 180 founding females were removed from the outbred stock and placed in 12 new media bottles, initiating generation 0 (15 females per bottle; two bottles per treatment). These bottles were kept in humidified 21% O_2 chambers until the generation 0 adults began to eclose. One hundred and eighty randomly chosen virgin females were mated and allowed to lay their eggs in 12 new bottles kept in the three different oxygen levels. For each subsequent mating in the OS experiments, 90 of the females (30 per oxygen level) were randomly chosen to found the next generation. For the AS experiments, 120 newly emerged females were removed from their respective oxygen levels, weighed and sorted. The 30 heaviest were mated and allowed to found the next generation. After 5–6 generations, flies were once again randomly chosen from each O_2 level and selection group and were reared for two generations in normoxia.

Collection of larvae

Larvae begin to search for a pupation site during the late third instar when they are approximately 120 h old (Ashburner, 1989b). This behavior facilitated the identification of larvae that were of the same developmental stage and also allowed them to be easily collected from the sides of the plastic bottles. In both the OS and AS experiments, 30 wandering-stage third-instar larvae were removed from each O_2 level and were washed in 100 mOsm sucrose/200 mOsm NaCl to remove food particles and debris. If measurements of the larvae were to be taken at a later date, the animals were kept in the solution and frozen. If the larval masses and tracheal dimensions were immediately analysed after collection, the larvae were gently rolled on a paper towel to dry. Larvae were weighed using a Mettler AE 240 microbalance (±0.01 mg; Mettler-Toledo, Inc., Columbus, OH, USA). Preliminary experiments indicated that freezing did not affect tracheal morphology so, in the second plasticity and multi-generation trials, larvae were frozen after collection and images taken later.

Dimensions of the DT

Individual larvae were placed in separate wells on a microwell plate. Each well contained two drops of isosmotic solution to improve visibility of the DT through the cuticle and

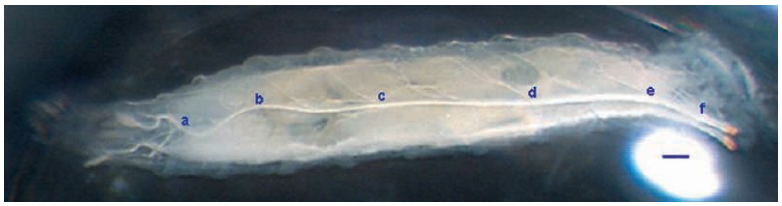

Fig. 2. Image of a third-instar larva. Diameters of the main dorsal tracheae (DT) were measured at (a) the anterior anastomosis, (b) the second, (c) fourth, (d) sixth and (e) eighth transverse connectives and (f) the posterior anastomosis. Scale bar, 0.1 mm.

to prevent desiccation and oxidation of the frozen animals. Images (26× and 70×) were taken with a Hitachi HV-C20M 3CCD camera (Hitachi Denshi, Ltd, Tokyo, Japan) on a Cambridge Instruments dissection microscope (Leica Microsystems Inc., Bannockburn, IL, USA), digitized with a Scion Image Grabber Card (model CG-7; Scion Corp., Frederick, MD, USA) and analyzed using Scion Image software (Scion Corp.). A small piece of 0.18 mm diameter Stren® fishing line placed at the bottom of a microwell was used to convert tracheal dimensions in pixels to μm.

Diameters were measured at six different positions along one of the DT during the first plasticity and multi-generation trials. Measurements were taken at the anterior anastomosis, at the second, fourth, sixth and eighth transverse connectives and at the posterior anastomosis (as defined by Manning and Krasnow, 1993; Fig. 2). Because results in our first trials indicated that most morphological changes occurred in the posterior regions (see Results), only the tracheal diameter near the posterior anastomosis was measured during the second plasticity and multi-generation trials (Fig. 2, between e and f). The lengths of the DT were measured from the posterior to anterior ends. While only the external tracheal diameters could be measured using this procedure, the thickness of the cuticle is assumed to be negligible as measurements of luminal diameters reported by Beitel and Krasnow (2000) are similar to those of this study.

