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Intraspecific variation in tracheal volume in the American locust, *Schistocerca americana*, measured by a new inert gas method

Hilary M. Lease[1,*], Blair O. Wolf[1] and Jon F. Harrison[2]

[1]*Department of Biology, University of New Mexico, Albuquerque, NM 87131, USA and* [2]*School of Life Sciences, Arizona State University, Tempe, AZ 85287-4501, USA*

*Author for correspondence (e-mail: hlease@unm.edu)

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Summary

The volume of a tracheal system influences breath-holding capacity and provides an index of an insect's investment in its respiratory system. Here, we describe a new, generally applicable method to measure tracheal volume that enables repeatable determinations on live animals. Animals are isolated in a closed chamber of a known volume and equilibrated with a helium:oxygen gas mixture. The chamber is then rapidly flushed with a nitrogen:oxygen gas mixture to eliminate the helium surrounding the animal, and sealed. After a period of time sufficient to allow equilibration of helium between tracheal system and chamber air, a gas sample is taken from the chamber, and tracheal volumes are calculated from the helium content of the sample, using a gas chromatograph. We show that relative investment in the tracheal system increases with age/size in the grasshopper; tracheal volume scales with mass to the power 1.3. This increased proportional investment in the tracheal system provides a mechanistic basis for the enhanced respiratory capacity of older grasshoppers. Tracheal volumes decrease strongly as grasshoppers grow within an instar stage, explaining reduced safety margins for oxygen delivery. Finally, tracheal volumes are smaller in gravid females than males, probably due to compression of air sacs by eggs.

Key word: respiration, trachea, scaling, American locust, *Schistocerca americana*.

Introduction

Respiratory systems play a crucial intermediary role in the delivery of oxygen from the atmosphere to an animal's cells, and the volume of a respiratory system can provide an index of the investment made by an animal in its respiratory system. In insects, which usually lack substantial oxygen-carrier pigments (Chapman, 1998) the volume of the tracheal system (V_T) also determines the amount of oxygen available during breath-holding, and thus may determine the duration of the closed phase of discontinuous gas exchange. Insects and many arthropods have developed complex tracheolar respiratory systems that deliver oxygen directly to tissues and cells (Chapman, 1998). The design and performance of these systems probably varies with animal size, body plan, and lifestyle, but relatively little is known about such variation, and how it affects V_T.

The relationship between V_T and other insect life history characteristics is almost certainly not a simple one. For example, insects with increased metabolic rate are likely to have more extensive gas delivery systems. Insect V_T may vary with organism size, metabolic rate, species type, mode of respiration (e.g. cyclic, non-cyclic, or discontinuous ventilation), presence of air sacs, and developmental stage. Tracheal volume may also change with reproductive or digestive status of an organism. For example, oogenesis or a full crop may cause temporary reduction in V_T in insects with air sacs. In addition, it has been previously shown that hemimetabolous insects accumulate mass within an instar, and that because of a relatively inelastic exoskeleton, this growth can reduce V_T and reduce oxygen delivery in some insects (Greenlee and Harrison, 2004a). However, in general, the magnitude of such effects on V_T and oxygen delivery capacity are unknown.

Tracheal volumes of insects have been estimated using water displacement (Wigglesworth, 1950), stereology (Schmitz and Perry, 1999) and inert gas-mass spectrometry (Bridges et al., 1980). The water displacement method is quick and inexpensive, but requires death of the animal, and may mis-measure V_T due to water adherence to the cuticle and lack of fluid infiltration of the tracheoles. Bridges, Kestler and Scheid developed an inert gas wash-out and mass spectrometry technique to assess V_T (Bridges et al., 1980). Their method provided the conceptual foundation for our approach, as it demonstrated the practical use of inert gases to measure V_T. However, the flow-through mass spectrometer system used is expensive, notoriously difficult to maintain, and not widely available. Also, their method relies on the insects exhibiting discontinuous gas exchange (DGC);

chamber flushing occurs during the closed DGC phase of the respiratory cycle. Since many insects do not predictably exhibit DGC (Lighton, 1998), comparative studies using this method are limited. Schmitz and Perry (Schmitz and Perry, 1999) developed a stereological approach to assessing V_T. Using light microscopy and electron microscopy, they made direct morphometric estimates of V_T. However, this approach is labor intensive and is challenging to use on large insects, hampering its utility for comparative studies. To date, no methods are available that allow repeated estimates of V_T on living insects that do not exhibit discontinuous gas exchange.

Here, we describe a new method to measure V_T using an inert gas volumetric (IGV) method that addresses all of these issues. We used helium as the inert gas with which to measure V_T, as its solubility in biological fluids is relatively low (see *Validation of use of helium* in the Materials and methods). The principal features of this method can be summarized as follows. Animals are isolated in a small closed chamber and equilibrated with a helium:oxygen gas mixture (the 'pre-equilibration phase'). The chamber is then rapidly flushed with a nitrogen:oxygen gas mixture, resulting in removal of helium from chamber air, and sealed ('flush phase'). After a period of time sufficient to allow equilibration of helium between tracheal system and chamber air ('wash-out phase'), a gas sample is taken from the chamber. The helium content of the sample is then measured using a gas chromatograph with a thermoconductivity detector. Tracheal volumes can then be calculated from chamber volume and helium concentrations.

