



## Review

# Issues of convection in insect respiration: Insights from synchrotron X-ray imaging and beyond<sup>☆,☆☆</sup>

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## ABSTRACT

While it has long been known that in small animals, such as insects, sufficient gas transport could be provided by diffusion, it is now recognized that animals generate and control convective flows to improve oxygen delivery across a range of body sizes and taxa. However, size-based methodological limitations have constrained our understanding of the mechanisms that underlie the production of these convective flows. Recently, new techniques have enabled the elucidation of the anatomical structures and physiological processes that contribute to creating and maintaining bulk flow in small animals. In particular, synchrotron X-ray imaging provides unprecedented spatial and temporal resolution of internal functional morphology and is changing the way we understand gas exchange in insects. This symposium highlights recent efforts towards understanding the relationship between form, function, and control in the insect respiratory system.

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## 1. Introduction

The template of the insect gas exchange system is simple. Respiratory gases enter and exit the body through spiracular valves, the gases are transported through air-filled tracheal tubes that ramify throughout the body, and diffusion occurs directly to and from the tissues. Although this basic pattern is conserved throughout the insects, tremendous variation exists in tracheal morphology and respiratory dynamics (e.g., Buck, 1962; Keister and Buck, 1974; Wasserthal, 1996; Harrison, 1997; Chapman, 1998; Bradley, 2007). Anatomically, variation is manifest in such features as spiracle number and location; tracheal size, shape, wall thickness, and branching pattern; density of tracheoles; and presence or absence of air sacs. Dynamically, time-dependent variation occurs due to coordinated opening and closing of the spiracles (e.g., Mill, 1985; Lehmann and Heymann, 2005; Wasserthal, 2001), movement of the fluid level at the terminal ends of the tracheoles (Wigglesworth, 1935; Woods et al., 2009) and, in some species, collapse and rein-

flation of the tracheal system itself (Herford, 1938; Westneat et al., 2003; Socha et al., 2008), including both tracheal tubes and air sacs (Greenlee et al., 2009a,b). This combination of morphological and behavioural variations results in a great variety of respiratory patterns and an overall general flexibility in the gas exchange system that may play a large role in the ecological and evolutionary success of insects. But despite over a century of study, our understanding of insect gas exchange systems—both pattern and process—can still be considered immature, largely due to this immense variation. Fundamental questions, such as the relative roles of convection and diffusion as a function of body size, phylogeny, development, and life history, remain unanswered.

Most research on gas exchange dynamics has focused on measurements made externally to the animal. These include measurements of CO<sub>2</sub> and H<sub>2</sub>O emission (Lighton, 2008), airflow patterns at the spiracles (Slama, 1988), frequency and duration of spiracle opening and closing (Lighton and Garrigan, 1995; Terblanche et al., 2008), and magnitude of exoskeleton movements (Slama, 2008). These techniques have been and still serve as an invaluable source of information on insect respiration, but they do not provide direct knowledge of internal gas exchange processes. Studies of morphology include description by dissection (Hartung et al., 2004; Harrison et al., 2005), endocasting using low-viscosity polymers (Meyer, 1988), and stereology (Schmitz and Perry, 1999; Hartung et al., 2004), which involves sectioning of the animal. These techniques are effective for determining tracheal system morphology, but they are relatively time consuming,

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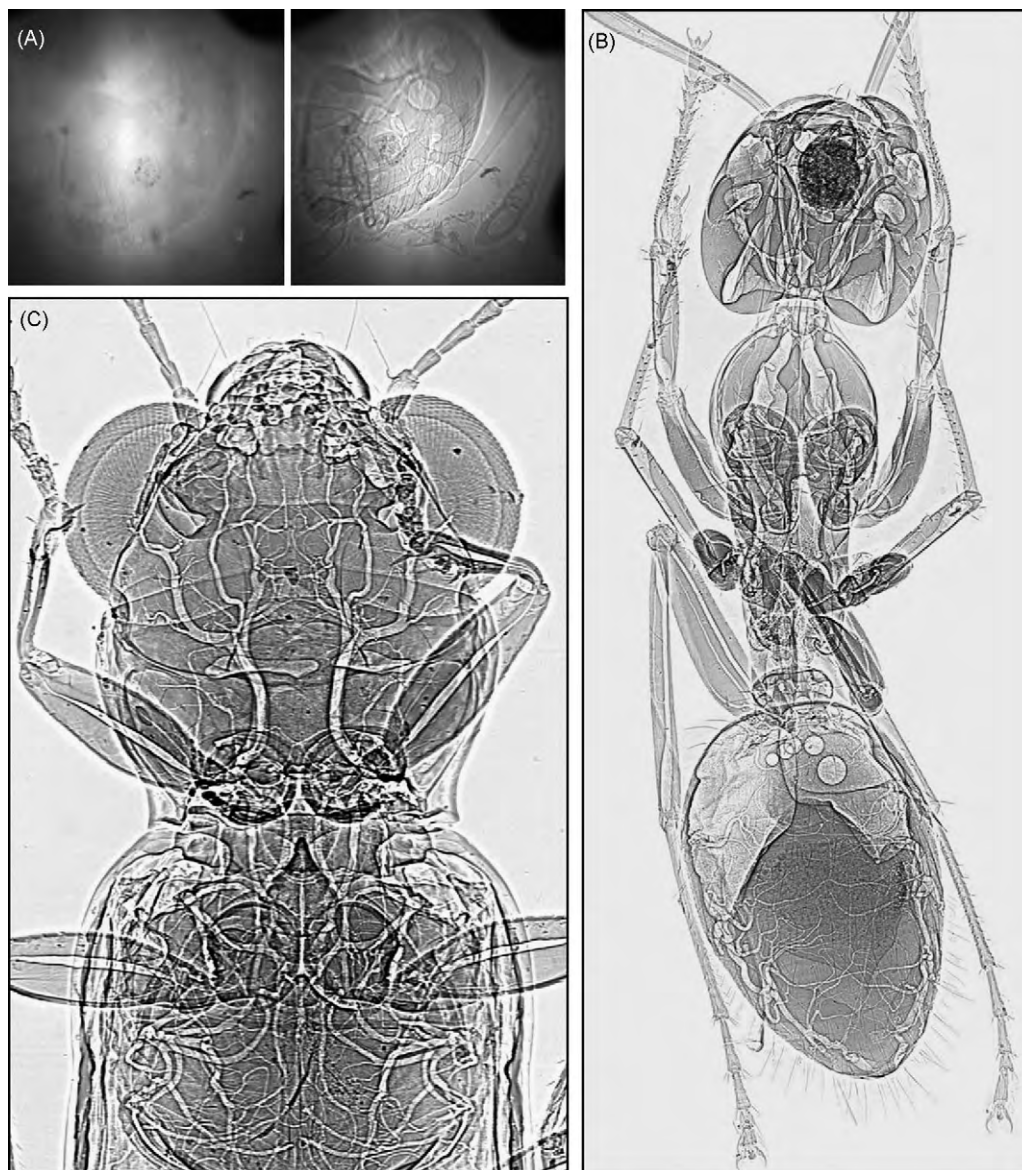
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require skillful preparation, or involve altering the system in some way, either by breaching the exoskeleton and relieving the native hemolymph pressure around the tracheal system, or by cutting the system itself. Perhaps resulting from these difficulties, it is not surprising that there are relatively few quantitative descriptions of entire tracheal systems (e.g., Clarke, 1957; Lease et al., 2006), and little is known about the variation of tracheal morphology across phylogeny.