Calculation of diffusing capacity, and P_{O_2} gradients

The mean diffusing capacity (G_{DT}; μmol kPa^{-1} s^{-1}) for oxygen moving longitudinally down a single DT was calculated as:

$$G_{DT} = \pi r^2 \times D_{O_2} \times \beta g_{O_2}/L \ , \quad (1)$$

where r is the mean DT radius, L is DT length, D_{O_2} is the oxygen diffusion coefficient at 25°C (0.178 cm^2 s^{-1}; Lide, 1991) and βg_{O_2} is the capacitance coefficient of oxygen in the air (404 μmol l^{-1} kPa^{-1}; Piiper et al., 1971).

We calculated the mean partial pressure gradient (ΔP_{O_2}; kPa) along the entire length of both DT if gas exchange occurred totally by diffusion as:

$$\Delta P_{O_2} = \dot{M}_{O_2}/2G_{DT} \ . \quad (2)$$

The molar rate of oxygen consumed ($\dot{M}_{O_2}$; μmol s^{-1}) for an active larval fly was estimated as:

$$\dot{M}_{O_2} = MR \times m \times 20.86 \ , \quad (3)$$

where MR is the mass-specific metabolic rate (7.571×10^{-3} J g^{-1} s^{-1}) measured for actively moving third-instar *D. melanogaster* (Berrigan and Pepin, 1995), m is the mass (g) and 20.86 is the conversion factor used to convert J s^{-1} to μmol s^{-1} (21 400 J l^{-1} and 2.24×10^{-6} l μmol^{-1}) assuming a respiratory quotient (RQ) of 1.0 (Berrigan and Lighton, 1993; Berrigan and Partridge, 1997).

Statistical analyses

Statistical analyses were carried out using SYSTAT 10.2 (SPSS, Chicago, IL, USA) software with our experimental type I error less than or equal to 5%. We used analysis of variance (ANOVA) to compare larval responses to different oxygen treatments. *Post-hoc* multiple comparisons of responses within an oxygen treatment over multiple generations were done using a Bonferroni correction. Analysis of covariance (ANCOVA) and linear regressions were also used in the analysis of mass effects on tracheal diameter. Values are shown as means ± S.E.M. unless otherwise noted. *P*-values are not reported when not significant.

Results

Comparing OS and AS effects on larval mass and tracheal diameters

Although artificial selection for large adult female mass (AS protocol) increased adult mass (data not shown), the AS and OS third-instar larvae did not differ in either mass or tracheal diameter. In both the first and second multi-generation trials, the tracheal diameters of AS and OS larvae were very similar (no significant effect of experiment type on tracheal diameter in ANOVA), only differing significantly in two of 18 comparisons, in a non-systematic manner. Body masses of the OS and AS larvae were also not significantly different except in 3 of 18 comparisons, and there was no significant effect of experiment type on larval body mass in ANOVA. Thus, for the

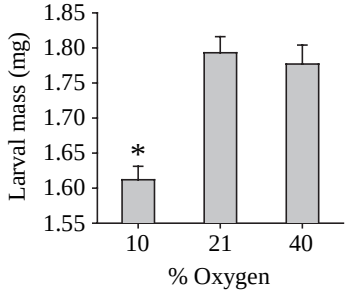

Fig. 3. Larval mass *vs* % O_2 in the plasticity trials. The asterisk denotes significant difference from 21% values (*post-hoc* test, $P<0.05$).

remaining analyses, data from the OS and AS treatments were pooled.