We first establish helium equilibration times for large and small grasshoppers, and characterize the system conditions that allow chamber flushing without significant loss of helium from the trachea. We then use this method to investigate how V_T varies with body size, developmental stage, gender, and reproductive status in *S. americana*.

Materials and methods

Animals

Schistocerca americana Drury were raised at Arizona State University as previously described (Harrison and Kennedy, 1994). For between-instar comparisons of *S. americana*, early instar animals and adult males were used. For within-instar comparisons of *S. americana*, fifth instar animals ranging from early to late instar were used. The instar stage of each animal was determined by taking the ratio of femur length to abdomen length, which decreases with age within an instar (S. D. Kirkton, and K. J. Greenlee, personal communication); all animals designated as early stage had femur to abdomen ratios of greater than 1. Wings were removed with scissors from sixth instars and adults prior to inert gas volumetry, because preliminary tests showed that they may hinder chamber flushing of heliox during the rapid nitrox flush period.

Apparatus

Gas mixtures were supplied from pre-mixed tanks. These flowed *via* Bevaline tubing and two computer-controlled solenoid valves (Clippard EVO-3; Clippard Instrument Laboratory, Cincinnati, OH, USA) through a glass animal chamber, and exited the chamber *via* a third computer controlled solenoid valve (Fig. 1). Sable Systems Data Acquisition hardware and software (Sable Systems International, Las Vegas, NV, USA) were used to control the valves. In most cases, the volume of the animal chambers used was approximately 7 ml, but for trials with larger insects we used chambers with volumes up to 100 ml. Threaded aluminum end caps with o-rings and vacuum grease (Dow Corning, Midland, MI, USA) were used to seal both ends of animal chambers. Clippard barb fittings (Clippard 1/8 inch to 10/32 inch) at the proximal ends of the chambers allowed gas entry and exit.

First, the chamber containing the insect was ventilated with heliox gas mixture (21% oxygen, 79% helium) at a flow rate of 1 l min^{-1} (Brooks mass flow controller; Brooks Instruments, Hatfield, PA, USA) until the insect respiratory system had equilibrated with heliox (0–10 min; see Results). The chamber was then flushed with a nitrox gas mixture (21% oxygen, 79% nitrogen) at a flow rate of 5 l min^{-1} until essentially all helium had been eliminated from the chamber (0–3 s; see Results), after which time the chamber was sealed. After a period of time (0–10 min; see Results), sufficient to allow at least 99% of the helium to evolve from the insect respiratory system, a 2 ml sample of air was withdrawn from the chamber using a gas-tight Hamilton syringe, and then injected into a gas chromatograph (Varian Inc., Palo Alto, CA, USA; Model 3400) to measure helium concentration (C_{He}, mol l^{-1}). The carrier gas used was ultra high purity N_2 (30 ml min^{-1}). Helium was separated from oxygen and carbon dioxide using a Gas Chrom MP-1 column (Varian), and the helium peak measured with a thermo-conductivity detector. Sable Systems Data Acquisition hardware and software (Sable Systems International, Las Vegas, NV, USA) were used to collect the gas chromatograph output and integrate the areas under the helium peaks. Calibration injections of helium (0–1000 μl), which overlapped the range of V_T values we measured yielded linear calibration curves (R^2=0.99) relating peak areas to moles of helium injected.

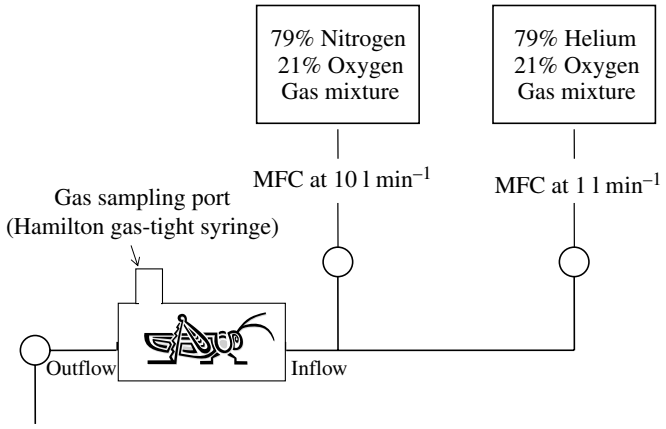

Fig. 1. Experimental set-up. MFC, mass flow controllers; computer-controlled solenoid valves.