This symposium was inspired by a new tool used to study insect physiology, synchrotron X-ray imaging, which is opening up a whole new window into the world of insect respiration. This tool provides the ability to visualize the tracheal system in an intact, living animal, and is motivating new work that revisits long-standing questions of insect respiratory physiology. Because the tracheal system can be observed in real time, this resource is particularly powerful for understanding internal dynamics involved in respiration.

### 1.1. Synchrotron imaging of insects

Synchrotron imaging has changed the way that we understand insect respiration. Although a few isolated X-ray images of insects appeared in the physical literature in the early 2000s (e.g., Matsui et al., 2000; Hwu et al., 2002), synchrotron X-ray imaging of insects began in earnest in 2001 when Wah-Keat Lee, a physicist at Argonne National Laboratory's Advanced Photon Source, became curious about what an insect would look like when viewed at his X-ray beamline (1-ID). Lee took an X-ray snapshot of the head of a carpenter ant (Fig. 1a) caught outdoors and, with it, sought the interest of biologists. He contacted Mark Westneat, a biomechanist at the Field Museum and University of Chicago, and they teamed together to explore the diversity of internal movements and morphology that can be seen with this powerful tool. As a necessary first step, their experimental approach was exploratory—they captured insects from the woods just beyond the doors of the synchrotron source



**Fig. 1.** Samples of synchrotron X-ray images of insects. (A) Demonstration of the capability of phase-contrast imaging with synchrotron X-rays. The two images show the head of a carpenter ant (*Camponotus pennsylvanicus*) in standard absorption-only configuration (left) and in phase-contrast configuration (right). Images were recorded in 2001 at beamline 1-ID-C at the Advanced Photon Source, Argonne National Laboratory. Images courtesy of Wah-Keat Lee. (B) and (C) Depict high-quality projection images of an ant worker (*Camponotus pennsylvanicus*) and carabid beetle (*Notiophilus* spp.), respectively. Large-scale differences in tracheal system architecture between the two insects can be easily discerned, including the ant's abdominal and head air-sacs and sac-like main tracheal trunks in the thorax. The round structures in the ant's abdomen are air bubbles.

and observed what could be seen inside their living bodies. Quite quickly, it became evident that the internal insect was far more dynamic than expected. Indeed, many of the videos they created were puzzling, because what they were seeing had not been previously documented. Within two years, they published data showing massive collapsing movements in the tracheal system of beetles, crickets and ants (Westneat et al., 2003), movements that either had not been described or were under-appreciated. Since then, synchrotron imaging of insects has been improved and refined to address more sophisticated and specific questions of insect respiration (Lee, 2009).

The power of synchrotron imaging is twofold: (1) it enables the direct visualization of the insides of living animals with high spatial and temporal resolution, and (2) methodologically, data collection can be relatively quick and simple. Once the imaging parameters have been set, all that is required is to restrain the animal in the X-ray beam. Imaging is not limited by insect size. Although the exact field of view is determined by the synchrotron source and beam-line apparatus, the X-rays can shoot through a giant beetle as easily as a fruit fly larva. A scintillating crystal converts the X-rays to a visible light image, and the researcher has the flexibility to record using whatever device is most appropriate for the question of interest, ranging from high-resolution still images to high-speed videos recorded at thousands of frames per second. Inherent between these two extremes is a tradeoff between image quality and, in general, survival of the insect (Socha et al., 2007).

