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RESEARCH ARTICLE

Coordinated ventilation and spiracle activity produce unidirectional airflow in the hissing cockroach, *Gromphadorhina portentosa*

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SUMMARY

Insects exchange respiratory gases *via* an extensive network of tracheal vessels that open to the surface of the body through spiracular valves. Although gas exchange is known to increase with the opening of these spiracles, it is not clear how this event relates to gas flow through the tracheal system. We examined the relationship between respiratory airflow and spiracle activity in a ventilating insect, the hissing cockroach, *Gromphadorhina portentosa*, to better understand the complexity of insect respiratory function. Using simultaneous video recordings of multiple spiracular valves, we found that abdominal spiracles open and close in unison during periods of ventilation. Additionally, independent recordings of CO_2 release from the abdominal and thoracic regions and observations of hyperoxic tracer gas movement indicate that air is drawn into the thoracic spiracles and expelled from the abdominal spiracles. Our video recordings suggest that this unidirectional flow is driven by abdominal contractions that occur when the abdominal spiracles open. The spiracles then close as the abdomen relaxes and fills with air from the thorax. Therefore, the respiratory system of the hissing cockroach functions as a unidirectional pump through the coordinated action of the spiracles and abdominal musculature. This mechanism may be employed by a broad diversity of large insects that respire by active ventilation.

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Key words: insect, ventilation, respiration, spiracle, tracheae.

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INTRODUCTION

The insect respiratory system consists of a network of air-filled tracheal vessels that extend from the external surface to the tissues. Early studies demonstrated the importance of diffusion in transporting gases within the tracheal system (Krogh, 1920; Weis-Fogh, 1964b; Wigglesworth, 1983). However, insects have additional processes for regulating gas exchange, including the use of complex respiratory patterns, active ventilation (Bailey, 1954; Levy and Schneiderman, 1966; Sláma, 1988; Duncan and Byrne, 2002; Chown and Nicolson, 2004; Chown et al., 2006; Hetz, 2007) and the activity of spiracular valves along the lateral sides of the body. Although it is clear that gas exchange patterns within an insect are regulated by internal gas partial pressures (P_{CO_2} and P_{O_2}) and metabolic rate (Lighton, 1996; Chown and Holter, 2000; Hetz and Bradley, 2005; Contreras and Bradley, 2009; Förster and Hetz, 2010), there are large variations across species in the amount of time an insect maintains spiracular closure, the use of ventilatory movements, and the frequency and volume of CO_2 released during a spiracular opening (Miller, 1960a; Miller, 1960b; Sláma, 1988; Nation, 2002; Marais et al., 2005).

Considerable attention has been paid to understanding spiracular control mechanisms. Studies of internal gas pressures and their effects on spiracle activity have enhanced our knowledge of spiracle function (Burkett and Schneiderman, 1974; Förster and Hetz, 2010). Spiracular valves are controlled by interacting feedback loops that trigger spiracles to open in response to high internal P_{CO_2} or low P_{O_2}, as well as close in response to high P_{O_2} (Levy and Schneiderman, 1966; Förster and Hetz, 2010). The interaction of these control mechanisms produces the discontinuous pattern of CO_2 release observed in many insects during periods of low metabolic demand (Punt, 1950; Schneiderman and Williams, 1953; Punt et al., 1957; Burkett and Schneiderman, 1974; Chown et al., 2006; Förster and Hetz, 2010). This pattern is characterized by a period of complete spiracular closure followed by a series of brief spiracular openings that allows bulk inward flow of oxygen, and finally an open phase during which CO_2 is released. While the details of discontinuous gas exchange have been studied extensively, ventilation and its effect on respiratory patterns and spiracular control have received less attention. The goal of our study was to observe ventilation, spiracular activity and respiratory patterns to determine: (1) how spiracles across the entire body are coordinated and (2) whether coordinated spiracle activity and ventilatory movements produce directional airflow through an insect.