Validation of method parameters

It was necessary to optimize and standardize the time periods of the method to ensure: (1) that helium was fully equilibrated in the insect respiratory system during the pre-equilibration phase; (2) that nitrogen flushing was long enough to remove helium traces from the chamber (and outside surface of the animal), yet short enough that the insects had not begun to eliminate significant amounts of helium from their respiratory systems; and (3) that all of the helium had evolved from the insects during the wash-out phase. We did this by varying heliox pre-equilibration time, nitrogen flushing time and wash-out duration, while holding the other two variables constant. We also tested whether these phase durations varied with size of organism by running the optimization tests on both large (0.9–1.5 g) and small (0.04–0.20 g) grasshoppers.

Calculation of tracheal volume

The gas chromatograph measures helium concentration, C_{He}, in the sample of air taken from the chamber. This concentration can be converted to a quantity of helium evolved from the tracheal system (V_{THe}, μl), when corrected for displacement of air within the chamber by the animal. The method requires minimization of chamber volume to maximize chamber helium concentration and reduce flush time, so a relatively small chamber volume was selected. To calculate the effect of displacement of air within the chamber by solid components of the animal, we placed dead grasshoppers of various sizes (with sealed spiracles) into the chambers. 100 μl of helium was injected into the chamber, and after equilibration, the concentration of helium in the chamber was measured. As the size of animals in the chamber increased, the measured helium concentration increased linearly, consistent with a simple displacement mechanism. For example, with our most commonly used 7 ml chamber, we calculated a displacement correction factor (DCF, unitless) that varied with mass and corrects a measured helium concentration back to that which would be calculated using an empty chamber as:

$$\text{DCF} = \text{helium actual} / \text{helium measured} = 1 - 0.073\ (\text{animal mass});\ R^2=0.73\ . \quad (1)$$

Therefore, the volume of helium in the chamber (V_{CHe}, μl) was calculated as:

$$V_{CHe} = (C_{He} \times \text{DCF})\ . \quad (2)$$

In addition to the problem of animal displacement of space, the presence of animals in the chamber can increase residual helium left after flushing due to trapping of air in animal boundary layers, and the quantity of such trapped helium is expected to increase with animal size. Therefore, we measured the amount of residual helium after a 2 s nitrox flush for 14 animals, ranging in body mass from 0.1 to 2.5 g, and found that residual helium volume (RHV, μl) could be predicted by animal mass (g):

$$\text{RHV} = 3.92 \times \text{mass} + 8.46\ (N=14;\ R^2=62\%)\ . \quad (3)$$

RHV must be calculated for each combination of chamber, flush rate, and flush time, and DCF should be calculated for each chamber.

We calculated the volume of helium in the tracheal system (V_{THe}, μl) as:

$$V_{THe} = V_{CHe} - \text{RHV}\ . \quad (4)$$

We calculated tracheal volume (V_T, μl) as:

$$V_T = V_{THe} / 0.79\ . \quad (5)$$

Experimental design

We measured V_T using the IGV method on early-instar second, third, fourth, and fifth instar *S. americana*, mid to late-instar fifth instar *S. americana*, and adult male and female *S. americana*. Based on preliminary data, a standardized IGV method protocol of 3 min of helium:oxygen wash-in, 2 s of nitrogen:oxygen chamber flushing, and 5 min helium wash-out in the sealed chamber was used for each animal. For each individual, the wet mass (g), dry mass (g), femur length (mm), abdomen length (mm), and, where relevant, sex and egg mass were measured. For comparative purposes, V_T was also estimated for a subset of individuals using the water displacement method (Bartholomew and Barnhart, 1984). Animals were placed in a 60 ml plastic syringe filled with soapy water. The plunger was moved back and forth to draw air out of the tracheal system and replace it with fluid. The outside of the insect was blotted dry, and the increase in insect mass was used as a measure of respiratory system volume, assuming water density=1 g ml^{-1}.

Results

Validation of method parameters

Determination of time period required for helium equilibration within insects

Helium concentration in an empty chamber previously filled with air nears a saturation point after 30 s of heliox perfusion at 5 l min^{-1} (data not shown). To measure the time required for helium equilibration within adult *S. americana*, we varied the time allowed for pre-equilibration in heliox (*x* axis; Fig. 2A); while all other parameters (e.g. 2 s for nitrox flush, 5 min for helium wash-out from the animal) were held constant. The amount of helium evolved from the animal stabilized when pre-equilibration times were 1 min or longer (Fig. 2A), suggesting that a 1 min helium wash-in period was sufficient to allow heliox equilibration between air and the respiratory system.

Duration of nitrox perfusion required to minimize helium residue

We also needed to determine the flushing period required to lower helium concentrations in the chamber to methodologically insignificant levels. For the method to be successful, this time must be less than the period required for loss of significant amounts of helium from the insect tracheal system. To determine how flush time affected residual helium volume (RHV), we flushed an empty chamber with heliox for 30 s, and then flushed the chamber with nitrox for periods

ranging from 0–30 s. In each case, after the flush, we sealed the chamber for 5 min, after which a 2 ml air sample was withdrawn and injected into the gas chromatograph. We found that 2 s of nitrox flushing removed the vast majority of the helium from an empty chamber (Fig. 2B), so a 2 s nitrox flush was used for all subsequent experiments. The RHV for an empty chamber ranged from 2.0 to 5.9 μl. When animals with plugged spiracles were added to the chamber, the RHV increased with mass (range: 8–13 μλ, Eqn 2); this was probably because of helium trapped in boundary layers of the animal cuticle. This was a minor correction (<5%) for adult grasshoppers, but was a major correction (~50%) for the smallest, first instar grasshoppers.