Oxygen effects on larval mass

In general, higher rearing oxygen levels produced larger larval mass in the plasticity trials but did not produce consistent heritable effects on larval mass. In the plasticity trials, there was no effect of trial on larval masses (ANOVA, $F_{1,538}$=0.445, P>0.05); therefore, the data were pooled across trials. After one generation, atmospheric oxygen had significant effects on larval mass (Fig. 3; ANOVA, $F_{2,528}$=10.85, P<0.001). Pair-wise comparisons of the larval masses showed that those raised in 21% and 40% O_2 were different from those raised in 10% O_2 (*post-hoc* Bonferroni corrected, ANOVA, P<0.001) but not different from each other (Fig. 3).

Because the larval body masses from the multi-generation trials were significantly different (ANOVA, $F_{2,1137}$=2.38, P<0.001), these data were not pooled. In the first multi-generation trial including data for all generations, there was no significant effect of oxygen on mass (Fig. 4A). For the second multi-generation trial, there was a significant effect of oxygen on larval mass (Fig. 4B; ANOVA, $F_{2,701}$=9.56, P<0.001), but the directional effect of O_2 differed each generation (ANOVA, oxygen × generation, $F_{8,689}$=6.16, P<0.001). Linear regression analyses showed that tracheal diameters did not significantly scale with mass in any generation or trial.

Position effects on tracheal diameters

The DT were narrower in the anterior portion of the larva (further from the functional spiracles; Fig. 5). Oxygen level affected tracheal diameters in the posterior but not anterior regions of larvae (ANOVA, oxygen × position, $F_{10,466}$=3.03, P=0.001; Fig. 5). Therefore, tracheal diameters were measured only near the posterior anastomosis in the second plasticity and multi-generation trials.

Scaling of tracheal diameters with larval mass

Because the larval masses and tracheal diameters differed significantly by trial, the data could not be pooled. Linear regressions showed that tracheal diameters did not significantly scale with mass in any generation or trial. Within any given trial or generation, there was no significant effect on tracheal diameters in different oxygen levels with mass as a covariate.

Developmental plasticity: effect of single-generation exposures to different atmospheric oxygen levels on tracheal diameters

Tracheal diameters were negatively correlated with rearing oxygen levels in the plasticity trials. Because the second trial used only measures from the posterior region while the first trial averaged all regions, the data were not pooled across trials.

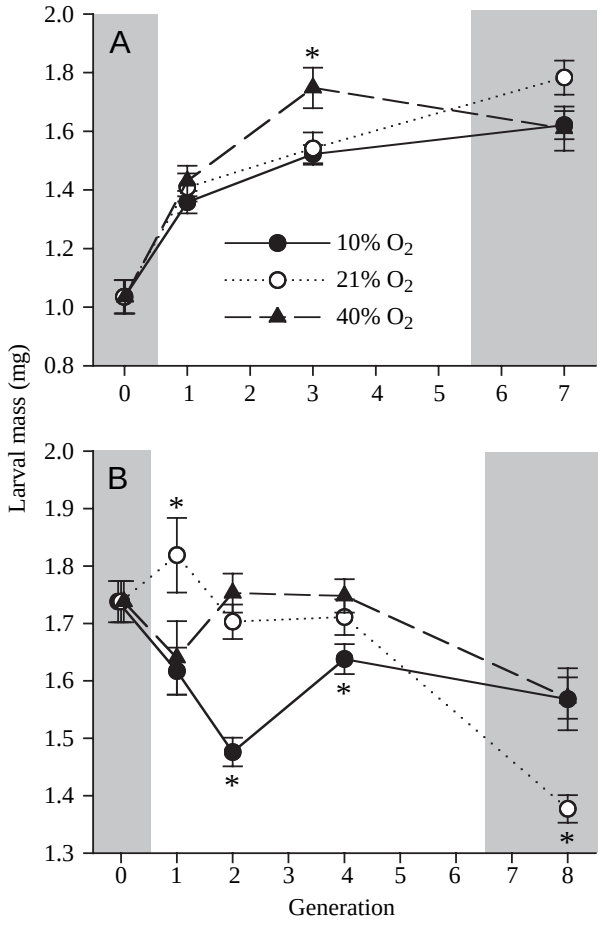

Fig. 4. (A) Larval mass *vs* generation for the first multi-generation trial. Shaded regions indicate generations raised in normoxia. Asterisks indicate significant difference from the normoxic group (*post-hoc* test, P<0.05). (B) Larval mass *vs* generation of the second multi-generation trial. Shaded regions indicate normoxic conditions. Asterisks indicate significant difference from the normoxic group (*post-hoc* test, P<0.05).

