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Developmental plasticity and stability in the tracheal networks supplying *Drosophila* flight muscle in response to rearing oxygen level

Jon F. Harrison[a,*], James S. Waters[a,b], Taylor A. Biddulph[a], Aleksandra Kovacevic[a,c], C. Jaco Klok[a,d], John J. Socha[e]

[a] School of Life Sciences, Arizona State University, PO Box 874501, Tempe, AZ 85287-4501, USA
[b] Department of Biology, Providence College, Providence, RI 02918, USA
[c] School of Biological Sciences, Monash University, Clayton, Victoria 3800, Australia
[d] Sable Systems International, 3840 N. Commerce St., North Las Vegas, NV 89032, USA
[e] Department of Biomedical Engineering and Mechanics, Virginia Tech, 332 Norris Hall, Blacksburg, VA 24061, USA

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ABSTRACT

While it is clear that the insect tracheal system can respond in a compensatory manner to both hypoxia and hyperoxia, there is substantial variation in how different parts of the system respond. However, the response of tracheal structures, from the tracheoles to the largest tracheal trunks, have not been studied within one species. In this study, we examined the effect of larval/pupal rearing in hypoxia, normoxia, and hyperoxia (10, 21 or 40 kPa oxygen) on body size and the tracheal supply to the flight muscles of *Drosophila melanogaster*, using synchrotron radiation micro-computed tomography (SR-μCT) to assess flight muscle volumes and the major tracheal trunks, and confocal microscopy to assess the tracheoles. Hypoxic rearing decreased thorax length whereas hyperoxic-rearing decreased flight muscle volumes, suggestive of negative effects of both extremes. Tomography at the broad organismal scale revealed no evidence for enlargement of the major tracheae in response to lower rearing oxygen levels, although tracheal size scaled with muscle volume. However, using confocal imaging, we found a strong inverse relationship between tracheole density within the flight muscles and rearing oxygen level, and shorter tracheolar branch lengths in hypoxic-reared animals. Although prior studies of larger tracheae in other insects indicate that axial diffusing capacity should be constant with sequential generations of branching, this pattern was not found in the fine tracheolar networks, perhaps due to the increasing importance of radial diffusion in this regime. Overall, *D. melanogaster* responded to rearing oxygen level with compensatory morphological changes in the small tracheae and tracheoles, but retained stability in most of the other structural components of the tracheal supply to the flight muscles.

1. Introduction

Many insects experience hypoxia during development due to larval or pupal location underground, or within organic material including other animals (Callier et al., 2015; Hoback and Stanley, 2001; Schmitz and Harrison, 2004). Additionally, as juvenile insects develop, growth of oxygen-requiring tissues may lead to localized regions of hypoxia (Callier and Nijhout, 2014), and in some cases, intense aerobic activity or high-altitude environments may also induce tissue hypoxia (Dillon and Dudley, 2014; Harrison et al., 1991; Marden et al., 2012). An emerging body of research has demonstrated that many aspects of the morphology of the tracheal system change in response to hypoxia or hyperoxia, producing compensatory changes in gas exchange capacity that help ensure appropriate local levels of oxygen (Centanin et al., 2010; Harrison et al., 2006; Henry and Harrison, 2004; Klok et al., 2016; Loudon, 1989; VandenBrooks et al., 2012). The insect tracheal system consists of multiple components that vary greatly in size, including the large (up to 2 mm) multicellular conducting tracheae and the small tracheoles (as small as 90 nm in diameter) that are formed from elaborately branched portions of single tracheolar cells. The relative importance of morphological changes in these different sub-sections of the tracheal system to compensatory responses to varied oxygen remains unclear. To date, no studies have examined the effect of rearing oxygen level on both large tracheal and fine-scale tracheolar supply to

Abbreviations: SR-μCT, synchrotron radiation micro-computed tomography; RVLFMT, right ventro-lateral flight muscle trachea; P_{O2}, partial pressure of oxygen; DLFM, dorsal-longitudinal flight muscles

* Corresponding author.
E-mail address: j.harrison@asu.edu (J.F. Harrison).