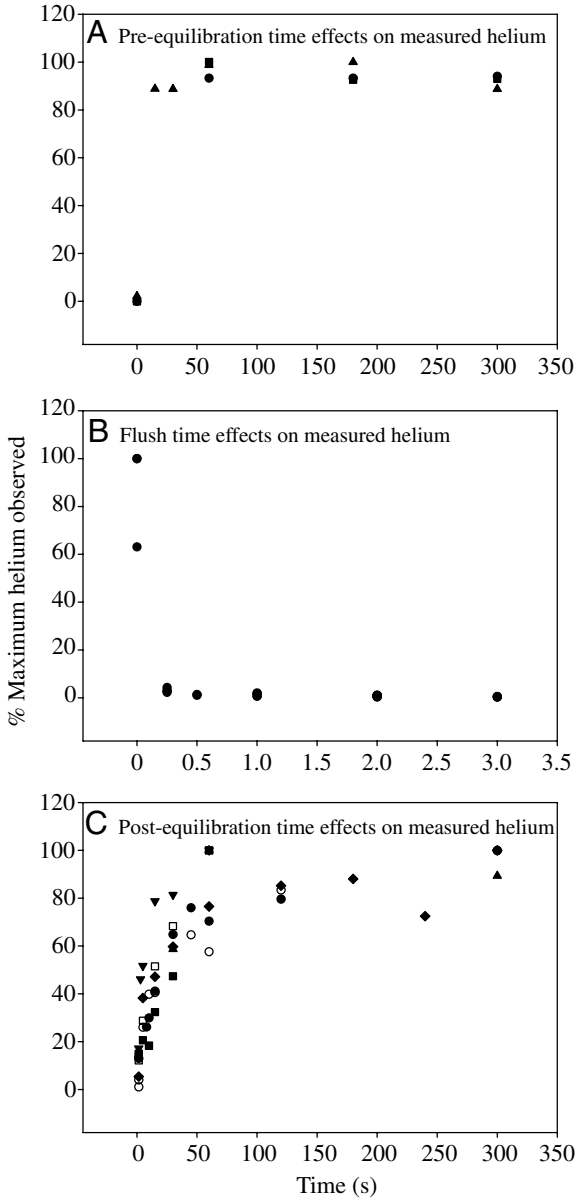

Fig. 2. Data showing validation of method parameters. Each data point represents measurement of helium emitted from a single animal in one trial after completing the experimental protocol (i.e. an entire sequence of heliox exposure, nitrox flushing of chamber, and sealed chamber equilibration; helium assessed by gas chromatography of an air sample from the chamber). Different symbols indicate different animals. (A) Helium yield from animals varied with the duration of pre-exposure to heliox if exposure durations were less than 1 min, but were relatively constant with exposures greater than 1 min. (B) Residual helium in empty chamber fell with flush time duration. (C) Helium yield from the animal increased until wash-out times exceed 2 min.

Determination of time period required for helium wash-out from insects

To determine the length of time required for helium to wash-out of the animal and equilibrate with the chamber air, we varied helium wash-out periods (*x* axis; Fig. 2C) while maintaining other parameters constant (3 min heliox exposure, 2 s nitrox flush time). This was calculated using seven animals, ranging from second instar to adult; six to ten different wash-out time periods were tested independently for each animal. We found that a period of 2 min was sufficient to allow for complete elimination of helium from the tracheal system of *S. americana*, regardless of body size (Fig. 2C).

One of the advantages of the IGV method is that it allows repeatable measurements of V_T without damage to the animal. Six repeated measurements yielded coefficients of variation of 0.076 for a 0.5 g *S. americana*, and 0.12 for a 1.1 g animal.

Inert gas volumetric method parameters for Schistocerca americana of variable size

Differences in wash-out and wash-in times between grasshoppers of different sizes were relatively small, although there was a trend towards shorter times in smaller animals (e.g. ~100 s to plateau in third instars *vs* ~180 s to plateau in adults). Thus, throughout the remaining studies (i.e. for all age classes of *S. americana*) we used method times established as sufficient for the larger animals, to ensure helium equilibration.

Comparison of inert gas and water displacement methods for assessing insect V_T

Tracheal volumes estimated using the water displacement method increased each time the syringe plunger was pumped (Fig. 3). This suggested that repeated compression and expansion stretched the abdomen, allowing more water to enter the body. To minimize this problem, for subsequent analyses, we stopped applying a vacuum to the system when air bubbles stopped leaving the spiracles. With both methods, V_T increased with animal mass (Fig. 4A). Within individuals, there was a curvilinear relationship between V_T measured with IGV and water displacement (WD) methods (Fig. 4B). In general, V_T measured for smaller animals were similar with both methods, but the WD method provided V_T estimates that were 15% to 250% higher than those obtained through the IGV method for larger animals (Fig. 3C).