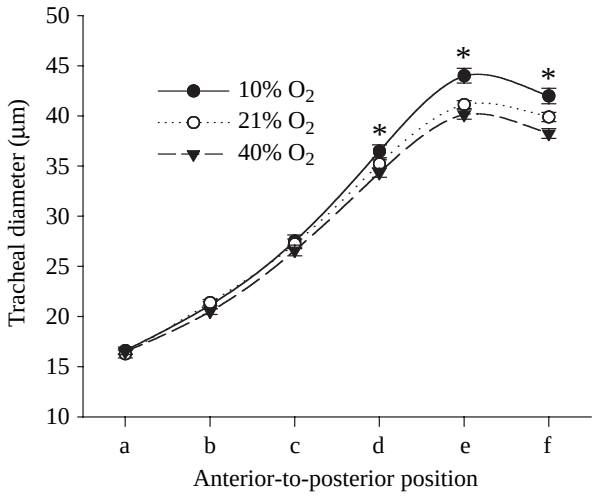

Fig. 5. Tracheal diameters *vs* position in the first plasticity trial (similar patterns were observed in the other trials). Alphanumerical values along the *x*-axis correspond to the six measurement positions described in Fig. 2, with a being the diameter at the anterior anastomosis and f being the diameter at the posterior anastomosis. Lines are defined using spline curves. Asterisks indicate a significant effect (P<0.02) of oxygen on diameter.

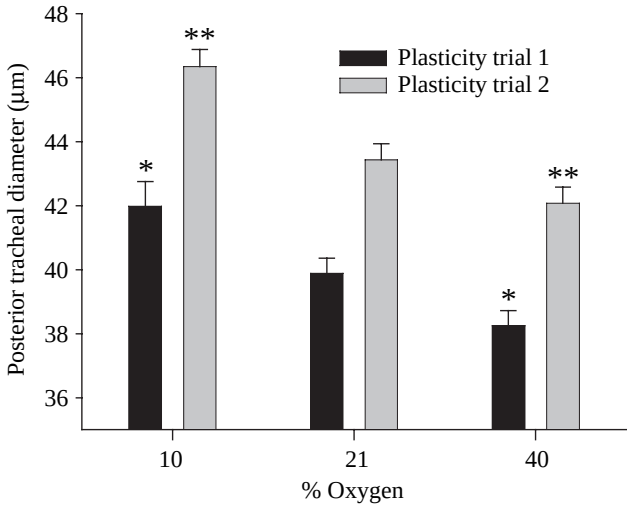

Fig. 6. The posterior tracheal diameters of fruit fly larvae in the two plasticity trials after one generation of exposure to different oxygen levels. * shows a significant difference (P<0.05) from normoxic diameters in trial 1 and ** shows significance (P<0.05) from normoxic diameters in trial 2.

In the first plasticity trial, mean tracheal diameters from hypoxic larvae were 5% larger than those raised in normoxia, and those from hyperoxic larvae were 4% smaller. In the second trial, hypoxic larval tracheal diameters from the posterior region of the animal were 7% larger, and hyperoxic larval tracheal diameters were 3% smaller than their normoxic counterparts (Fig. 6). These changes in the tracheal diameters with oxygen were significant in both trial 1 (ANOVA, $F_{2,83}$=9.92, P<0.001) and trial 2 (ANOVA, $F_{2,164}$=17.76, P<0.001).