Active ventilation of the respiratory system is a common behavior observed in most large or highly active insects, including locusts, cockroaches, beetles and honey bees (Miller, 1960a; Weis-Fogh, 1967; Duncan and Byrne, 2002; Duncan et al., 2010; Groenewald et al., 2012), as well as lepidopteran and dipteran pupae (Sláma, 1976; Sláma, 1984; Sláma and Rembold, 1987; Wasserthal, 2001; Lehmann and Schützner, 2010). Despite oxygen's high rate of diffusion in air, insects utilize multiple methods for ventilation. These methods include abdominal pumping (Weis-Fogh, 1967), thoracic pumping associated with wingbeats, as described in locusts and dragonflies (Weis-Fogh, 1956a; Weis-Fogh, 1967), and the compression and expansion of airsacs and tracheae by either body wall movements or pressure transmission from the hemolymph

(Wigglesworth, 1963; Sláma, 1984; Wasserthal, 2001; Socha et al., 2008; Lehmann and Schützner, 2010; Socha et al., 2010; Harrison et al., 2013; Waters et al., 2013). Ventilatory motions have been shown to produce anterior to posterior unidirectional airflow, as well as tidal air flow in locusts (McArthur, 1929; Fraenkel, 1932; Weis-Fogh, 1967). Conversely, airflow from posterior to anterior has been demonstrated in the honey bee (Bailey, 1954) and the dung beetle (Duncan and Byrne, 2002).

It remains unclear how spiracular activity is coordinated across the entire insect or how the spiracles contribute to the production of airflow patterns through the body. Individual spiracles have been examined visually in non-ventilating insects (Levy and Schneiderman, 1966), and the activity of spiracular motor neurons has been examined in *Gromphadorhina portentosa*, *Blaberus discoidalis* and *Periplaneta americana* (Kaars, 1979; Nelson, 1979). Based on their neurological observations, Nelson and Kaars suggested that air may flow from the anterior to posterior of the body through the coordinated action of spiracles in *G. portentosa* and *B. discoidalis*. In the present study, we empirically tested this hypothesis by visually observing spiracle valve activity and inspecting for anterior–posterior movement of respiratory gases in an intact insect.

We used the Madagascar hissing cockroach [*Gromphadorhina portentosa* (Schaum 1853)] to examine spiracular activity and airflow patterns produced during ventilation. This cockroach is a well-studied insect, known for the hissing sound it produces. Like most cockroaches, this species relies heavily on ventilation, even at low metabolic rates when discontinuous gas exchange cycles (DGC) are employed. The hissing cockroach provides an additional experimental advantage in that the abdominal spiracles are large and easily observed under a macro camera lens. Finally, the neurophysiology of *G. portentosa* has been described in detail by Nelson (Nelson, 1979), who studied tracheal morphology and spiracular innervation in this species to determine how they produced the hissing sound.

Because *G. portentosa* and other cockroaches utilize ventilation while also producing discontinuous patterns of CO_2 release, we suspect that there may be more complex respiratory control systems at work than have been described for the discontinuous cycle alone. To better understand the relationship between spiracular control, gas exchange and ventilation in this insect, we observed the activity of all abdominal spiracles, measured CO_2 release using flow-through respirometry, and followed the movement of a tracer gas through the insect. We observed and quantified spiracular valve movements in spiracles located along the entire length of the body to understand how all spiracle pairs (Fig. 1) function together in time. Simultaneous video observations of spiracle movements and abdominal contractions and recordings of CO_2 release provided insight into the relationships between CO_2 release patterns and spiracular activity in ventilating insects. Additionally, we used independent recordings of CO_2 release from the abdominal and thoracic regions and observations of hyperoxic tracer gas movement to detect the direction of airflow.