Synchrotron X-ray imaging using a phase-contrast configuration is particularly effective for respiratory problems because air-filled tracheae produce a high degree of contrast compared to more dense body tissues, and because it enables edge enhancement. The raw data are two-dimensional (2D) projection images, with all structures within the field of view in focus. Depending on angle of view, anatomical structures that may be non-contiguous can appear to overlap, requiring some discrimination to analyze features of interest. Movements, both internal and external to the animal, are readily apparent, making it easy to investigate dynamic processes, such as the deformation of a tracheal tube or an air sac.

Two-dimensional projections can also be used to visualize and quantify morphology in three dimensions by using computed tomography. With the animal stabilized (either through sacrifice or chilling) and mounted on a rotation stage, multiple X-ray projection images are taken as the animal spins about the vertical axis. These images are computer-processed within minutes to reveal a stack of virtual slices of the intact animal, from which the three-dimensional (3D) morphology of soft tissue structures can be reconstructed. Aside from its speed, the virtue of this technique is that it can preserve the natural 3D anatomy of the animal. The size, shape, and connectivity patterns of the tracheal system can be analyzed without deformation of the tracheal walls and without massively altering the animal's natural hemolymph pressure environment.

The spatial resolution of 2D and 3D synchrotron imaging techniques used in this paper is on the micron scale, which means that much of the insect tracheal system can be analyzed, but the smallest tubes (tracheoles) are below the resolution limit and therefore cannot be seen. Alternatively, transmission X-ray microscopy (TXM) is capable of reaching 30 nm spatial resolution (Chu et al., 2008); however, this extreme resolution comes with a tradeoff of a very limited field of view (on the order of 25  $\mu\text{m}$ ). To date, there have been few, if any, studies of insect morphology using TXM, likely owing to the very small field of view.

Overall, imaging with synchrotron X-rays is currently the most effective, and in some cases only, method of studying insect tracheal morphology and dynamics. Its ability to directly observe the tracheal system provides a powerful method for re-examining issues

in insect respiration. Broad questions that are under current investigation include:

What explains the diversity of tracheal architecture and organization?

How does the respiratory system respond to changing metabolic needs during development and the transition from rest to activity?

What are the rules of convection—under what circumstances is it required or employed?

How are convective airflows created, and what airflow patterns are used?

Below (in Sections 1.2–1.6), we highlight symposium contributions that aimed to answer some of these questions involving pattern and process of insect gas exchange.

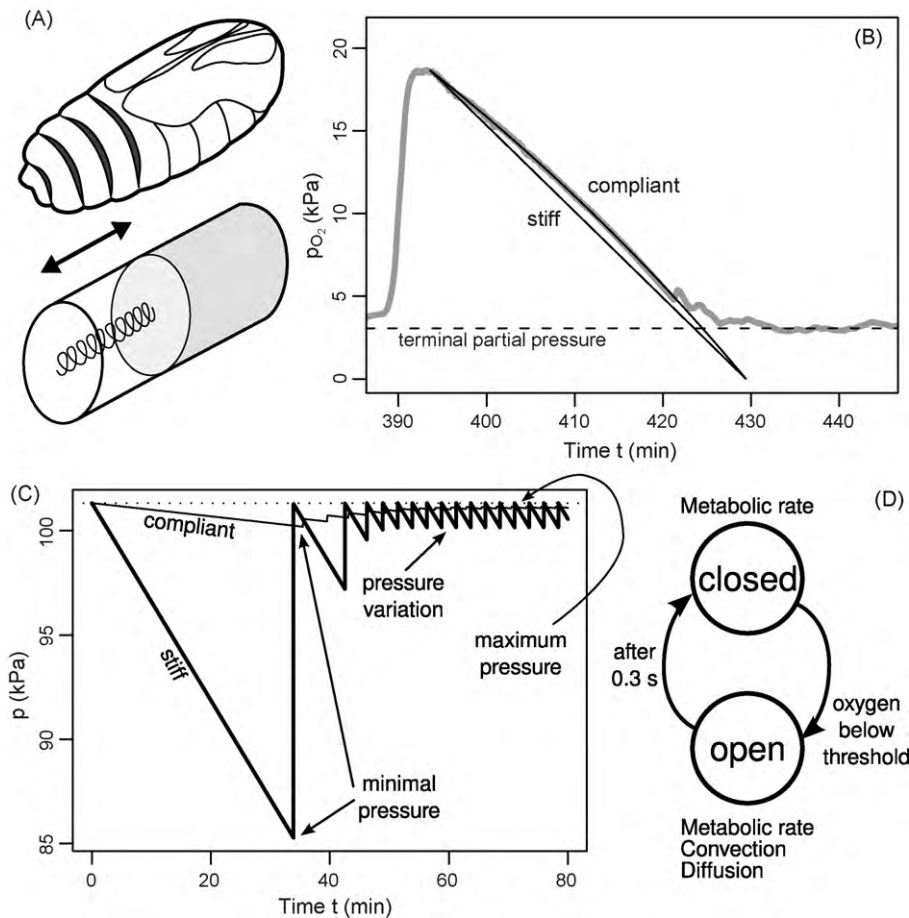
### 1.2. Airflows in insects at rest

Traditionally, respiration research in insects has focused on resting individuals, which has both experimental and theoretical underpinnings. For one, it is usually simpler to make measurements on insects that are not moving. Moreover, some resting insects exhibit an intriguing and complex pattern of gas exchange called “discontinuous gas exchange cycles” (DGC, see Lighton, 1996 for review), of which the physiological basis remains controversial (Chown et al., 2006). Lastly, there is the influence of August Krogh's famous calculations that diffusion alone should be sufficient to deliver enough oxygen to the respiring tissues (Krogh, 1920a).

