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RESEARCH ARTICLE

Mass and volume growth of an insect tracheal system within a single instar

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SUMMARY

Organisms must accommodate oxygen delivery to developing tissues as body mass increases during growth. In insects, the growth of the respiratory system has been assumed to occur only during molts, whereas body mass and volume increase during the larval stages between molts. This decouples whole-body growth from the growth of the oxygen supply system. This assumption is derived from the observation that the insect respiratory system is an invagination of the exoskeleton, which must be shed during molts for continued growth to occur. Here, we provide evidence that this assumption is incorrect. We found that the respiratory system increases substantially in both mass and volume within the last larval instar of *Manduca sexta* larvae, and that the growth of the respiratory system changes with diet quality, potentially as a consequence of shifting metabolic demands.

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INTRODUCTION

The balance between oxygen demand and the oxygen supply system is a key physiological factor underlying nearly every aspect of organismal function. In most large animals, physiological supply networks distribute oxygen for metabolism throughout the body, while energy from metabolism is used to fuel key processes such as maintenance, development and growth. Whole-organism growth during the juvenile period in particular is energetically demanding in part because it is expensive to grow new tissues, and metabolic demand increases with total body mass (Glazier, 2005; da Silva et al., 2006; Biswas et al., 2008). As an organism grows, its respiratory capacity must also increase so that new tissues can be supplied with oxygen.

In all insects, oxygen delivery occurs through a ramifying series of hollow tubules, called tracheae and tracheoles, which branch inward from external openings in the exoskeleton called spiracles (Fig. 1) (Chapman, 1998). Tracheae develop embryonically from invaginations of the epidermal tissue of the exoskeleton, subsequently decreasing in diameter with each branching so that the smallest tracheoles supply oxygen directly to tissues and cells (Chapman, 1998; Affolter and Caussinus, 2008; Samakovlis et al., 1996). Tracheae and tracheoles develop by distinct developmental mechanisms that drive branching tubulogenesis during embryogenesis and later as a consequence of oxygen deprivation signaling (Affolter et al., 2003; Uv et al., 2003; Samakovlis et al., 1996; Affolter and Caussinus, 2008; Centanin et al., 2008; Centanin et al., 2010). For simplicity, we collectively refer to the entire tracheal system as ‘tracheae’, though there are systematic physical and structural differences as tracheae branch and decrease in diameter.

The current model of insect respiratory growth and physiology assumes that major changes in the structure and size of the tracheal system are constrained to occur during molts, as tracheae are lined with a layer of cuticle, developmentally homologous with the insect exoskeleton and epidermis (Chapman, 1998; Klowden, 2007; Callier and Nijhout, 2011). During the larval period, all insects undergo a number of discrete stages termed ‘instars’, separated temporally by molts. During a larval molt, the exoskeletal and tracheal cuticles are shed to allow growth of a larger exoskeleton and tracheal system. This molting cycle results in discrete jumps in tracheal branch diameters and volume capacity between juvenile instars (Chapman, 1998; Beitel and Krasnow, 2000; Callier and Nijhout, 2011). However, growth that occurs within a larval instar is assumed to be decoupled from the growth of the respiratory system, and may thus result in oxygen supply limitation (Callier and Nijhout, 2011).

Holometabolous insects in particular (e.g. Lepidoptera, Coleoptera, Diptera and Hymenoptera) can undergo substantial increases in body mass within a single larval instar, with most of the absolute growth occurring in the instar preceding metamorphosis (Davidowitz et al., 2004; Shingleton et al., 2008). Body size increases within all larval instars in both mass (Nijhout et al., 2006; Sears et al., 2012) and volume (Wielgus and Gilbert, 1978; Lin et al., 2011), while tracheae are assumed to remain size-fixed, such that growing larvae may become increasingly oxygen limited as growth progresses (Chapman, 1998; Callier and Nijhout, 2011). However, in the final larval instar of the tobacco hornworm, *Manduca sexta* (Linnaeus 1763) (Lepidoptera: Sphingidae), the conductance of oxygen through the tracheal system has been shown to be higher at the end of the instar than at the beginning, and growing larvae are resistant to effects of reduced oxygen availability (Greenlee and Harrison, 2005; Socha et al., 2010). While behavioral mechanisms may allow for some compensation in oxygen delivery as the larva grows, these mechanisms cannot explain how oxygen is adequately supplied to growing tissues, for example larval fat

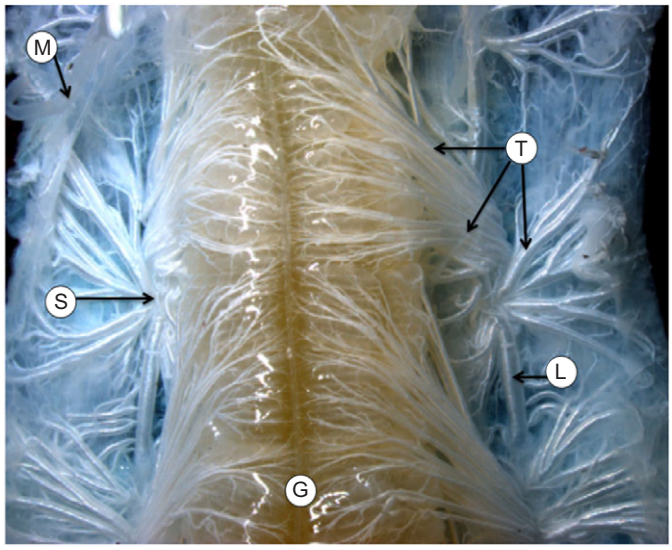

Fig. 1. The tracheae (T; only a subset labeled) of a small (2.2 g, day 2) fifth instar *Manduca sexta* caterpillar, exposed *via* dissection and saline wash. The tracheae are seen as the white–silver branching tubules extending from bundles along the outer body wall. The bundles originate at the spiracle (S; only one labeled) openings and are interconnected by longitudinally running tracheal branches (L; only one labeled). The tan central structure running the length of the larva is the gut (G), which is heavily tracheated. A clear tubular structure in the upper left portion of the picture is a Malphigian tubule (M), and is not a component of the tracheal system.

body. An alternative hypothesis is that tracheal systems may lengthen continuously throughout larval development to accommodate oxygen delivery *via* 'tracheole sprouting' or general increases in the amount of tracheae (Samakovlis et al., 1996; Jarecki et al., 1999; Greenlee and Harrison, 2005); however, quantification of tracheal growth has remained generally inconclusive (Socha et al., 2010).