MATERIALS AND METHODS

Animals

Adult *G. portentosa* were obtained from a laboratory colony maintained at 24°C under a 12 h:12 h light:dark cycle. Cockroaches were housed in a 38 l terrarium with a coconut husk substrate and clay pots for shelter. They were fed dry rabbit food (ProLab HF Rabbit 5P25, Lab Diet, Richmond, IN, USA), fresh fruits and vegetables, and had continuous access to water. The enclosure was sprayed with water daily to increase the relative humidity. The mean body mass of the cockroaches used for this study was 7.04±0.89 g (mean ± 1 s.d., *N*=7). Six females and one male were examined as a result of availability within the colony. Adult cockroaches were chosen because of the larger size of their spiracles, because they are easily handled during preparations, and to ensure that effects of molting on cuticular and tracheal morphology did not affect the observed spiracular and ventilatory activity.

CO_2 release patterns

We used flow-through respirometry to determine the pattern of CO_2 release from *G. portentosa* at rest. CO_2 release was measured with a Li-Cor model 6262 infrared CO_2 analyzer (Lincoln, NE, USA). Data were collected every second with Expedata PRO data

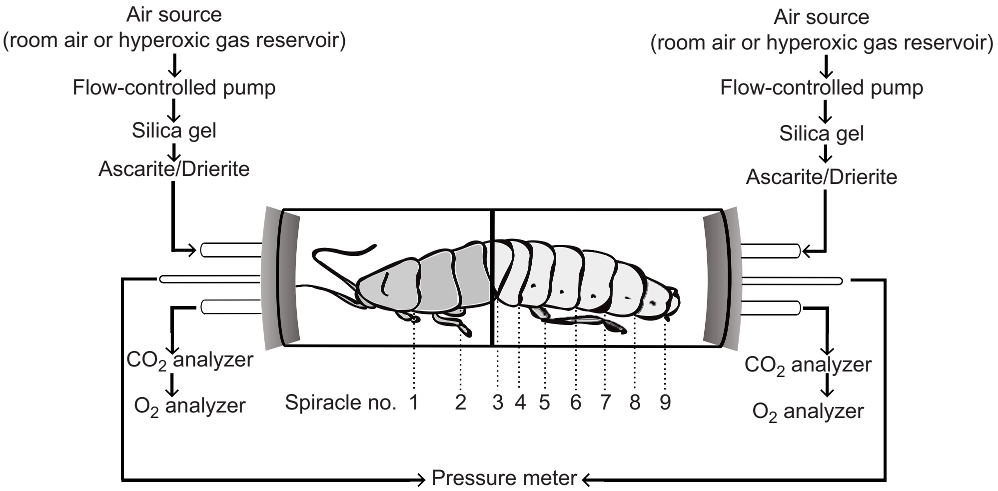

Fig. 1. To test for directional airflow, the thorax and abdomen of *Gromphadorhina portentosa* were separated into two chambers with a latex diaphragm. The first two pairs of legs were restrained within the thoracic chamber and the third pair of legs was restrained along the abdomen. The diaphragm was located just anterior to the third spiracle pair and was secured tightly around the insect to prevent leaks between chambers. CO_2 and O_2 content in each chamber were recorded simultaneously. One chamber was maintained at 70% oxygen. If the insect moved air from thorax to abdomen, then bursts of oxygen would be detected in the abdominal chamber while the thoracic chamber was maintained at 70% O_2, and *vice versa*. Spiracle identification numbers and locations are provided for reference. Each number refers to a spiracle pair, including left and right spiracles on the same segment.

acquisition software (Release 1.3.4, Sable Systems International, Las Vegas, NV, USA). An MFS mass flow system (Sable Systems) pushed air at a rate of 600 ml min^{-1} through a column of silica followed by a column of Ascarite (Thomas Scientific, Swedesboro, NJ, USA) and Drierite (W. A. Hammond Drierite Co., Xenia, OH, USA) to remove CO_2 and water. The air then passed through the experimental chamber containing the insect and finally through the CO_2 analyzer. To observe resting respiratory patterns, adult cockroaches were placed into 25 ml cylindrical plastic chambers (~2.85×4.45 cm) within a temperature-controlled cabinet maintained at 20°C by a PELT-5 temperature controller (Sable Systems). Recordings were taken from an empty chamber for 5 min before and after each trial to obtain baseline CO_2 readings.