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an insect tissue. Here, we use two imaging techniques to study structural characteristics of the tracheal system—synchrotron radiation micro-computed tomography (SR-μCT) to assess the morphology of tracheal structures larger than ten microns in diameter, and confocal microscopy to assess the smaller tracheolar structures. These tools enabled us to examine the integrated morphological responses of the tracheal supply to the flight muscle of *Drosophila melanogaster* reared in hypoxia (10 kPa·O_2), normoxia (21 kPa·O_2), and hyperoxia (40 kPa·O_2).

Insects transport and exchange respiratory gases using a tracheal system of branching and anastomosing, thin-walled, air-filled epithelial tubes. Oxygen enters via (usually) occludable spiracles, and then travels via a branching network of tracheal trunks to the metabolically active tissues throughout an insect's body. The tracheae vary greatly in size, structure, and organization within an individual, between developmental stages, and across the great diversity of insect taxa (Harrison et al., 2013a). The terminal tracheal cells form cytoplasmic extensions (tracheoles), which are blind-ended, air-filled tubes that are considered to range from 90 nm to 2.0 μm in diameter, the surface areas of which provide the primary sites for respiratory gas exchange between the environment and active tissues (Hartung et al., 2004; Meyer, 1989; Schmitz and Perry, 1999; Snelling et al., 2011; Wigglesworth, 1983). Transport of gases in the insect respiratory system often occurs by both advection and diffusion, though the relative importance of each process across the various components of tracheal networks remains poorly understood (Harrison et al., 2013b; Huang et al., 2014; Scheid et al., 1981; Socha et al., 2010).

It is now well documented that developmental plasticity of the major conducting tracheae can facilitate physiological compensation for variation in ambient oxygen availability; however, there is substantial variation across species and tracheae within species. In the larvae of the beetle *Tenebrio molitor*, the diameter of several major longitudinal tracheae vary such that diffusive capacities perfectly compensate for changing atmospheric oxygen (10–21 kPa) experienced during ontogeny (Loudon, 1989). Conversely, the spiracular tracheae of larval *T. molitor* (Loudon, 1989), and the transverse tracheae of the adult grasshopper, *Schistocerca americana,* show no changes in morphology when reared at 5–40 kPa O_2 (Harrison et al., 2006). In general, intermediate, partial compensatory changes are the most common response. Compensatory changes (smaller diameters in hyperoxia, larger diameters in hypoxia) occur for leg and abdominal tracheae of adult *D. melanogaster* (Klok et al., 2016), the dorsal-longitudinal tracheae of *D. melanogaster* larvae (Henry and Harrison, 2004), and the sub-cuticular abdominal tracheae of the larval lepidopteran, *Calpodes ethlius* and the larval hemipteran, *Rhodnius prolixus* (Locke, 1958). Although piecemeal, these studies provide some basis to support the hypothesis that distal tracheae, which may be less effectively ventilated (e.g. leg tracheae), exhibit more morphological variation than tracheae located near spiracles that may be more easily ventilated. Diversity in responses across insects and tracheae might occur because some tracheae may have primarily diffusion-based gas exchange and strong compensatory morphological response, whereas others may exhibit stronger ventilatory responses to atmospheric oxygen and thus may not require morphological compensation.

Insect tracheoles can also exhibit compensatory responses to rearing oxygen level. Hypoxia stimulates greater branching and density of tracheoles, whereas hyperoxia reduces tracheolar density (Jarecki et al., 1999; Locke, 1958; Wigglesworth, 1983). Oxygen-mediated developmental responses of tracheae and tracheoles result from hypoxia-inducible factor (HIF)-mediated control of growth factor pathways (Acevedo et al., 2010; Centanin et al., 2010; Jarecki et al., 1999; Lavista-Llanos et al., 2002). While we know that both the tracheae and the number of tracheoles can respond in a compensatory fashion to varied oxygen availability, we currently lack the information necessary to estimate how effective this compensation is, as we lack quantitative information on how hypoxia or hyperoxia affect the diameter, length, and diffusing capacities of the entire tracheal supply to a tissue.