Effect of multi-generation exposures to different atmospheric oxygen levels

Rearing oxygen level produced heritable, compensatory changes in tracheal diameters. In all but one generation across both trials, flies raised in 10% O_2 had larger tracheal diameters than those raised in 21% O_2, while those raised in 40% O_2 had the thinnest tracheae (Fig. 7). The negative relationship between rearing oxygen level and tracheal diameter persisted after two generations of rearing in 21% O_2, indicating that rearing in 10% or 40% O_2 for 5–6 generations produced heritable changes in tracheal diameters. In both the first and second trials, there was a significant negative linear relationship between oxygen content and diameter that persisted after all flies were reared in the common-garden conditions (trial 1 – diameter=42.97–0.20O_2, r^2=0.26, P<0.0001; trial 2 – diameter=47.90–0.24O_2, r^2=0.20, P<0.0001).

Effect of rearing oxygen level on tracheal diffusing capacities and required P_{O_2} *gradients*

The diffusing capacities of the DT in the first plasticity and multi-generation trials decreased linearly with similar slopes as

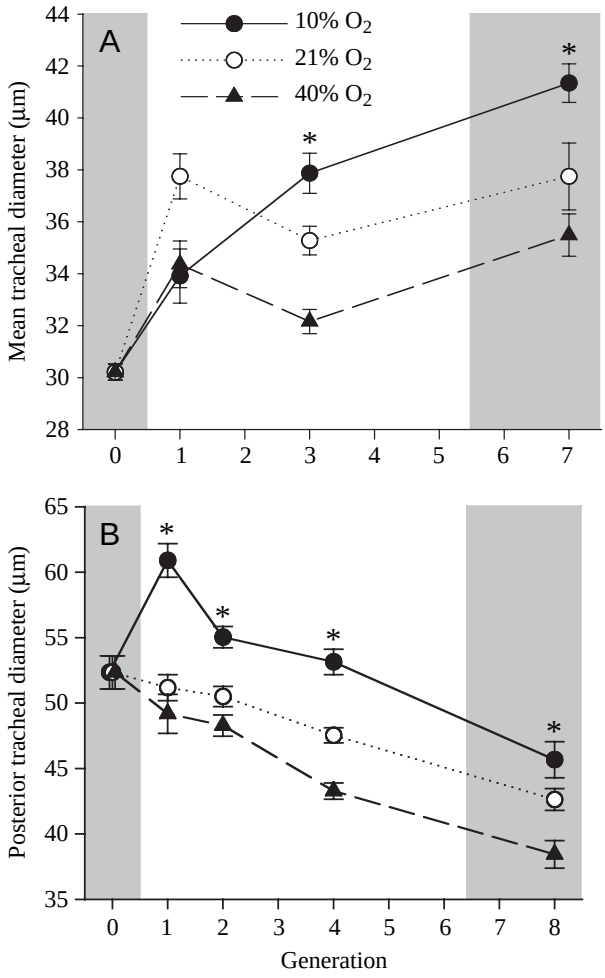

Fig. 7. (A) Mean tracheal diameters over multiple generations during the first trial. Shaded areas indicate the generations during which all treatment groups were raised in normoxic conditions. (B) Tracheal diameters at the posterior anastomosis over multiple generations during the second trial. Shaded areas indicate the generations that were raised in normoxic conditions regardless of treatment line. Generations in which O_2 significantly affects tracheal diameter are marked with an asterisk (P<0.05).

atmospheric P_{O_2} increased (Fig. 8; slopes not significantly different). The changes in diffusing capacity partially compensate for the change in air P_{O_2} (Table 1). In hypoxia,

Table 1. *Oxygen partial pressure gradients required for purely diffusive gas exchange along the length of the main dorsal tracheae (DT) in third-instar* Drosophila *larvae in generation 1 of the first plasticity trial and generation 7 of the first multi-generation trial*