In this study, we test the assumption that the tracheal system is size-fixed during larval growth in *M. sexta*. We measured dry tracheal mass and volume during the growth phase of fifth instar of *M. sexta* larvae. We predicted that if tracheae are size-fixed within a larval instar, tracheal volume and mass should both remain constant as body mass increases during the growth phase of the instar, i.e. that the slopes of these relationships would not be significantly different from zero. Previous studies have shown that lowered diet quality causes a reduction in metabolic rates (Cruz-Neto and Bozinovic, 2004; Jeyasingh, 2007). If the tracheal system is responding to the demand for oxygen of the body tissues, then altering diet quality should alter the relative demand for oxygen during whole-body growth. This should alter the relative investment in tracheal growth with body size, when diet quality is lowered. Therefore, we assessed dry tracheal mass scaling of larvae when diet quality was reduced. We test for effects of nutritional quality on tracheal growth by rearing larvae on high quality diet continuously, low quality diet continuously or switched from high to low quality diet at the beginning of the fifth instar. If tracheal growth is a product of metabolic demand, then tracheal allometries should reflect changes that would be consistent with changes in diet quality. If tracheal scaling patterns are a consequence of within-instar changes in the tracheal system rather than changes at molting, then the tracheal scaling of the switched diet treatment should be different from individuals reared on constant nutritional conditions.

MATERIALS AND METHODS

Study organism and rearing conditions

We assessed body mass, dry tracheal mass and tracheal volume of *M. sexta* during the feeding phase of the fifth (last) larval instar. Over 90% of larval growth occurs during this last larval instar (Davidowitz et al., 2003; Davidowitz et al., 2004). For tracheal mass measurements, *M. sexta* larvae were fed an artificial wheat germ-based diet *ad libitum* that was either of standard (high) or reduced (low) nutritive quality [see Davidowitz et al. (Davidowitz et al., 2003) for diet ingredients]. Low quality artificial diet differed from high quality diet in two ways. First, nutritive components of the diet (wheat germ, protein, lipid and carbohydrate) were reduced in quantity by 40% of their value in the high quality diet, so that the low quality diet had 60% of the nutritional value of the standard rearing (high) diet. Second, non-nutritive cellulose (Alphacel non-nutritive bulk, MP Biomedical, Solon, OH, USA) was added as mass filler for removed ingredients. For the tracheal mass experiments, caterpillars were hatched from eggs obtained from our laboratory colony at the University of Arizona and reared in an environmental chamber under a 16 h:8 h light:dark photoperiod at 25°C. Cohorts of caterpillars with common egg-laid and hatch dates were kept in 35.5×22×10 cm lidded metal trays. Artificial food and caterpillars were suspended above the bottom of the trays on hardwire mesh platforms (6 mm mesh for instars 1 to 4, 12 mm mesh for instar 5). Cohorts of caterpillars were further subdivided as they progressed from fourth to fifth instar to reduce crowding. Cohorts of caterpillars were checked daily to ensure adequate food supply and cleanliness. For tracheal volume measurements, caterpillars were not reared from eggs, but rather were obtained as fifth instar caterpillars directly from our laboratory colony. Caterpillars in both experiments experienced similar rearing regimes.

Tracheal mass measurements

We sampled 80 fifth instar *M. sexta* caterpillars ranging in mass from 1.50 to 10.73 g. Each individual was weighed (Denver Instrument MXX-123, Bohemia, NY, USA) and euthanized by a brief (<5 s) immersion in boiling water. Samples were then placed in a 2% NaOH solution to dissolve non-chitinous soft-tissues. Samples were submerged in the NaOH solution for a total of 4 days, and the solution was refreshed 2 days into the procedure. Prior to dissolution, small incisions were made on the prothoracic dorsum and abdominal segment 8–10 between the horn and anus to allow solution to enter the body cavity. At the end of the 4 days, samples were gently and thoroughly rinsed with deionized water and then placed in a 30% alcohol solution until dissection.

The tracheal system of each sample was exposed by cutting through the exoskeleton between previously made incisions for tissue dissolution (see above). Longitudinally running tracheae were severed between each body segment, and tracheal branches of each abdominal body segment were removed by gently pulling the tracheal branches and spiracles from the exoskeletal cuticle at the spiracular suture. Tracheae from each segment were then dried for a minimum of 48 h in a 50°C drying oven and weighed (Sartorius Microbalance, Goettingen, Germany). Abdominal tracheal mass was determined by summing the tracheal mass from each abdominal segment.

Tracheal volume measurements

To estimate tracheal volumes of fifth instar *M. sexta* caterpillars, we used a volume displacement method (Wigglesworth, 1950; Callier and Nijhout, 2011). We sampled 30 fifth instar *M. sexta* caterpillars, ranging in mass from 0.96 to 12.69 g. Each individual was weighed

(Metler Toledo ML54, Columbus, OH, USA) and then anesthetized briefly under a carbon dioxide flow (~1 min). Individual caterpillars were placed into a 60 ml plastic syringe, to which a valve stem had been attached. Thirty milliliters of a 0.5% soap solution were drawn into the syringe so that the larva was submerged and excess air bubbles released. With the valve stem closed, the syringe was drawn to the 60 ml mark line and held, displacing air from the tracheal system. Once air bubbles were no longer observed exiting the spiracles, pressure in the syringe was released slowly, allowing the soap solution to enter the tracheae. This process was repeated until no new air bubbles were seen escaping from the spiracles (four to five cycles), and slight pressure was applied to force solution into tracheal tubes. Caterpillars were removed from the syringe. Surface moisture was wicked away. The caterpillars were then weighed a second time. We calculated the relationship between mass and volume of the soap solution (R^2=0.99, N=20) and used the resulting regression equation (tracheal volume=1056.67Δmass–0.2876) to estimate tracheae volume from change in mass.