Narrowing down Krogh to the claim that insects rely solely on diffusion, however, is a gross misinterpretation. Krogh was well aware of the presence and necessity of active ventilation during activity (Krogh, 1920b). Based on tracheal cross-sectional shape, he tried to distinguish the parts of the tracheal system that serve ventilatory purposes, “ventilation tracheae”, from those that could be supplied via diffusion only, “diffusion tracheae” (Krogh, 1920b). The characteristic difference between these two types of tracheae is their compliance, which is the ratio between the resulting volume change to the applied pressure change. Classically, diffusion tracheae with circular cross section are assumed to experience little deformation, whereas the elliptic ventilation tracheae may collapse and thereby pump air. However, recent work with X-rays is challenging these assumptions (Westneat et al., 2003; Socha et al., 2008; Greenlee et al., 2009a,b).

DGC consist of a tripartite cycle differentiated by the behavior of the spiracles (closed, flutter, open). In the closed phase, all spiracles are hermetically sealed from the environment (Bridges et al., 1980). Hemolymph buffering of  $\text{CO}_2$  (Buck and Keister, 1955, 1958; Buck and Friedman, 1958) as well as disparate rates of oxygen depletion vs. carbon dioxide generation then lead to development of subatmospheric pressure within the tracheal system (Schneiderman and Schechter, 1966; Brockway and Schneiderman, 1967). Because of this negative pressure, it has been argued that convection plays a role in preventing water loss by passive suction ventilation (PSV) during the subsequent flutter phase (Buck and Keister, 1958; Kestler, 1985). However, the mechanism of gas exchange during the flutter phase is still debated (Förster and Hetz, 2009). Diffusive and convective models differ in their predictions of the effect of decreasing the ambient oxygen partial pressure on flutter phase duration. If gas exchange during flutter occurs by convection, the increased accumulation of  $\text{N}_2$  due to the higher net inward flow of fresh air would prematurely disrupt the necessary pressure gradient and the flutter phase duration is shortened. In contrast, more  $\text{CO}_2$  would be lost during hypoxia in a diffusive flutter phase.

Förster and Hetz (2008) have recently shown that tracheal compliance is an important parameter not only for convection, but also for the DGC pattern itself, for which transport processes are thought to be primarily diffusive (Lighton, 1996). Given that compliance shaped the time course of endotracheal  $p\text{O}_2$  during the constrict-



**Fig. 2.** Summary of the flutter phase modeling study of Förster and Hetz (2009). (A) Conceptual model of tracheal compliance. (B) Model fit to the oxygen partial pressure during a constriction phase showing the effect of altered compliance. Modified from Förster and Hetz (2008). (C) The effect of altered compliance on endotracheal pressure during the flutter phase. Main effects are highlighted. (D) Schema of the flutter phase hybrid state model identifying the processes in every state and transition condition.

tion phase (Fig. 2b) the authors decided to extend the model to also include the tracheal dynamics when the spiracles open (Fig. 2d) and thus were able to model the flutter phase (Förster and Hetz, 2009).

Several interesting results arose from the model. First, when one or the other of the transport processes (convection or diffusion) was turned off in the model, the predicted pressure traces were very different from actual observations in insects (Wobschall and Hetz, 2004; Terblanche et al., 2008). These limiting cases of pure convection and pure diffusion resembled the predictions of Kestler (1985) based on partial differential equation modeling of mixed convective-diffusive transport. Only if this mixed transport was allowed could traces similar to the observations be obtained from the model. Looking at the relative contributions of convection and diffusion, it became clear from the model that there was a change from convection at the beginning of the flutter phase (the so-called “pressure rise period”; Schneiderman and Schechter, 1966) to diffusion during the later part of the F-phase when endotracheal pressure is near ambient values (“microcycles”; Schneiderman and Schechter, 1966). The model numerically supports the qualitative predictions of Chown et al. (2006) that PSV would be effective only during the relatively short pressure rise period. However, acknowledging the fact that convection and diffusion are not mutually exclusive is just a first step. Further modeling as well as experimental work needs to be undertaken to settle this question. Despite this new work, the tongue-in-cheek term “confusive” (Lighton, pers. comm.) for the mixed convective-diffusive mode of transport still describes our current understanding of the flutter phase.

### 1.3. Respiration in active insects

If the respiratory dynamics can be complex in resting insects, this is even more true for insects in motion. Owing to higher metabolic demand, convection naturally should play a much more pronounced role during activity than at rest. But how different is ventilatory control during activity?

Lighton (2009) examined the transition from rest to running activity in the beetle *Cryptoglossa verrucosa* using flow-through respirometry with high temporal resolution. As expected, the beetles appeared to switch from DGC to continuous  $CO_2$  release with the change from rest to activity. However, when their gas exchange rates were examined at high temporal resolution, their apparently “continuous” nature was shown to consist of rapid, discrete, pulsatile emissions (active ventilation). In contrast to the lepidopteran pupae that are commonly used for DGC investigations, *C. verrucosa* also exhibited active ventilations during the O-phase when at rest. Surprisingly, this active ventilation pattern during rest was indistinguishable from ventilation patterns during activity, and gas exchange rates were equivalent when locomotory and O-phase rates were compared. Furthermore, manual sealing of the thoracic spiracles indicated that airflows are tidal and chiefly involve the subelytral spiracles.