Video recordings and analysis of spiracle activity

We used video recordings of spiracular valves on adult cockroaches (*N*=7) to observe patterns of spiracle activity across the entire body and to relate these movements to recorded CO_2 release from the whole insect (supplementary material Movie 1). Individuals had their legs restrained with medical tape and were secured to a platform attached to a micromanipulator. Video cameras (models Marlin F131B and Pike F032B, Allied Vision Technologies, Newburyport, MA, USA) were used to record two spiracles on contralateral sides. The camera fields of view were ~0.75×1 mm and 1.5×2 mm, respectively. Video was captured with Fire-i software (Unibrain, San Ramon, CA, USA), which triggered both cameras to record simultaneously. Videos were recorded as 640×480 pixel grayscale images at 15 frames s^{-1}.

Video recordings were taken from same-segment pairs 1 through 9, as well as the following different-segment pairs: 8–3, 8–4, 8–5, 8–6 and 8–7 (see Fig. 1 for spiracle identification). As described in the Results, we found that same-segment pairs were significantly coordinated in their pattern of opening. Therefore, our findings for different-segment pairs from contralateral views may be assumed to apply to different segments on the ipsilateral side of the body. For all measurements, a random 20 s segment of video was analyzed. Repeated, non-invasive recordings of spiracle pair 9 were not possible because the dorsal and ventral cuticle surfaces close tightly around the spiracle opening, obscuring the view of the valve underneath. To observe spiracle 3, a small portion of the cuticle on the posterior end of segment 3 was clipped to allow a clear field of view during recordings.

Simultaneous video and CO_2 release recordings were collected by placing a restrained cockroach in a 25 ml cylindrical glass chamber attached to a micromanipulator, which positioned the chamber within the focal range of the cameras. Cameras on both sides recorded video of the spiracles while 1 l min^{-1} of dry, CO_2-free air was pumped through the chamber and CO_2 analyzer. Upstream from the insect, air passed through a metal coil that was submerged in an ice bath to reduce the temperature of the airstream experienced by the insect to ~15°C. This was done to promote discontinuous gas exchange during observations. We corrected for the time lag in the CO_2 trace caused by the tubing length between the glass chamber and the analyzer by recording the latency that followed injections of a constant volume of CO_2 into the chamber at multiple locations. A lag of ~2.48±0.84 s (mean ± 1 s.d., *N*=30) was observed.

Spiracular activity was quantified manually in ImageJ (version 1.45s, National Institutes of Health, Bethesda, MD, USA) by scoring the state of the spiracle as opened or closed for each frame of a video recording. Spiracles were considered open when the cuticular valve detached from the flexible membrane on the opposite end of the spiracle atrium, or in the case that this membrane was not visible, when the valve moved inward enough that the inside of the spiracle atrium was visible. Spiracles were considered closed if the valve was attached to the membrane or if the entire internal spiracle atrium was covered. Periods of abdominal contraction and expansion were also manually identified from these recordings in order to correlate abdominal ventilatory movements with spiracular activity. Video images of spiracular valves included footage of same-segment dorsal and ventral cuticle. The start of abdominal contractions was defined by the frame in which the ventral and dorsal cuticular segments first began to move toward one another from a stationary resting position, and the end as the frame in which the cuticular segments reached their closest point before moving apart again. Abdominal relaxation was defined by the first frame in which these cuticular segments began to move apart and the frame in which the cuticular segments returned to a stationary position.