	Diffusion ΔP_{O_2} (kPa)	
% Oxygen	1st Plasticity	1st Multi-generation
10	5.73±0.17	4.53±0.17
21	7.17±0.21	4.82±0.32
40	8.05±0.16	5.47±0.27

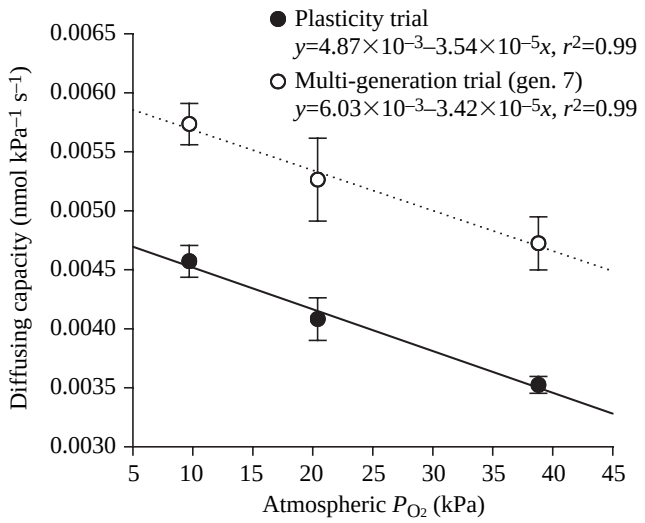

Fig. 8. Diffusing capacities of the main dorsal tracheae (DT) in the first plasticity and multi-generation trials *vs* rearing oxygen partial pressure (P_{O_2}).

diffusing capacity increased 8% in the multi-generation trial and 12% in the plasticity trial, while in hyperoxia it decreased 11% in the multi-generation trial and 15% in the plasticity trial. In both trials, there was a significant effect of oxygen on calculated ΔP_{O_2} across the length of the DT, with the % change in ΔP_{O_2} being similar to the % change in diffusing capacity (plasticity trial – ANOVA, $F_{2,82}$=27.6, P<0.0001; multi-generation trial – ANOVA, $F_{2,57}$=4.51, P<0.02).

Discussion

Phenotypic plasticity: oxygen effects on tracheal dimensions and diffusing capacities

Fruit fly larvae can partially acclimate to altered atmospheric oxygen levels by changing DT diameters. These plastic changes in G_{DT} compensated by 8–15% for the changes in atmospheric oxygen from normoxia (Fig. 8; Table 1). The fact that tracheal diameters change in response to rearing oxygen level in distantly related mealworms (Loudon, 1989) and fruit flies suggests that this is a general response in larval insects, though studies of more basal lineages would be useful to test this hypothesis. In mealworms, only the secondary and tertiary tracheae, not the main longitudinal tracheae, showed compensatory changes in diameter. However, longitudinal tracheae in mealworms may be less directly related to oxygen delivery since in these insects there is approximately one pair of spiracles for each segment. The degree of compensation in G_{DT} of the fruit fly was much less than reported for the mealworm secondary and tertiary tracheae (Loudon, 1989). If all the tracheal compensation for rearing oxygen level occurred in the DT, the G_{DT} should be approximately twice as large in 10% O_2 as seen in normoxia, while in 40% O_2, G_{DT} should be approximately half. However, the regulation of tracheal branching by oxygen (Jarecki et al., 1999) suggests that morphological changes in secondary or tertiary tracheae or tracheoles provide further compensation.