The physiology of flying *D. melanogaster* is particularly well known among insects, facilitating analysis of gas exchange through the flight muscle tracheal system. As for other insects, oxygen consumption rate increases dramatically during flight, mostly due to the activity of the flight muscles, allowing estimation of flight-muscle-specific oxygen consumption rate (Dickinson and Lighton, 1995). *Drosophila melanogaster* is one of the very few insects for which maximal flight oxygen consumption rate has been determined (Lehmann and Dickinson, 1997). The gas transport capacities of the thoracic spiracles, estimated from water vapor flux, maintain low gradients (~1 kPa) for oxygen diffusion across the spiracle; therefore, we know that during flight, P_{O2} in the major trachea should be about 19.9 kPa (Lehmann, 2001). Despite the small gradient for oxygen across the spiracles, blocking even a single thoracic spiracle significantly reduces flight power, demonstrating that there is little safety margin for oxygen delivery (Heymann and Lehmann, 2006). Given the small oxygen gradient across the spiracles, lower conductance steps must occur elsewhere within the tracheal system. During flight, *D. melanogaster* circulate hemolymph within the body cavity and potentially air within the tracheal system of their thorax using a pumping proboscis (Lehmann and Heymann, 2005; Westneat et al., 2008), so oxygen transport to the flight muscle results from both diffusion and bulk flow of air. In contrast to adults, larval *Drosophila* may experience hypoxia, as the culture medium becomes strongly anoxic during development (Callier et al., 2015). However, as late-instar larvae breathe using abdominal spiracles held near the medium-air interface, they may be substantially protected from this type of hypoxia. Despite research spanning more than a century with *D. melanogaster* used as a model organism in biology, only the major tracheal trunks and air sacs of the tracheal system in the thorax of *D. melanogaster* have been previously described (Whitten, 1957). Here, we investigated how the structure of the tracheal supply of adult *D. melanogaster* to the dorsal-longitudinal flight muscles (DLFM) is affected by the partial pressure of oxygen experienced during development, combining the measurement approaches of SR-μCT and confocal microscopy, allowing us to examine tracheal structures from the large conducting tracheae to the small tracheoles. A central hypothesis tested, based on the prior comparative studies, was that most morphological compensation will occur in the small, distal tracheae and tracheoles, rather than in the large, likely well-ventilated primary and secondary tracheae.

2. Methods

2.1. Animals and general conditions

Approximately 80 1–3 day old adult *D. melanogaster* (Oregon R strain) taken from lab cultures maintained as previously described (Klok et al., 2009) were placed into 237 ml plastic bottles with 50 ml of yeasted media to lay eggs for 24 h. These eggs were then transferred at equal, low densities (50–100 eggs per 237 ml bottle) to chambers that were regulated at 10, 21, or 40 kPa oxygen environments using a controller that monitored and adjusted chamber oxygen level every few minutes (ROXY-8, Sable Systems) to regulate the oxygen partial pressure in each chamber (Klok et al., 2009). The oxygen chambers were kept in a walk-in environmental room maintained at 24.5 °C. Adults were removed daily and transferred to a new vial containing standard media. Males used for confocal microscopy were four days past eclosion.

For SR-μCT work, adult flies were placed in fresh vials to lay eggs for 2 h and then removed in order to synchronize larval development. Once the larvae reached the second instar, the vials were packed in Teflon bags filled with 10, 21 or 40 kPa oxygen atmospheres. Immediately upon sealing the bags, they were overnight shipped in a thermally insulated box from Arizona State University to Argonne National Laboratory. Upon arrival, the bags were re-perfused with the required oxygen mixtures from premixed compressed cylinders (during