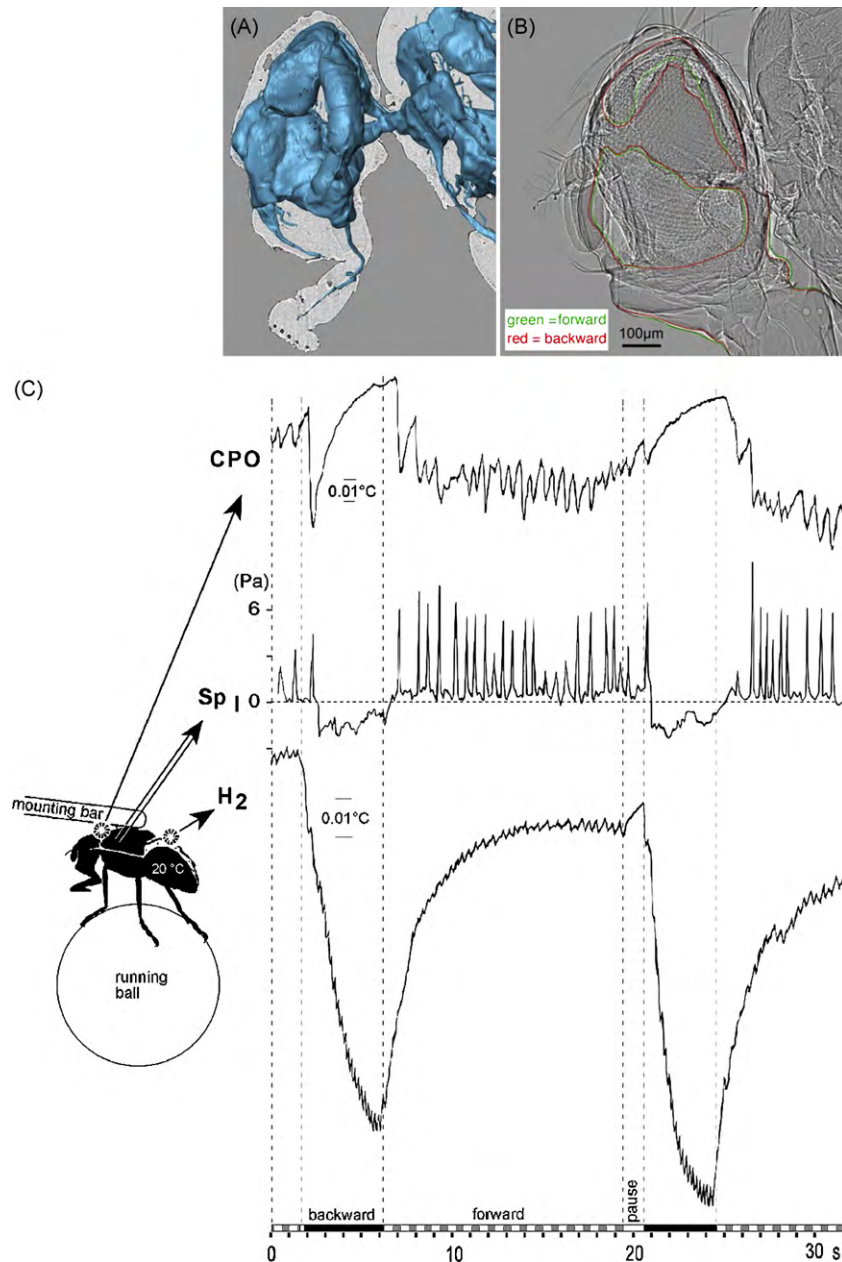
These data suggest that maximal energetic flux rates may be limited by the beetle’s ability to move air tidally in and out of the posterior body. In contrast, flying hawkmoths create a unidirectional airflow whereby fresh air enters via anterior spiracles and stale air exits via posterior spiracles (Wasserthal, 2001).

This directed airflow, which is a byproduct of flight movements with coordinated action of the metathoracic spiracles, creates an extremely high, almost atmospheric  $pO_2$  in the flight muscles of the insect (Komai, 1998). However, for the vast majority of insects that use convection, it is not known if tidal or unidirectional flows are created, or indeed if it is possible to modulate between the two for specific needs.

#### 1.4. Tracheal ventilation via alternating movement of hemolymph

In vertebrates, circulation and respiration are tightly coupled. Insects, however, show no strong dependence of oxygen deliv-

ery upon respiratory pigments in the hemolymph, and the link between cardiac function and respiration has been largely ignored (Wasserthal, 1996). While insects commonly mechanically ventilate tracheae and air sacs with visible muscular contractions of abdominal or thoracic cavities, some adult insects use an additional mechanism for ventilation. In several species of flying insects, much of the body and head are filled with light-weight air sacs (Wasserthal et al., 2006). As the heart pumps forward, a net flow of hemolymph to the head and thorax occurs, which is compensated by a volume reduction in the air sacs of the anterior body. When the heart reverses the direction of pumping, hemolymph is delivered to the abdomen to the same effect. During retrograde



**Fig. 3.** Respiratory–circulatory coupling in flies. (A) Three-dimensional tomographic rendering of the tracheal system of a fruit fly (*Drosophila melanogaster*). (B) Volume changes in air sacs resulting from hemolymph pumped in or out of the head, indicated by green and red, respectively. Images are 2D synchrotron X-ray projections. (C) Periodic heartbeat reversals and coordinated periodicity at the cephalic pulsatile organ (CPO) and their effect upon intratracheal pressure recorded at the thoracic spiracle (Sp I) of the fly *Calliphora vicina*. Heart pulses (measured on the tergite above the 2nd heart segment = H2) and CPO-pulses (measured at the occipital cuticle of the head) were inferred from convective cooling effects upon slightly heated thermistor sites. (A) and (B) from (Wasserthal et al., 2006), (C) from Wasserthal (1999); reprinted from International Journal of Insect Morphology and Embryology, L.T. Wasserthal, Functional morphology of the heart and of a new cephalic pulsatile organ in the blowfly *Calliphora vicina* (Diptera: Calliphoridae) and their roles in hemolymph transport and tracheal ventilation, pp. 111–129, 1999, with permission from Elsevier.