Test for unidirectional airflow

Gromphadorhina portentosa, like many cockroaches, actively ventilates during the open phase of the DGC (Contreras and Bradley, 2010). We were interested in determining whether unidirectional airflow through the animal occurs during these ventilatory bursts. For this purpose, we separated the abdomen and thorax of the insects into two separate chambers and used oxygen as a tracer gas to visualize directional airflow. This method has been described previously (Duncan and Byrne, 2002). CO_2 release from the abdomen and thorax were recorded separately by gently restraining the cockroach's legs with medical tape and placing a latex diaphragm anterior to the first abdominal spiracle to separate the abdominal and thoracic segments into two chambers (Fig. 1). To secure the diaphragm, a small hole was cut in the center of a latex sheet, which was then stretched over the insect. The material was flexible enough to allow small movements of the insect without producing leaks. The forelegs and midlegs were restrained within the thoracic chamber and the hindlegs were pulled down along the abdomen and secured within the abdominal chamber. The cockroaches were then placed into a 25 ml cylindrical plastic tube with the diaphragm fastened through the center of the chamber to separate the two regions. Dry, CO_2-free air was pumped at 600 ml min^{-1} into each region. CO_2 release and oxygen content of the air from each region were recorded simultaneously using two CO_2 analyzers and an Oxzilla differential oxygen analyzer (Sable Systems), respectively.

In order to observe active movement of air from one region of the body to another, we employed hyperoxic air as a tracer gas. We introduced supplemental oxygen into one region and recorded the oxygen concentration in the other, normoxic region. A reservoir of hyperoxic air (~70% O_2) was created within a large Mylar balloon, from which air was pulled by either the thoracic or abdominal chamber pump. An oxygen content of 70% was required in order detect small bursts of tracer gas in the normoxic chamber. An increase in oxygen above normoxic values in the non-perfused chamber was interpreted as a product of directional airflow through the tracheal system. Furthermore, a burst of oxygen that coincided with a burst of CO_2 was considered to result from a specific spiracular opening event. Multiple recordings with the tracer gas on the abdominal or thoracic segments were taken on the same cockroach during one 2–3 h session (two 30–45 min observations per region). These tests were performed on five of the adult cockroaches used for the spiracle recordings after completion of video observations.

Secondary recordings were also performed after the initial experiments to verify that observed tracer gas movements were not

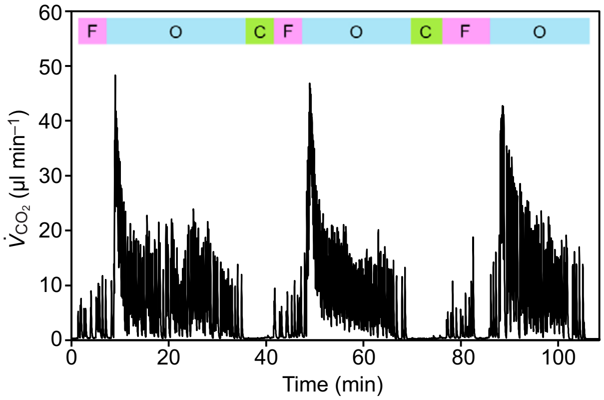

Fig. 2. An example of discontinuous CO_2 release from an unrestrained adult male *G. portentosa* at 20°C in normoxia, measured at 600 ml min^{-1}. Periods of ventilation are separated by 5–10 min of spiracular closure. The discontinuous pattern consists of a flutter phase (F), in which micro-openings of the spiracles allow oxygen to enter the tracheal system by bulk flow while some CO_2 is released; an open phase (O), in which the spiracles open to release accumulated tracheal CO_2; and a spiracular closed phase (C). Note that in this species, the open phase is marked by frequent bursts of CO_2 release associated with active ventilation.

produced by pressure variations between chambers. In these tests, a PT Series pressure meter (Sable Systems) measured pressure differences between the thoracic and abdominal chambers. In addition, high vacuum grease (Dow Corning, Midland, MI, USA) was added to the seal between the insect and the diaphragm, and Parafilm (Pechiney Plastic Packaging, Chicago, IL, USA) was wrapped tightly around the entire chamber to provide extra protection from potential leaks.