Exoskeletal mass and body volume measurements

So that we could place dry tracheal mass measures in terms of chitinous growth and tracheal volume measures in terms of whole body mass, we measured the exoskeletal mass and whole body volumes of *M. sexta* in the fifth instar. We sampled 60 *M. sexta* larvae across the size range of the fifth instar and treated them with the soft tissue base dissolution as before (see above). Exoskeletons that remained after the base dissolution were then stretched to full distension and flattened between microscope slides until dried. Each exoskeleton was weighed and then photographed with a Canon DSLR EOS 20D (Melville, NY, USA), affixed to a camera stand. We then measured the length and circumference of exoskeletons digitally using the MATLAB 2012a Image Analysis Toolkit (The MathWorks, Natick, MA, USA). We estimated body volume by modeling the measures as a cylinder, $l\pi r^2$, where l represents axial length and r represents radius calculated from the exoskeleton circumference.

Mass scaling of tracheal system on different quality diet treatments

We reared caterpillars on three artificial diet treatments. Groups of caterpillars were reared on either high or low quality artificial diet treatments for all five larval instars. A third group of caterpillars was reared on high quality diet for instars one through four and then switched to low quality diet for the final fifth instar. Eighty individuals were sampled from each group, and tracheal masses were measured as described above with the exception that all tracheae were extracted (including thoracic tracheae). Tracheal systems were dried and weighed as before.

Statistical analyses

Tracheal mass and volumes were analyzed using an allometric approach, wherein log-transformed values of mass and volume are regressed against log-transformed values of body size. Allometric analysis was performed and the relationship between size measures were described with the allometric equation $y=mx^b$, linearized as $\log(y)=b\log(x)+\log(m)$. The coefficient (b) indicates the scaling coefficient, or slope, describing the relationship between traits x and y. A scaling coefficient value of one indicates an isometric relationship between the traits, i.e. that both traits increase proportionally with one another as body size increases.

For all scaling regressions, we fitted Type II standardized major axis (SMA) regressions to log-transformed values of tracheal size (mass and volume) and body mass (mass and volume) using the package SMATR in R for Linux (v. 2.15.1) (Warton et al., 2006; R Core Development Team, 2012). We compared the fitted scaling coefficients with an expectation of isometry using a slope test for each regression, and we tested for differences in the slope of scaling relationships in cases with multiple comparisons using SMATR.

We estimated the tracheal scaling relationship of dry abdominal tracheal mass against whole body mass. Because the tracheal mass of each abdominal segment was measured, we also determined the tracheal scaling relationship of each abdominal body segment individually. Tracheal cuticle only represents a small proportion of the total body mass, so we also determined the tracheal scaling relationship with exoskeletal mass, which placed tracheal growth in terms of chitinous mass. For each group of caterpillars, exoskeletal mass (M_e) was regressed against whole body mass (M_b) and the resulting equation (M_e=12.53M_b–4.52) was used to predict exoskeletal mass of samples for which the tracheal mass had been measured. Then, log-transformed tracheal mass was regressed against predicted log-transformed exoskeletal mass to determine the allometric relationship of tracheal and exoskeletal growth. The ratio of dry tracheal mass and exoskeletal mass were analyzed as a function of whole body mass to determine how tracheal investment varies in terms of chitinous investment during growth.

We estimated the tracheal volume scaling by regression of tracheal volumes against whole body mass. Then, we regressed body volumes (V_b) estimated from exoskeletons and used the equation V_b=1030.65M_b+888.61 to predict body volumes for samples for which tracheal volumes had been measured. Log-transformed values of tracheal volumes were then regressed against predicted log-transformed body volumes to determine the allometric relationship between tracheal and body volumes. The ratio of tracheal volume to body volume was subsequently analyzed as a function of whole body mass to determine how tracheal volumes vary in terms of total body volume during growth.

Finally, we determined the tracheal mass scaling for larvae that had been reared on the standard diet quality (100%), reduced diet quality (60%) or switched from high to low quality diet at the beginning of the fifth instar. We performed an ANOVA to determine whether body mass, development time and diet quality had effect on tracheal mass. Then, we regressed total dry tracheal mass against body mass for each treatment and compared the slopes using SMATR.

Microscopy of tracheae

We imaged each of the tracheal bundles from one small (day 2, 2.25 g) and one large (day 5, 10.31 g) caterpillar. Each sample was processed with tissue dissolution and tracheal dissection as before. At the time of dissection, the tracheal branches from each spiracle were dissected individually and mounted on 25.4×76.2 mm microscope slides. Tracheae were placed gently onto the microscope slides, and the branches were spread by dripping small amounts of water on the samples. Excess water was wicked away, and samples were allowed to dry, fixing them to the microscope slide. Each bundle of tracheae was then imaged using a Leica M205A dissecting microscope with a Leica DFC400 digital camera (Wetzlar, Hesse, Germany) at 7.8× magnification.