What mechanisms might be responsible for the capacity of tracheal diameters to respond during development to rearing oxygen level? Tracheal diameters generally enlarge during larval growth in insects (Manning and Krasnow, 1993), so oxygen mediation of tracheal diameters may represent modulatory control of a normal morphogenic process. In *Drosophila*, no cell division is observed when tracheal diameters grow during normal development, suggesting that changes in tracheal diameter may be due to changes in tracheal cell size (Madhavan and Schneiderman, 1977) or cell shape. It has been shown that, during the embryonic dilation of the DT, the luminal diameter increases while there is little change in the basal surface of tracheal cells (Beitel and Krasnow, 2000). How might oxygen affect tracheal cell growth? Fruit flies exhibit a heterodimeric basic helix-loop-helix/Per-Arnt-Sim hypoxia-inducible factor (HIF) system analogous to that seen in vertebrates (Lavista-Llanos et al., 2002). Hypoxia induction of levels of the Similar protein (analogous to HIF1-α) is greatest in tracheal cells, demonstrating that the tracheae have cellular mechanisms for sensing and responding to oxygen (Lavista-Llanos et al., 2002). Increases in tracheal length are induced by a nitric oxide/cyclic GMP pathway (Wingrove and O'Farrell, 1999), suggesting that activation of Similar may produce changes in tracheal dimensions *via* this pathway. Increases in tracheal diameter only occur during molts (Beitel and Krasnow, 2000), suggesting that exposure to hypoxia in prior developmental stages and the subsequent activation of Similar may be responsible for the observed hypertrophy of the DT in 3rd instars.

Heritable effects on tracheal diameters and diffusing capacities

Rearing fruit flies for 5–6 generations in atmospheres of 10, 21 or 40% O_2 produced heritable changes in tracheal diameters observable after two generations of rearing in 21% O_2 (Fig. 7). These heritable changes allowed 8–15% compensation for atmospheric P_{O_2} (Fig. 8; Table 1); again, it should be noted that changes in other parts of the tracheal system are likely to have increased the magnitude of compensation by the entire tracheal system. Perhaps due to small differences in experimental protocol such as the use of different humidities and initial fly stocks, diameters seem to increase in one trial and decrease in the other regardless of rearing oxygen. Despite these differences, hyperoxia produced the smallest DT diameters and hypoxia produced the largest diameters in both trials.

Heritable effects of rearing oxygen levels on tracheal diameters suggest that atmospheric oxygen level caused differential mortality or growth rates of flies. Our data suggest that flies that had small-diameter tracheae were more likely to reach adulthood and successfully reproduce in 40% O_2 atmospheres, while flies with larger-diameter tracheae were more likely to reproduce successfully in 10% O_2 atmospheres.

Rearing *D. melanogaster* in 10 kPa P_{O_2} at 25°C reduces

survival by 15% and growth rates by 30% and increases time to eclosion by ~5% (Frazier et al., 2001). Moderate hypoxia may exert these effects by reducing aerobic ATP production and/or by limiting feeding. Hyperoxia also has deleterious effects on flies. Oxygen levels above 45 kPa inhibit development in *D. melanogaster*, with stage sensitivity to O_2 being first instar > later instars > pupae > eggs (Smith and Gottlieb, 1975; Kloek et al., 1976). Reproduction can be inhibited at a P_{O_2} of 38 kPa (Kloek, 1979), though obviously these flies reproduced. Hyperoxia is believed to cause toxicity by stimulating the production of reactive O_2 species that induce teratogenesis and generalized cellular damage (Lane, 2002). For example, in *D. melanogaster*, exposure to pure O_2 at 33 kPa, or O_2 above 45 kPa in nitrogen, induces striking brain degeneration and accumulation of dense bodies in neurons (Philott et al., 1974; Kloek et al., 1978) due primarily to degradation of mitochondria (Miquel, 1998). While the role of reactive O_2 species in aging remains controversial, there is considerable evidence that O_2 consumption and hyperoxia induce reactive O_2 species such as superoxide, hydrogen peroxide and hydroxyl radicals in *D. melanogaster* (Sohal and Weindruch, 1996; Miquel et al., 1975). Prior studies have shown that fruit flies can adapt to hyperoxia (Kloek and Winkle, 1979); our studies indicate that compensatory changes in tracheal diameters are a component of this adaptation.