Dissections

Dissections were performed to determine the internal morphology of the spiracular apparatus and trachea leading to the spiracular opening in the thorax. Adult cockroaches were anesthetized with CO_2 and the heads were removed. Individual thoracic spiracles and surrounding tissue were excised. An incision was made through the major tracheal tube attached to the spiracle in order to display any internal valve structures medial to the spiracular surface opening. Video recordings of the internal tracheal morphology were collected during dissections.

Data analysis

To analyze flow-through respirometry data, CO_2 was recorded at 1 s intervals in parts per million and zeroed in Expedata analysis software (Sable Systems) using baseline values from empty chambers. Data were exported to Excel 2010 (Microsoft, Redmond, WA, USA) and converted to units of microliters per minute.

Statistical analyses on spiracular activity were performed in RStudio (RStudio, Boston, MA, USA). Coordination of spiracle pairs was determined using a Pearson's chi-squared test with Yates' continuity correction on four possible states (left and right open, left and right closed, left open and right closed, or left closed and right open). This test allowed us to determine whether the state of the left spiracle in a pair was independent of the state of the right spiracle. Seven cockroaches were observed, with 300 frames analyzed per pair, resulting in a total of 2100 observations per test. A final overall test of independence including data from all abdominal spiracle pairs was also completed.

Comparisons were also made of the observed *versus* expected proportions of time that a spiracle pair was found in a particular position (both open, both closed, left open and right closed, left closed and right open). Observed proportions were calculated as the total number of frames in which a pair was found in a given position divided by the total number of observations for that pair (approximately 2100 observations per test). Expected proportions for the four possible pair positions were calculated as the product of the fraction of time that the left spiracle was in a given position (open or closed) and the fraction of time that the right spiracle was in a given position (open or closed).

RESULTS

Respiratory pattern in *G. portentosa*

At rest, *G. portentosa* exhibits discontinuous gas exchange (Fig. 2). These episodes of rhythmic bursts of CO_2 release (labeled 'O' in Fig. 2) are interrupted by 5–10 min spiracular closed phases (labeled 'C' in Fig. 2), in which no CO_2 is released. Active ventilation causes the rate of CO_2 release to rapidly rise and fall throughout the open phase. Simultaneous recordings of spiracular activity, ventilatory movements and CO_2 release demonstrate that individual peaks of CO_2 release are often associated with an abdominal contraction and spiracular opening (Fig. 3).

Dissections and microscopic observations

Dissections of the first and second thoracic spiracles and underlying tracheae revealed no internal valve structure, which would have appeared as either two sclerotized regions on either side of the spiracle atrium, or as a fold of the wall of the atrium (Snodgrass, 1935). Snodgrass (Snodgrass, 1935) described four possible spiracle structures. The thoracic spiracles of *G. portentosa* resemble what he refers to as an atriate spiracle with a lip-type closing apparatus. This morphology is common to thoracic spiracles, while internal valves are typical of abdominal spiracles (Snodgrass, 1935). Spiracles with a lip-type closing apparatus have been described as closing with different degrees of efficiency (Snodgrass, 1935), indicating that some valves may not hermetically seal the spiracular opening during contraction of the closer muscle. As such, we were unable to detect closed positions in thoracic spiracles with microscopic observations or video recordings. Although the closing