RESULTS

Dry tracheal mass and tracheal volumes increased with whole body mass in fifth instar *M. sexta* caterpillars. Tracheal mass increased from ~1 mg dry mass in the smallest and youngest fifth instar caterpillars to ~9 mg dry mass by the end of larval growth (SMA,

b=0.91, R^2=0.84, P<0.001; Fig. 2A). The slope of this relationship was found to be significantly less than isometry (slope test, r=–0.23, P=0.04), indicating that tracheal mass increased at a slower rate than body mass during growth. This increase in the dry tracheal mass was a consequence of tracheal mass growth in all of the abdominal segments independently, though the scaling relationship observed varied among the abdominal segments (Table 1). Exoskeletal mass increased steadily as whole body mass increased during the growth period: ~12 mg exoskeletal growth per 1 g body mass growth (SMA, b=12.43, R^2=0.93, P<0.001; Fig. 2B). Using this regression, we calculated that tracheal mass scaled with exoskeletal mass hypoallometrically (SMA, b=0.83, R^2=0.84, P<0.001; Fig. 2C) with a slope significantly less than 1 (slope test, r=–0.43, P<0.001). The chitinous tracheal mass only represented a very small proportion of the total body mass, ~0.001%; however, it comprised ~9% of the total chitinous mass at the beginning of the growth period and declined by ~0.6% with each additional gram of body mass growth (SMA, b=–0.0059, R^2=0.26, P<0.001; Fig. 2D).

Additionally, tracheal volumes increased from ~100 µl in small *M. sexta* caterpillars to over 1000 µl in individuals nearing the end of larval growth (SMA, N=30, b=0.94, R^2=0.72, P<0.001; Fig. 3A). The slope of the volume to body mass scaling relationship was indistinguishable from isometry (slope test, r=–0.11, P=0.55). Body volume increases steadily with body mass, such that a 1 g fifth instar caterpillar would have a volume of ~2000 µl that would increase ~1000 µl for each gram increase of body mass (SMA, b=1030.65, R^2=0.89, P<0.001; Fig. 3B). Using this regression to estimate body volumes for tracheal volume measurements, we estimated the tracheal volume to body volume scaling relationship to be indistinguishable from isometry (SMA, b=0.94, R^2=0.72, P<0.001; slope test, r=0.0059, P=0.96; Fig. 3C). Tracheal volume on average makes up 7.5±0.57% (mean ± s.e.m.) of the total body volume; however, the ratio of tracheal volume relative to body volume does not correlate with changes of body volume during growth (SMA, b=–0.008, R^2<0.01, P=0.88; Fig. 3D). Two of the samples appear to have especially high ratios, which skewed the results; however, removal of these points also yielded no correlation between tracheal volume ratio and body mass growth (SMA, b=0.005, R^2=0.07, P=0.18). Therefore, as body size increased during growth of the fifth instar, tracheal volume increased proportionally, such that at the end of growth, larvae had an equivalent volume of tracheae per unit body mass and volume as those at the beginning of the instar.

The increases in tracheal mass and volume within the instar were mirrored by morphological differences between large and small fifth instar caterpillars (Fig. 4, supplementary material Fig. S1). Though the fine details of the tracheal images are difficult to compare quantitatively, there appears to be more tracheae in all body segments in the larger caterpillar than in the small (supplementary material Fig. S1). When comparing the details specifically between homologous sets of tracheae from large and small caterpillars, there appear to be differences in the morphology of the terminal ends of visible tracheae rather than the major tracheal trunks more proximal to the spiracle (Fig. 4).

On all three diet treatments, high quality, low quality and high→low quality, tracheal mass increased with larval growth. However, the scaling relationship differed significantly with diet quality (multiple comparison of slopes, r=9.877, P=0.007). Reduction in diet quality does prolong development time, but development time had little influence on dry tracheal mass variance after the effects of body mass and diet quality treatments were accounted for (ANOVA, F_{236}=0.004, P=0.97; Table 2). Caterpillars reared on a high quality diet showed a similar hypoallometric scaling relationship as the first tracheal mass measurement (above) with an increase in tracheal mass (SMA, b=0.89, R^2=0.74, P<0.001; Fig. 5A) and a slope significantly lower than isometry (slope test, r=–0.22, P=0.04). This hypoallometric relationship between dry tracheal mass and body mass was consistent in both experiments in which