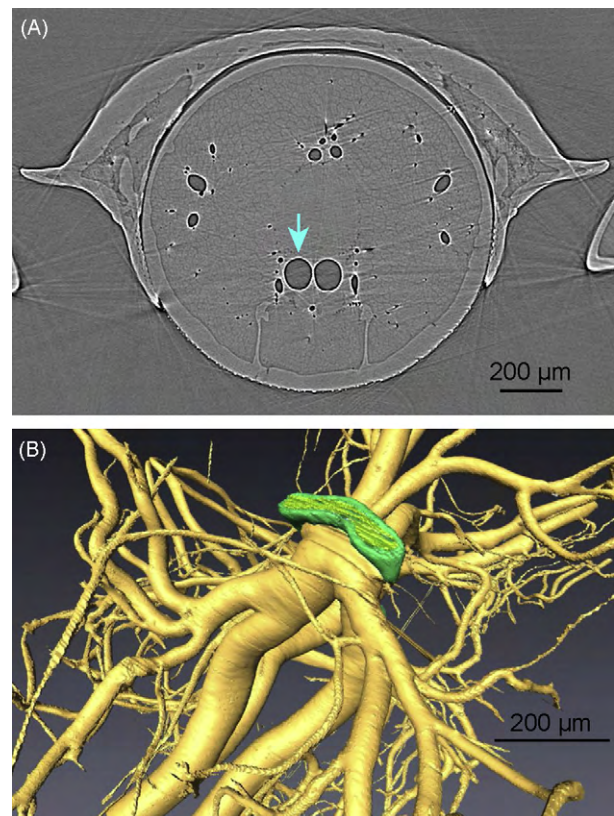
heartbeat, the elytral air sacs of scarabaeid beetles and the supercoiled wing tracheae in giant silk moths respond by compensatory volume increase and store elastic energy in their compliant walls for a tidal backflow of hemolymph into the hemocoelic space of the appendages. In effect, the air sacs work as antagonists of the heart and thoracic pulsatile organs. Using this pattern of reversing hemolymph flow, these insects are able both to create respiratory convection and to possess a reduced hemolymph volume, which decreases body mass and facilitates flight (Wasserthal, 1998).

Adult flies (*Calliphora* sp. and *Drosophila* sp.) exhibit this type of mechanical ventilation in the cephalic and thoracic air sacs. Heartbeat reversals occur at regular intervals and have been shown to correlate with periodic pressure changes at the spiracles in *Calliphora*, which suggests that heartbeat reversals regularly affect ventilatory gas exchange (Fig. 3 and Wasserthal, 1999). Support for this hypothesis has been obtained by synchrotron X-ray visualization of air sac movements in the head and parallel measures of intratracheal pressure changes in the thorax, which accompany the periodically reversed heart peristalsis (Wasserthal et al., 2009). Additionally, parallel measurements using O<sub>2</sub> micro-optodes and pressure sensors applied at the punctured thoracic air sacs confirmed the correlation of pressure decrease and a rise in O<sub>2</sub> during periodic heartbeat reversal. Accordingly, the X-ray videos demonstrated that air sacs in the heads of *Drosophila* and *Calliphora* expand during intratracheal pressure decreases in the thorax (Fig. 3b). In addition, cyclic CO<sub>2</sub> emission has been shown to occur mainly during forward beating of the heart, using flow-through respirometry. The heart action is supported during forward beating by intermittent activity of a cephalic pulsatile organ (Wasserthal, 1999) and of pleuro-cervical muscles, representing a new prothoracic pulsatile organ. X-ray tomographs enabled the reconstruction of the tracheal system (Fig. 3A) to calculate its volume and assess the effects of the circulatory–respiratory coupling. X-ray videos in combination with measurements of physiological parameters prove to be a promising tool for investigating air sac volume changes and gas exchange mechanisms induced by hemolymph shifting between thorax and abdomen.

### 1.5. Tracheal architecture in insects that use convection

Mechanisms of insect respiration are recognized to include both passive and active means of gas transport via diffusion and convection through tracheal tubes and air sacs. However, the role of active mechanisms of creating convection may be underestimated historically, due to the inability to visualize movements through opaque exoskeletons in intact, living insects. The X-ray identification of a periodic form of tracheal collapse and reinflation in some insects (Westneat et al., 2003), known as rhythmic tracheal compression, has opened new inquiries into the mechanisms, origins, and functions of internal airflows in insects. Rhythmic tracheal compression in some carabid beetles is characterized by the periodic, rapid collapse and reinflation of parts of the tracheal system (Socha et al., 2008).