Intraspecific variation in tracheal volume

We found that across early-stage instars, V_T increased with increasing age and body size [Fig. 3A, Fig. 4; $\log V_T = 2.544 + 1.296 \times \log(\text{mass})$, where V_T is in μl and mass in g; $P<0.0001$, $R^2=0.72$]. By contrast, within the fifth instars, V_T

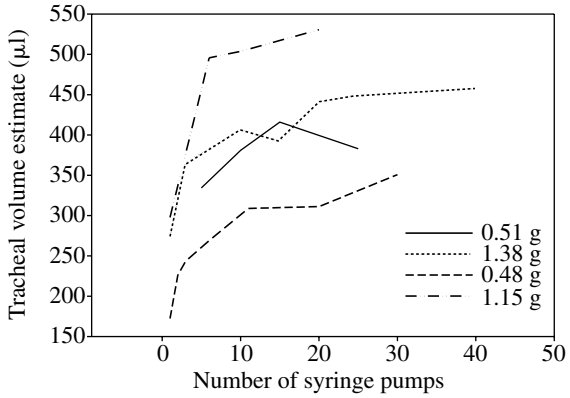

Fig. 3. Magnitude of estimated tracheal volume, using the water displacement method, increases with number of pumps of the syringe plunger.

decreased with increasing age and body size [Fig. 5; $\log V_T = 0.609–2.370 \times \log(\text{mass})$; $P<0.0001$, $R^2=0.65$]. Although gravid females are larger than adult males (mean mass for females=2.26 g; males=1.19 g), V_T in gravid females was significantly less than that of adult males (mean V_T for gravid females=145 μl; males=293 μl; Fig. 6). Sexually mature females show decreased V_T in comparison to adult males and adult females lacking eggs (Fig. 7; $V_T=2.602–1.344 \times \text{mass}$; $P<0.001$, $R^2=0.43$).

Discussion

We developed a method for measuring V_T of insects that is relatively simple, inexpensive, and repeatable on the same individuals. We found that, in contrast to the isometric scaling of lung volumes in vertebrates, older grasshoppers have proportionally larger V_T. This partially explains the ontogenetic increase in tracheal conductance in this species (Greenlee and Harrison, 2004a). Within an instar, however, V_T decreases dramatically with tissue growth due to the constraints of a semi-rigid exoskeleton. In adults, we found that gender has a strong effect on V_T; males and gravid females had V_T that were 40% and 7% of body volume, respectively. Both ontogenetic and reproductive developmental changes thus have the capacity to impact V_T in insects, and may have strong effects on respiratory function and life history variables.

Critique of inert gas volumetric method

The most significant potential error in the IGV method seems to be the correction for residual helium in the chamber after the nitrox flush. For the smallest insects we used (mass about 0.06 g, V_T=13 μl), helium remaining in the chamber after the nitrox flush was more than 50% of V_T. Although repeatability of V_T estimates was relatively good, even for small grasshoppers, it seems probable that this could provide an important, difficult to resolve error. Thus we feel that our system was pushed to near its limit of resolution for measuring V_T, which is unfortunate as most insects are under 1 mg in size. However, this system could likely be adapted to function for very small insects by substantially reducing chamber and tubing size, as we did not challenge the resolution of the gas chromatograph.