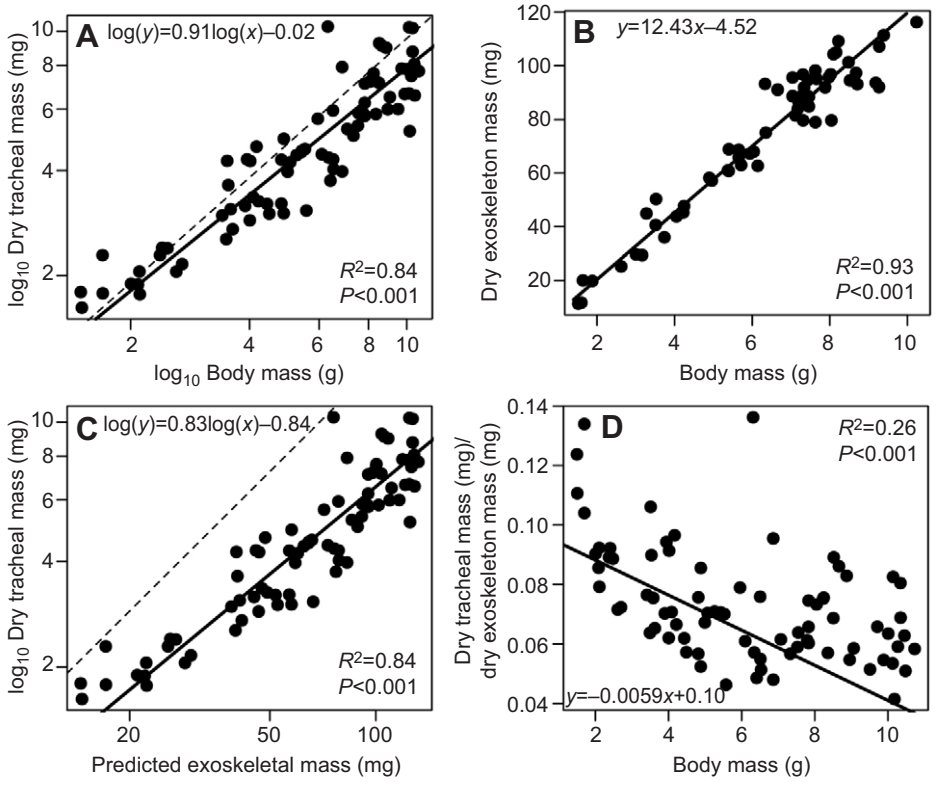

Fig. 2. (A) Tracheal scaling relationship between abdominal tracheal mass (summed from each abdominal segment) and body mass under standard laboratory rearing conditions. (B) Mass of the exoskeleton as body mass increases during growth. (C) Tracheal mass scaling against predicted exoskeletal mass. (D) The ratio of tracheal mass to exoskeletal mass as body mass increases during the growth. For A and C, the solid line represents the observed scaling patterns, while the dashed line represents a predicted isometric slope (b=1.0).

Table 1. Standardized major axis regression of log-transformed dry tracheal mass and body mass for each abdominal segment individually

	Regression statistics								Test of isometry		
Segment	Slope	Lower 95% CI	Upper 95% CI	Intercept	Lower 95% CI	Upper 95% CI	R^2	P	Test statistic	P	MC
1	0.74	0.67	0.83	–0.85	–0.91	–0.79	0.78	<0.0001	–0.52	<0.0001	A
2	0.91	0.81	1.02	–0.99	–1.07	–0.91	0.75	<0.0001	–0.19	<0.0001	B
3	0.96	0.87	1.06	–0.92	–0.97	–0.85	0.82	<0.0001	–0.094	0.41	B,C
4	1.00	0.91	1.10	–0.90	–0.99	–0.82	0.82	<0.0001	–0.0077	0.95	B,C
5	1.07	0.96	1.20	–0.98	–1.07	–0.88	0.75	<0.0001	0.13	0.24	C
6	0.98	0.88	1.10	–0.96	–1.04	–0.87	0.75	<0.0001	–0.042	0.71	B,C
7	0.93	0.84	1.03	–1.00	–1.08	–0.93	0.78	<0.0001	–0.16	0.16	B
8–10	0.91	0.82	1.00	–0.97	–1.04	–0.93	0.81	<0.0001	–0.22	0.05	B

Summary statistics are presented for each abdominal segment with a test of isometry (b=1) and multiple comparisons (MC) between groups. Different letters for multiple comparisons indicate statistical differences between treatments.

A comparison of slopes showed statistically significant differences between the slopes of scaling regressions between the various body segments (likelihood ratio test, D=24.91, P<0.001). This was primarily accounted for because the slope of the scaling relationships for segment 1 was significantly lower than all other segments (critical P=0.05). Abdominal segment 5 had a significantly higher slope than segments 2, 7 and 8.

caterpillars were reared on high quality diet. To assess this, we compared the regressions from the two experiments and found that there were no differences between mass scaling relationships when larvae were reared on 100% diet quality, even with the thoracic tracheal masses excluded (comparison of slopes, r=2.63, P=0.11). Caterpillars reared on low quality diet showed a positive scaling relationship (SMA, b=1.19, R^2=0.70, P<0.001; Fig. 5B), and the slope was significantly greater than isometry (slope test, r=0.26, P=0.02). Caterpillars that had been reared on a high quality diet for instars 1 through 4 and then switched to low quality diet for the final instar showed an isometric relationship between tracheal mass and body mass (slope test, r=0.0059, P=0.96), and an intermediate scaling relationship to those reared on either high or low quality diets continuously (SMA, b=1.00, R^2=0.84, P<0.001; Fig. 5C). Generally, there was an increase in the slope of the mass-scaling relationship as diet quality was reduced and as the duration of feeding on the low quality diet was increased (Table 3). There appear to be potential outliers, which could skew the regression estimates; however, their removal did not change the observed scaling relationships on any of the diet treatments or between diet treatments.

DISCUSSION

Contrary to the assumption that tracheal size cannot increase within a larval instar because cuticle must be shed for continued growth, both tracheal mass and tracheal volume increased with body size within the fifth instar *M. sexta* caterpillars. These results challenge the conventionally held assumption that respiratory systems are size-fixed in larval insects during feeding and growth. Our results show