Currently, it is not understood how and why compression occurs in the insect tracheal system during this form of active respiration. An important mechanical question regarding rhythmic tracheal compression is how efficient is the system at producing flows at the microscale? One method of addressing this question is to examine the architecture of the tracheal system itself to determine how network design contributes to the effectiveness of the flow system. For example, in a system that obeys Murray's law, energy loss is minimized at sites of branching; at each branch in such a system, the diameter cubed of the parent vessel will equal the sums of the diameter cubed of the daughter vessels (Murray, 1926; Sherman, 1981). Physiological flow networks that obey Murray's law have been observed across a broad range of systems, ranging from water



**Fig. 4.** Three-dimensional tracheal analysis of a carabid beetle (*Platynus decentis*) using synchrotron X-ray microtomography (SR- $\mu$ CT). (A) Sample two-dimensional cross-sectional slice from the head–prothorax junction. Tracheal tubes are round and oval structures; a representative tube is indicated (arrow). (B) Three-dimensional rendering of the tracheal system in the thorax. Stacked two-dimensional slices were volume-rendered using Amira software. Tracheal tubes (yellow) were highlighted using image segmentation. A thoracic spiracle is indicated in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

channels in sponges (LaBarbera, 1990) to vascular and respiratory networks in mammals (Kassab, 2006). However, this general principle has never been tested in an insect physiological system. Socha (2009) tested the hypothesis that the tracheal system of the beetle *Platynus decentis* is optimized for convective flow by determining if tracheal dimensions match those prescribed by Murray's law. Tracheal dimensions were measured from 3D renderings of the beetle's tracheal system (Fig. 4) using synchrotron X-ray microtomography (SR- $\mu$ CT) (Socha and De Carlo, 2008). Preliminary analyses indicate that the system is not optimized via Murray's law. If true, this suggests that energy efficiency related to convective flow is not an important factor in the design of the tracheal system of this carabid beetle, or simply that convection plays a minor role in respiratory function. Conversely, it is possible that energy efficiency is relevant, but that the assumptions used to formulate Murray's law do not hold for this carabid beetle.

### 1.6. Development of respiratory patterns in insects

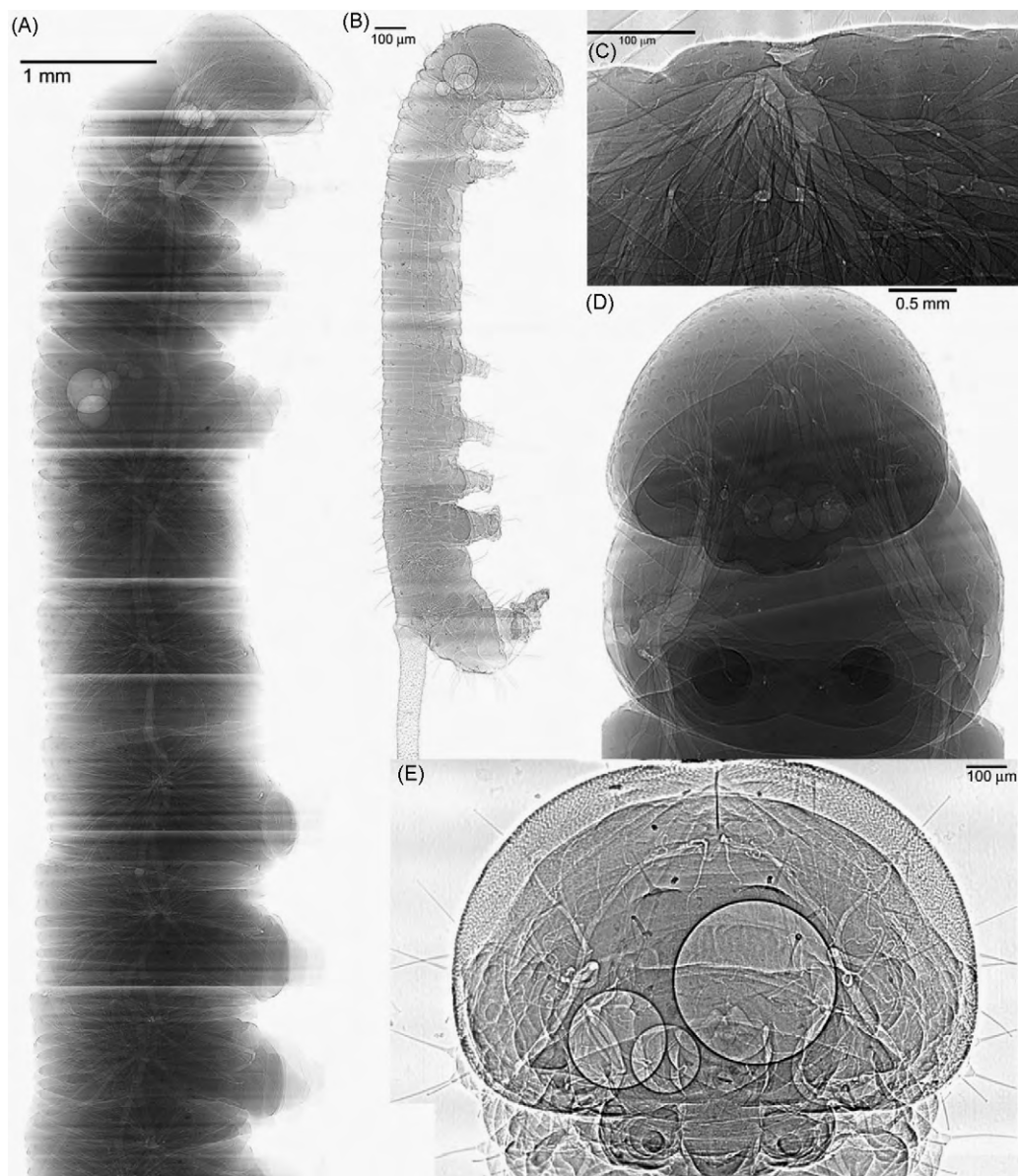
Insects often exhibit huge increases in body mass from hatching to adulthood (Elmes et al., 2001; Greenlee and Harrison, 2004, 2005; Kingsolver, 2007; Tammaru and Esperk, 2007). Because the increased mass is likely accounted for by increases in the amount of metabolically active tissue, these changes must naturally be accompanied by increases in metabolic rate and, hence, oxygen demand. In addition to increases in tissue mass, adult insects may use locomotory strategies that require high levels of oxy-