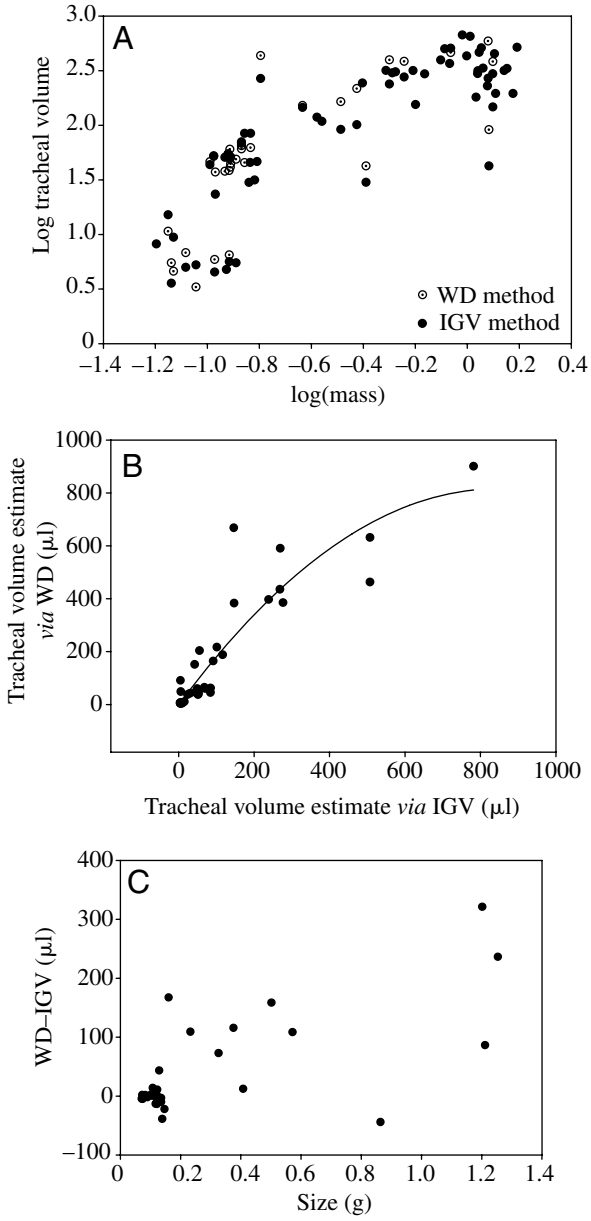

Fig. 4. Water displacement (WD) method and inert gas volumetric (IGV) method comparisons. (A) Both methods predict a similar increase in tracheal volume with mass. (B) Estimate of tracheal volume using the WD method *versus* IGV method for the same individuals. (C) The difference between the tracheal volume estimates using the WD method versus the IGV method as a function of body size. The WD method predicted larger tracheal volumes than the IGV method, with absolute difference increasing with animal size.

A related source of error in the IGV method is increased helium in boundary layers of the insect due to structures such as wings and hair. We found that wings needed to be removed from adult grasshoppers in order to reduce residual helium to acceptable levels. Potentially this would be a major issue with insects with substantial cuticular hair such as bumblebees. We

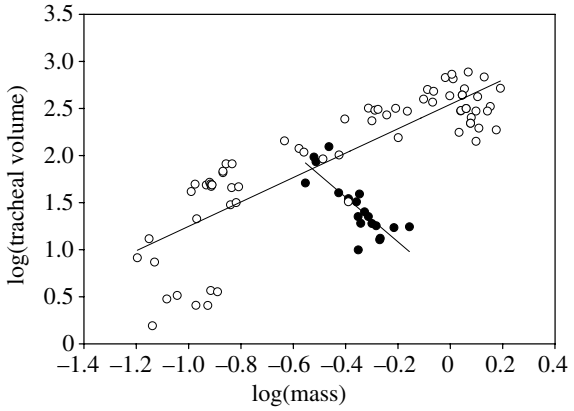

Fig. 5. Relationship between tracheal volume and mass within fifth instar stage (filled circles) for *S. americana*, overlaid on between-instar data (open circles). Between-instar data includes only early-instar sub-adults and male adults. Tracheal volume increases between instars (slope=1.30) and decreases within an instar (slope=–2.37).

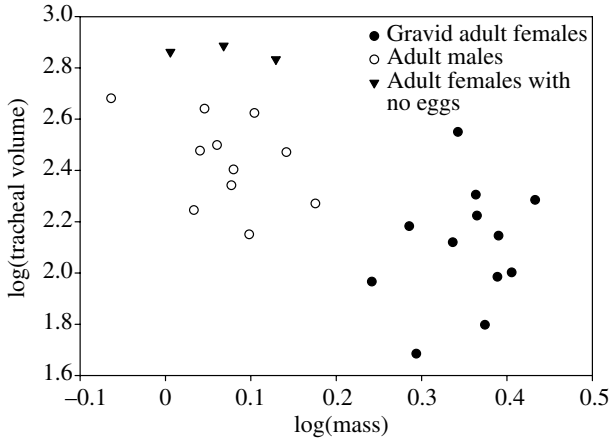

Fig. 7. Relationship between tracheal volume and mass for adult *S. americana*. Tracheal volume decreases with body size in adults (slope=–1.34).

assessed the magnitude of helium adherence to the outside of the animal by measuring V_{CHe} for dead animals whose spiracles had been sealed with wax, and we recommend this approach to gauge the magnitude of this potential error.

A final potential source of error in the IGV method is the correction for animal body displacement. When known quantities of helium are injected into chambers with animals (with plugged spiracles) and allowed to equilibrate with chamber air, a body displacement correction factor (DCF) can be calculated; this was done because animal body size will affect chamber helium concentration (*via* body displacement of total chamber volume). In our system, this turned out to be a minor correction factor for both large and small grasshoppers. The DCF ranged from 0.6 μl He (for a 0.07 g hopper; <5% V_T) to 10.2 μl He (for a 1.2 g hopper; also <5% V_T). However, reducing the chamber volume to body volume ratio might increase the importance of this correction factor.