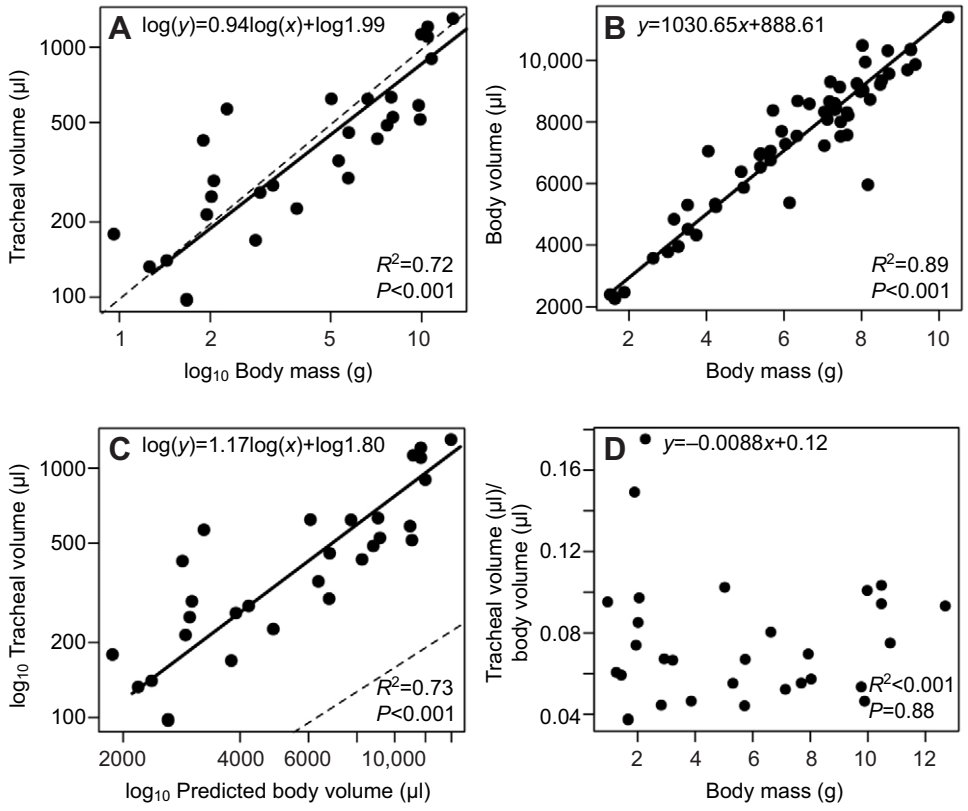

Fig. 3. (A) Tracheal volume scaling relationships with body mass. (B) Body volume calculated from exoskeletons as body mass increases during growth (see Materials and methods). (C) Tracheal volume scaling relationship with predicted body volume. (D) Tracheal volume to body volume ratio as body mass increases during growth. In A and C, dashed lines represent a predicted isometric relationship (b=1.0).

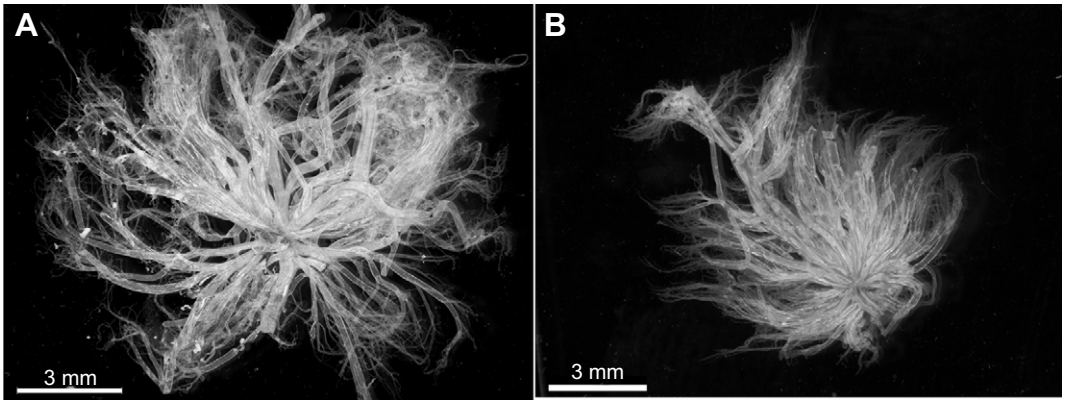

Fig. 4. Images of dissected tracheae of the first abdominal segment from one large (A) and one small (B) fifth instar *M. sexta*. The first abdominal segment was chosen for visualization because it is the body segment where dry tracheal mass increases the least relative to body mass (see Table 1).

that larval insect tracheae may be capable of responding to body growth with increases in both the structural investment and volume of the respiratory system where previously this was assumed not to be possible. Dry tracheal mass scaled hypoallometrically (b<1) with total body mass and exoskeletal mass, while tracheal volumes scaled isometrically with body mass and volume (b=1). Hypoallometric scaling of the dry tracheal mass and body size suggests a mismatch between growth of the respiratory system relative to the whole organism; however, isometric scaling of tracheal volume suggests that available respiratory volumes match growth during the fifth instar in terms of both body mass and body volume. Using body mass to combine the allometric equations for tracheal mass and volume scaling, we estimate that tracheal volume (V_t) scales with dry tracheal mass (M_t) as: $\log V_t = 1.03 \log M_t + \log 2.01$.

The increases in tracheal structure and volume shown here are consistent with studies of respiration in larval *M. sexta*. First, Greenlee and Harrison (Greenlee and Harrison, 2005) demonstrated that absolute tracheal conductance increases, though mass-specific tracheal conductance decreases, from the beginning to the end of the fifth instar. Our results show that tracheal volume increases with body size isometrically. Thus, total tracheal conductance could be higher as a consequence of increased tracheal volume; however, tracheal volume may not be the limiting factor for mass-specific decreases in tracheal conductance. Tracheal mass scaling was hypoallometric with body mass, suggesting that oxygen supply may not be sufficient to keep up with increases in whole body mass. This observation supports the argument that larvae are increasingly oxygen-limited as they grow (Greenlee and Harrison, 2005; Callier and Nijhout, 2011). Alternatively, increasing oxygen limitation may be a consequence of tracheal thickening during growth (Callier and Nijhout, 2011). A thickening tracheal system could cause decreases in mass-specific conductance through increased difficulty of gaseous diffusion, irrespective of tracheal volumes. Second, a recent study showed that tracheal volumes exhibited a slight positive trend within the fifth instar of *M. sexta* that was divergent from the negative trend in the third and fourth instars; however, the within-instar increase in tracheal volume was not significant when tested across the final three larval instars (Callier and Nijhout, 2011). Here we show that when looking only within the fifth instar, tracheal volume increases with larval body size, and follows the isometric tracheal volume relationship observed across instars (Callier and Nijhout, 2011; Greenlee et al., 2013).