gen delivery (reviewed in Harrison and Roberts, 2000). Together, these life history traits suggest that there may be some form of respiratory compensation. While research on respiration in adult and pupal insects is abundant, studies on larval stages of both hemimetabolous and holometabolous insects are limited.

One possible compensatory mechanism is increasing the quantity or capacity of tracheal system structures throughout ontogeny. In grasshoppers, there is strong evidence that this occurs, as measures using water displacement (Clarke, 1957), histology (Hartung et al., 2004), direct measures of individual tracheae (Harrison et al., 2005) and inert gas volume (Lease et al., 2006) all show increases in tracheal volume with age. Using synchrotron X-ray imaging, Greenlee et al. (2009b) recently showed that not only does the fraction of tracheal structures increase, but it increases disproportionately relative to body size, exhibiting a pattern also seen in some adult beetles, termed “tracheal hypermetry” (Kaiser et al., 2007). While this pattern of increasing tracheal volume relative

to body size has been firmly established in developing grasshoppers and adult beetles, data are lacking for other insect taxa and developmental stages.

Modulation of active ventilation is another possible mechanism allowing insects to compensate for increases in body size and oxygen demand. Active respiration is described here as continuous, muscular-driven breathing. In terrestrial insects exhibiting active respiration, one may observe obvious, quantifiable ventilatory movements, such as abdominal pumping, neck ventilation or prothoracic ventilation (Miller, 1960). Recently, results from synchrotron X-ray imaging indicate that these external movements are highly coordinated with gas exchange and internal collapse of tracheae in grasshoppers (Greenlee et al., 2009b) and possibly in carabid beetles (Socha et al., 2008). Grasshoppers rely on abdominal pumping for gas exchange under most normal conditions and during exposure to X-rays. In conditions of normoxia, adult and juvenile grasshoppers had similar breathing frequencies



**Fig. 5.** Synchrotron X-ray images of larval *Manduca sexta*. Larvae were euthanized with ethyl acetate, then mounted and secured in polyimide tubing (Kapton, Dupont). Insects were chilled at 4 °C for 1 h and then allowed to return to room temperature prior to X-ray exposure. Lateral and dorso-ventral views of (A) and (D) a fourth instar larva (0.425 g) and (B) and (E) a first instar hatchling (0.0019 g) demonstrate increasing complexity of respiratory structures with age. Horizontal striping is an artifact of the X-ray. Dorso-ventral view of (C) an abdominal spiracle from the fourth instar larva reveals the spiracle atrium and spiracular valve. The large circular structures in (A), (B), (D), and (E) are air-filled bubbles within the body.

(Greenlee and Harrison, 2004; Greenlee et al., 2009b). However, in hypoxia, the smallest nymphs showed no response, while adults and larger nymphs doubled ventilation frequency. These changes were accompanied by increases in the frequency of tracheal collapse (Greenlee et al., 2009b).

Interestingly, the tobacco hornworm caterpillar also tolerates low levels of oxygen throughout its juvenile development. However, the mechanism of ventilation in hypoxia in these insects is unclear, because they lack air sacs and do not exhibit abdominal pumping during activities such as feeding or locomotion. Surprisingly, fifth instar caterpillars in 3–5% oxygen exhibit rhythmic body contractions that closely resemble abdominal pumping (Greenlee et al., 2009a). Synchrotron X-ray imaging in conjunction with high-speed respirometry reveals that in normal air, the caterpillars have no tracheal system collapse and CO<sub>2</sub> emission is not correlated with any particular body movement. However, in extreme hypoxia, tracheae rhythmically collapse, and the collapsing is highly correlated with the external body contractions and carbon dioxide emission peaks. Similar to observations in grasshoppers, the youngest caterpillars do not respond to hypoxia with an increase in respiratory movements. Overall, tracheation appears to be increased in larger caterpillars relative to smaller ones (Fig. 5). However, this parameter has not yet been quantified.

## 2. Conclusion

Wilhelm Conrad Röntgen won the first Nobel prize in physics for his exploration of “a new kind of ray” (Röntgen, 1896). Since their discovery in 1895, X-rays have been used to visualize a wide array of materials from bones (Spiegel, 1995) to molecular protein structures (Kendrew et al., 1958), and now synchrotron X-ray imaging enables the exploration of form and function in living insects. The recent application of this technique to the study of insects allows us to see how tracheal structures move in intact animals and to quantify 3D anatomy. While visualization of a structure is not required for testing hypotheses about function, the benefits of this ability are myriad.

As the application of X-rays has revolutionized many areas of science before, it is similarly revitalizing the study of insect respiration. These proceedings describe a few of the ongoing research programs using synchrotron imaging. However, more questions remain. For instance, in what ways does the design of the respiratory system impose constraints upon insect physiology, ecology, and evolution? Answering these questions will contribute to a greater understanding of basic physiological function in the most abundant group of animals on the planet.

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