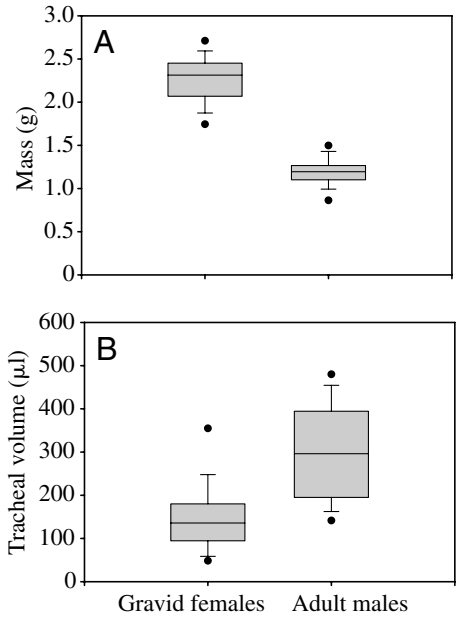

Fig. 6. Body size (A) and tracheal volume (B) vary with gender in adult *S. americana* (P<0.05). The boundaries of the boxes indicate the 25th and 75th percentiles, and the whiskers above and below the box indicate the 90th and 10th percentiles.

Like all gases, helium is soluble in body fluids, and so helium dissolved in body fluids has the potential to produce an overestimate of V_T. The solubility of helium in biological fluids is approximately 67% of that of N_2, and 33% of O_2, so tissue solubility is less of an issue than for other gases. Helium solubility in blood plasma is approximately 0.007 atm^{-1} (Hlastala et al., 1980), and this value provides a reasonable estimate of the solubility of helium in grasshopper body water. Thus, grasshopper body water equilibrated with heliox at STPD should contain 0.79×0.007=0.0055 ml of helium per ml of body water. Total body water is 66% of the wet mass of *Schistocerca* grasshoppers (Harrison, 1989). Because proportional V_T increases with mass (Figs 4, 5), the fraction of total animal helium localized in body water will be greatest for small grasshoppers. For the smallest grasshoppers (0.06 g, 13 μl V_T, 0.3 μl He in body water) helium dissolved in the body water represents 2.3% of the estimated V_T, whereas for the largest grasshoppers (1.6 g, 542 μl V_T, 5.8 μl He in body water), helium dissolved in body water represents only 1.1% of V_T. In either case, this is a small error compared to the variation among individual grasshoppers, although for insects with a very small V_T [e.g. stick insects have V_T only 1.3% of body volume (Schmitz and Perry, 1999)] this error could become relatively more important.

Finally, the various parameters of the IGV method should be checked when applying this method to different taxa, and animals of different sizes. In general, insects with higher metabolic and ventilation rates will require shorter, faster nitrox flushes to ensure that exhaled helium is not removed from the chamber during the nitrogen flush period. On the other

hand, animals with particularly low metabolic and respiratory rates may require longer helium equilibration periods and wash-out periods.

Comparison of inert gas volumetric and water displacement methods for assessing insect V_T

Wigglesworth developed (Wigglesworth, 1950), and others have since utilized (Clarke, 1957; Weis-Fogh, 1964; Bartholomew and Barnhart, 1984; Harrison, 1989), a water displacement method to estimate V_T. This method replaces tracheal air with fluid, and the subsequent increase in insect mass is used as a measure of respiratory system volume. The V_T may be underestimated by this method if tracheoles are not infiltrated with fluid because of their small size. The V_T may be overestimated by this method because water adheres to cuticle. The magnitude of these errors is difficult to estimate, but both are likely to be relatively greater issues for animals without air sacs (and lower V_T), whereas water-adherence seems likely to be a greater problem for smaller insects because of their higher surface/volume ratios. We also identified another potentially major problem with the water displacement method here. It is difficult to know whether one is actually expanding the animal when applying syringe pressure; this causes over-inflation of air sacs and over-estimation of V_T when multiple syringe pumps are used with grasshoppers (Fig. 3). Even when we attempted to control for this problem by ceasing syringe pumps when no further air was removed by suction from the animal, we found that this method can substantially overestimate V_T (Fig. 4C).

Despite the mismatch in V_T between measures made by the IGV and WD methods (Fig. 4C), across individuals, both the IGV and the WD methods predicted a similar increase in V_T with animal mass (Fig. 4A). Thus the inexpensive and field-portable WD method is still of use for comparative studies, especially for animals that lack air sacs.

The IGV method may be particularly useful for measuring within-individual (physiological) variation in V_T. For example, the effect of digestive status on V_T can be investigated using the IGV method. Reduction in V_T after a meal may be important since feeding increases metabolic rate (Zanotto et al., 1993), increasing the need for gas exchange. Feeding status effects on V_T may be particularly interesting for flying insects, since a full crop simultaneously increases body mass (increasing the metabolic cost of flying) and compresses air sacs (potentially impacting the safety margin for oxygen delivery). Inter-individual developmental assessment of V_T might also be interesting using the IGV method for holometabolous insects that have large and flexible trachea (but lack air sacs) during their larval stage, and have air sacs and thin, rigid trachea in their adult stages.