Table 2. ANOVA for the effects of body mass, development time and diet quality on dry tracheal mass of *M. sexta* reared on three diet treatments

Factor	d.f.	SS	MS	*F*	*P*
Body mass	1	781.30	781.30	344.19	<0.0001
Development time	1	0.0040	0.0040	0.0018	0.97
Diet treatment	2	25.90	12.95	5.70	0.0038
Error	236	536.70	2.27		

When tracheal masses from each abdominal segment were regressed against body mass independently, the tracheal scaling relationships varied from segment to segment. This suggests that there is differential tracheal growth across the body that could represent local responses of the tracheal system to oxygen needs (Greenlee et al., 2013). Functions of body tissues are dependent on adequate tracheation, and growth of various tissues throughout the body may require more or less additional tracheae during growth (Greenlee et al., 2013). For example, fat tissues grow tremendously during the fifth instar in particular, and may require increased tracheation to function in nutritional processing and storage. Differences in the relative amount of fat body that accumulates in each abdominal segment could lead to differences in the growth of the tracheal system throughout the body. Other body tissues that increase less in absolute mass, such as imaginal discs, may not require as much new tracheal support.

There are several caveats that must be considered alongside our results when relating these tracheal size measures back to the whole organism. First, it is possible that much of the increase we observed in the tracheal dry masses is due to a thickening of the tracheal cuticle during larval growth (Callier and Nijhout, 2011); however, we do not consider this likely because a trachea of a given size cannot add volume (Fig. 3) without a concomitant increase in length. Second, we applied light positive pressure to force solution into the tracheae in our volume displacement measurements. As a consequence, our results may represent a higher tracheal volume than is available *in vivo*. Morphological measurements may not relate directly to functional performance of respiration in living *M. sexta* caterpillars. Quantification methods of tracheal size that capture both tracheae and tracheole volumes in greater detail would be more optimal to tie respiratory size dynamics with growth in larval insects. Last, it is unlikely that tissue dissolution captures the size dynamics of the tracheoles during the fifth instar because they may have been dissolved with soft tissues. Thus, our results may be an underestimate of tracheal mass increase with body size.

Our methods do not allow an assessment of volume change at the level of the tracheoles. Nevertheless, larval insects may augment their respiratory systems during growth by continuously increasing the volume and delivery capacity of the tracheae *via* 'tracheole sprouting' or elongation of the terminal tracheal ends (Samakovlis et al., 1996; Jarecki et al., 1999; Beitel and Krasnow, 2000; Greenlee and Harrison, 2005). Genetic studies of tracheal tubulogenesis in *Drosophila melanogaster* have shown two

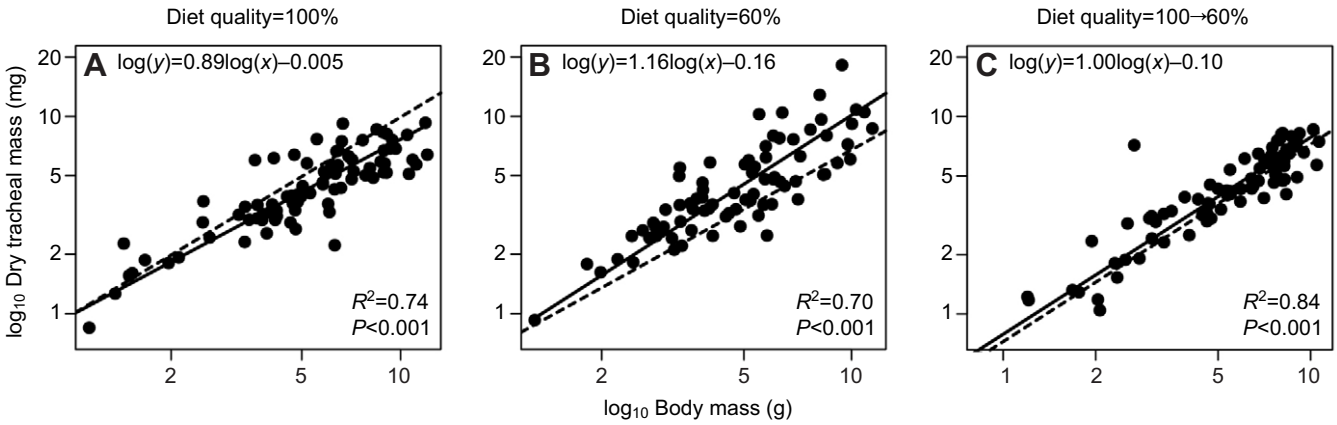

Fig. 5. Tracheal mass scaling with body mass for three diet treatments – (A) high quality, (B) low quality and (C) high→low quality – in fifth instar *M. sexta* caterpillars. Dashed lines represent a predicted isometric relationship for the three treatments (b=1). Summary statistics for each regression are presented in Table 3. The slopes of the regressions are significantly different from one another.

promising observations that support tracheal growth within an instar. First, the molecular signals responsible for signaling oxygen deficiency and growth responses in tracheal epithelium are active throughout post-embryonic development, indicating constant expansion of new tracheal systems (Jarecki et al., 1999; Centanin et al., 2008; Centanin et al., 2010). Second, the length of the tracheae increases steadily throughout all stages of *D. melanogaster* larval growth, while tracheal diameters make discrete jumps during molts (Beitel and Krasnow, 2000). Embryonic tracheal formation occurs in a developmentally fixed manner (Affolter and Caussinus, 2008), while genetic development of tracheae during the larval period is plastic in response to oxygen demand (Jarecki et al., 1999; Centanin et al., 2010). Fixed and plastic periods of tracheal development could potentially explain how tracheal conductance and tracheal volume scaling relationships could vary between instars in *M. sexta* (Greenlee and Harrison, 2005; Callier and Nijhout, 2011; Sears et al., 2012). Tracheal expansion may be limited to the portion of the larval growth period where growth is maximal – the fifth larval instar (Davidowitz et al., 2004). While there are no molecular developmental studies on tracheae deposition for *M. sexta*, it is likely that mechanisms similar to those in *D. melanogaster* are involved.