Oxygen effects on larval mass

In a previous study (Frazier et al., 2001), adult flies were significantly larger (heavier and with longer thorax lengths) in 40% O_2 than in 21% O_2, and similar results have been found for adults in our multi-generation experiments (J. F. Harrison, unpublished data). We found no effect of hyperoxia on larval body masses. The lack of a significant increase in fruit fly larvae masses when reared in hyperoxic conditions seems unlikely to be due to low statistical power since there was not even a trend in this direction. These results suggest that hyperoxic effects on adult body size are due to effects occurring during the pupal stage.

Rearing in hypoxic conditions did cause smaller larval masses in the developmental plasticity study, a result similar to that found for adults by Frazier et al. (2001). In hypoxia, adult flies were roughly 20% lighter than their normoxic counterparts at 24°C (Frazier et al., 2001); third-instar fruit fly larvae had 10% less body mass in hypoxia, again suggesting that effects on the pupal stage may be important in determining the magnitude of developmental plastic response of adult mass. Interestingly, multi-generation exposure to hypoxia produced no evolutionary effects on larval body masses despite strong effects on adult mass (J. F. Harrison, unpublished data), again suggesting that O_2 effects on pupae may be very important.

Diffusion in the DT

The calculated partial pressure gradient for oxygen along the DT if gas exchange occurs by diffusion ranged from 4 to 8 kPa (5–7 kPa for normoxic-reared flies), with the gradient decreasing at lower rearing oxygen levels (Table 1). Thus, in normoxic conditions, a 14–15 kPa oxygen gradient is available to drive oxygen transfer from the DT to the mitochondria. While a much smaller gradient, 4–5 kPa, is available to drive oxygen transfer in hypoxia, it is still sufficient to support metabolism. Moreover, since our calculations do not account for increased branching in the tracheoles, as found by Jarecki et al. (1999), the degree of compensation and, consequently, the available O_2 gradient may be larger.

Conclusions

The flexibility in tracheal diameters in response to atmospheric oxygen in fruit flies suggests that maintenance of tissue P_{O_2} within a relatively narrow range is an important physiological function for fruit flies and perhaps animals generally. Scientific literature suggests that insect tracheal systems are overbuilt relative to need, with gas-phase oxygen delivery allowing high oxygen consumption rates and very large safety margins for oxygen delivery. However, the fact that hyperoxia can increase insect size (Greenberg and Ar, 1996; Frazier et al., 2001) and moderate hypoxia strongly affects growth and survival (Loudon, 1988; Frazier et al., 2001) suggests that tracheal morphology and physiology must be tightly regulated to ensure proper tissue P_{O_2}. Modulation of tracheal diameters provides a mechanism for insects to strike a balance between adequate oxygen delivery and excessive, toxic tissue P_{O_2} levels.

The evolutionary effects of atmospheric oxygen on tracheal diameters that we observed in the lab suggests that variation in atmospheric P_{O_2} has influenced insect tracheal dimensions in nature. These variations in atmospheric P_{O_2} might be historical (Dudley, 1998) or associated with environmental exposure to hypoxic conditions such as high altitude, soils, water or burrows with limited oxygen availability (Hoback and Stanley, 2001). Fruit flies develop in rotting fruits, which may experience hypoxia due to microbial respiration. This capacity for evolutionarily flexible tracheal structures has probably aided the evolution of insect diversity in form and habitat.

List of symbols

AS	artificial selection
D_{O_2}	oxygen diffusion coefficient
DT	main dorsal tracheae
G_{DT}	diffusing capacity
L	length of main dorsal tracheae
m	mass
$\dot{M}_{O_2}$	molar rate of oxygen consumed
MR	mass-specific metabolic rate
OS	oxygen selection
P_{O_2}	oxygen partial pressure
r	mean radius of main dorsal tracheae
RQ	respiratory quotient
ΔP_{O_2}	oxygen partial pressure gradient
βg_{O_2}	capacitance coefficient of gaseous oxygen

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