Finally, the IGV method may allow determination of V_T variation as a function of mode and intensity of gas exchange. Gas-exchange patterns (e.g. discontinuous and continuous gas exchange) can change quickly within individual insects and are linked to variation in metabolic rate (Gibbs and Johnson, 2004). Average V_T measured over multiple cycles (e.g. discontinuous gas exchange cycles, or abdominal pumps) may vary with the magnitude of pressure differentials, and the IGV method provides a new and exciting method to assess such variation.

Scaling of tracheal volume with body size in Schistocerca americana

Proportional V_T increases as body size increases across instars, but falls dramatically as mass increases within an instar (Fig. 4). A similar pattern was found using the water displacement method (Clarke, 1957), and may partially explain why oxygen delivery becomes more problematic as insects progress through an instar stage (Greenlee and Harrison 2004b). We speculate that the decrease in V_T within an instar is a common phenomenon as many insects gain mass within each life stage and yet have relatively rigid cuticles.

Our data provide the strong quantitative data that relative investment in the tracheal system increases across instar with age and size in *S. americana* (Fig. 4). This is a particularly interesting result given that lung and cardiac volumes change isometrically during development in vertebrates (Altman and Dittmer, 1974; Weibel et al., 1981; Gehr et al., 1981), whereas mass-specific capillary densities decrease with age/size (Weibel et al., 1981). We found that V_T scales with mass to the 1.3 for this species. However, when we separate out the instars with the smallest V_Ts ($\log V_T<0.6$), we find that the slope of the line describing the relationship between V_T and body mass is no longer significantly different from 1 [$V_T=2.536+1.011\times\log(\text{mass})$, where V_T is in μl and mass in g; $P<0.0001$, $R^2=0.79$]. This '$\log V_T<0.6$' cut-off may be arbitrary; when we exclude individuals with log mass<0.9, we get a slope of 1.1 instead of 1.01 for the larger individuals. Regardless, the exclusion of small instars indicates a smaller slope for the relationship between V_T and mass than when all individuals are included. Our data thus suggest that very early instars have considerably reduced tracheal volumes relative to older animals, but that tracheal volume may change isometrically with body size for larger instars and adults. Despite this, we think that the higher slope of 1.3 more accurately reflects the overall trend of V_T scaling for this species. At this time we cannot determine to what extent the observed increase in V_T is due to increases in relative air sac and/or tracheole content. Hartung et al. (Hartung et al., 2004) showed a proportional increase with age in the tracheal volumes of *S. americana* legs, which indicate increased investment in tracheae, especially tracheoles. Greenlee and Harrison (Greenlee and Harrison, 2004a) showed that the percent compression of the abdomen during ventilation of *S. americana* increased with age (first instars and adults), strongly suggesting an increase in relative air sac content with body size. Additionally, recent X-ray synchrotron imaging suggests that first instars lack air sacs, and that second instars have very few air sacs (J.F.H., unpublished data from Argonne National Laboratory).

Increased relative investment in tracheal system structure in larger/older *S. americana* explains many developmental patterns in respiratory capacities. Tracheal system respiratory volumes do not appear to scale isometrically with resting metabolism, which scales with mass$^{0.8}$ (Greenlee and Harrison, 2004a). However, mass-specific metabolic rates during jumping increase strongly

with mass (Kirkton et al., 2005), and flying adults have metabolic rates much higher than juveniles (Rascón and Harrison, 2005). Increased relative investment in the tracheal system provides a partial mechanistic explanation for this increased metabolic capacity. Also, this increased relative investment in the tracheal system explains the greater respiratory capacity of older grasshoppers, observed as larger safety margins for hypoxia [lower critical oxygen partial pressures; tested across first, third and fifth instars (Greenlee and Harrison, 2004a)]. This trend of increased tracheal investment with increased age might allow insects in general to overcome diffusion limitations as age and size increases. If so, we should see such increases in proportional V_T in interspecific comparisons as well.

Effect of sex on tracheal volume

Female *S. americana* clearly show reduced V_T compared to males (Fig. 6), probably due to displacement of air sac space by eggs (Fig. 7). Currently it is unknown whether this reduced volume translates into a performance deficit. Egg mass displacement of respiratory volume is probably widespread in other species. It is also interesting to note that egg accumulation may temporarily increase the oxygen consumption needs of insects (Taylor and Leelapiyanart, 2001) and at the same time, as our data suggests, possibly compromise oxygen delivery capacity. Clearly an important next step is to examine how this phenomenon affects performance.

Conclusions

Variation in metabolic capacity in insects can be reflected by respiratory structure and volume in diverse ways. We have documented here a new, repeatable method to measure insect V_T. We have also shown here that proportional investment in the tracheal system increases with age/size in this species. The scaling exponent of 1.3 for V_T exceeds scaling relationships of 0.8 for metabolic rate, and partially explains the enhanced respiratory capacity found in larger grasshoppers (Greenlee and Harrison, 2004a). Both of these findings contradict the argument that gas exchange is more difficult for larger insects. The trend we found, of increased proportional investment to V_T in larger insects, probably interacts with the increased use of convection that has been observed in larger insects (Greenlee and Harrison, 2004a) to allow insect respiratory systems to compensate for increasing size.

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