Our study demonstrates that structural growth in the tracheal system is influenced by larval nutrition. Caterpillars reared on a low quality diet invested relatively more mass into their tracheal systems than those reared on a high quality diet during the fifth instar. This result is somewhat counterintuitive, given that any tracheal growth is likely responding to oxygen needs, and that lowered diet quality should result in a lower metabolic rate and, presumably, a lowered oxygen demand (Cruz-Neto and Bozinovic, 2004; Jeyasingh, 2007). Differences in tracheal scaling could be due to nutritional effects on tracheal growth; however, differences in development time do not account for variation in dry tracheal mass after it is separated from the effects of body mass and diet quality. This suggests that tracheal size increases are size-dependent rather than time-dependent, and interact with the physiological responses to nutrition. Changes in diet quality may alter the oxygen signaling mechanisms that drive increased tracheation, and responses in overall tracheal growth within the instar may reflect an optimization pattern between growth in oxygen supply and metabolic demand. Metabolic demand should be lower on poor quality diet, but this would also result in lower ATP production (Harrison and Haddad, 2011). Reduced oxygen availability should promote tracheal cell growth but should lower somatic growth because less ATP is available (Harrison and Haddad, 2011). This may result in the increases in tracheal scaling values seen when switched or reared continuously on a reduced quality diet. Alternatively, hypertrophic growth of the tracheal system on poor quality diet may be a consequence of other physiological factors. For example, the larva may respond to low quality diet by changing overall body composition. For example, mass-specific fat body growth is higher on reduced quality diet than on standard quality diet (B.R.H. and G.D., unpublished data). As a consequence, more or less tracheation may reflect the necessity to supply oxygen to growing larval fat bodies.

Regardless, the switched diet treatment yielded a pattern that was intermediate of either the high or low quality diet treatments, and this shows that response of the tracheal system to diet quality is influenced by both immediate and previous nutritional conditions. This provides strong evidence that the tracheal scaling patterns observed are a

Table 3. Standardized major axis regression of log-transformed dry tracheal mass and body mass for three diet treatments

	Regression statistics								Test of isometry		
Segment	Slope	Lower 95% CI	Upper 95% CI	Intercept	Lower 95% CI	Upper 95% CI	R^2	P	Test statistic	P	MC
100%	0.89	0.79	0.99	0.0052	–0.081	0.070	0.74	<0.0001	–0.22	0.04	A
100→60%	1.00	0.91	1.09	–0.10	–0.17	–0.035	0.84	<0.0001	0.0059	0.96	A,B
60%	1.19	1.03	1.31	–0.16	–0.23	–0.56	0.70	<0.0001	0.26	0.02	B

Summary statistics for each diet treatment are presented along with a test of isometry (b=1) and multiple comparisons (MC) between groups. Different letters for multiple comparisons indicate statistical differences between treatments.

A test of the slopes amongst regressions showed significant differences between diet treatments (likelihood ratio test, D=9.88, P=0.007). Individuals reared on 60% quality diet had a significantly higher slope than those reared on 100% quality (r=5.025, P=0.03). There were no significant differences found between individuals reared on the switched diet treatment and either 100% (r=1.42, P=0.23) or 60% (r=1.69, P=0.19) diet treatments.

consequence of within-instar changes, distinct from changes that may arise during a molt. If tracheal scaling patterns were unaffected by within-instar growth or nutritional conditions, then we would have expected the tracheal scaling pattern of individuals reared on a constant high quality diet to be the same as individuals switched to a low quality diet for the fifth instar. This was not the case. Individuals that experienced low quality diet conditions for just the fifth instar elevated their investment in tracheae.

The evidence presented here shows that the tracheal system of insects can and does increase in size within an instar. Why has this been missed given the long history of studies of insect respiration? While it is certainly true that physiological descriptions of the tracheae can be traced back over 100 years for *M. sexta* (Peterson, 1912), much of the foundational work is on the anatomy of insect respiratory systems in only a few hemimetabolous insects (Wigglesworth, 1954; Wigglesworth, 1981; Wigglesworth and Lee, 1982; Locke, 1958a; Locke, 1958b; Locke, 1958c). More recent studies have demonstrated the possibility of tracheal expansion between molts in holometabolous insects (Beitel and Krasnow, 2000). Generally, hemimetabolous insects have more juvenile instars, with less total body growth occurring within each instar than in holometabolous insects (Cole, 1980). The assumption of tracheal size fixation is derived originally from the observation that tracheae are lined with a layer of cuticle (Chapman, 1998). While sclerotized exoskeletal cuticle cannot increase in size, soft-bodied larvae of the holometabolous insects can increase the size of the exoskeleton without molting (Wielgus and Gilbert, 1978; Wolfgang and Riddiford, 1986; Kaznowski et al., 1985). Within-instar tracheal growth could be an adaptation that permits holometabolous insects to maximize growth within an instar and decrease the number of larval molts, which are energetically expensive and ecologically critical periods during larval development.

Determining the role that the tracheal system plays in larval growth of insects is crucial because of the central role that oxygen plays in aerobic metabolism and overall organismal function (Harrison et al., 2006). Atmospheric oxygen influences insect body size and induces size plasticity in the tracheal system: increases in oxygen concentration generally results in larger body size (Harrison et al., 2006; Harrison et al., 2010; Heinrich et al., 2011). Oxygen concentration has the strongest effects late in larval growth (Heinrich et al., 2011), where absolute growth is maximal (Davidowitz et al., 2004; Shingleton et al., 2008; Sears et al., 2012). The ability of the tracheal system to sufficiently deliver oxygen is a factor limiting maximum body size in insects (Kaiser et al., 2007), and it has been hypothesized as a limiting factor for growth as well (Callier and Nijhout, 2011). Thus, a mechanism that increases oxygen capacity during periods of increased larval growth should be strongly favored by selection. We suggest that this mechanism may be growth of tracheal mass and volume within the larval instar.

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AUTHOR CONTRIBUTIONS

B.R.H. designed and executed experiments, analyzed results, and was the primary writer preparing the manuscript. G.D. advised over all aspects of the project and assisted with manuscript preparation.

COMPETING INTERESTS

No competing interests declared.

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