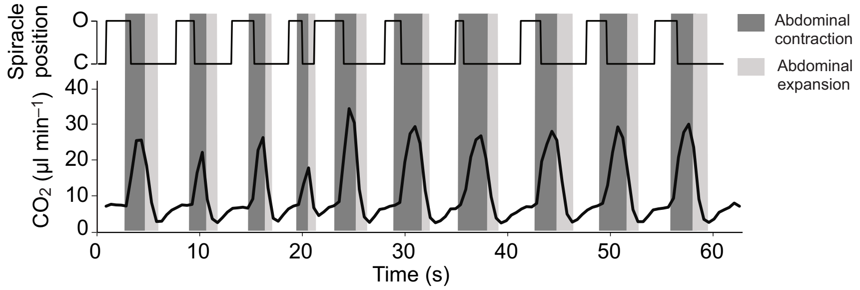

Fig. 3. The position of spiracle 7 (right side) over time, recorded as open (O) or closed (C), is associated with ventilatory movements and CO_2 release. Data were recorded from a restrained female *G. portentosa* at 15°C. This pattern was typical in individuals utilizing ventilatory movements. Spiracles open for a period of time during abdominal contraction and remain closed throughout abdominal expansion. CO_2 peaks are detected at the same frequency as abdominal contractions.

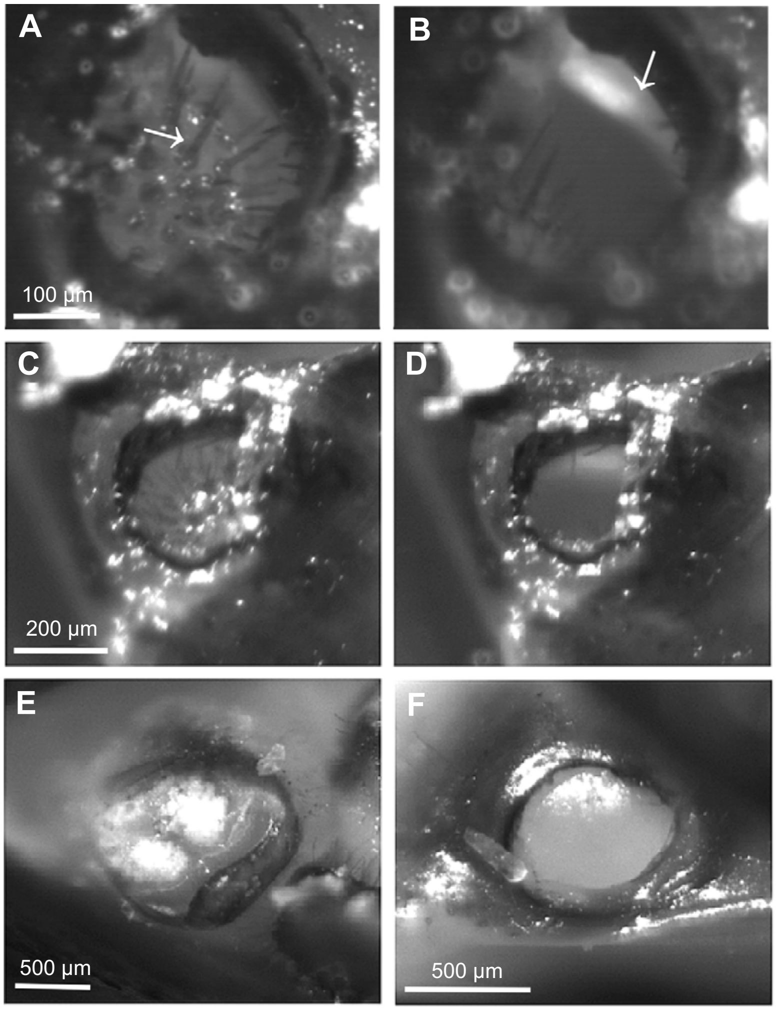

Fig. 4. Single frames from video recordings showing closed abdominal spiracles (A,C), open abdominal spiracles (B,D), an open thoracic spiracle (E) and the closed first abdominal spiracle (hissing spiracle) (F). Bristles are visible on the valve structure of abdominal spiracles (labeled with an arrow in A), and an internal membrane is visible in the atria of abdominal spiracles during opening (labeled with an arrow in B). Spiracular valves are framed by a circular opening in the structural cuticle. During abdominal spiracular closure, the distal portion of the valve moves laterally and attaches to the flexible membrane, presumably to hermetically seal the opening. During opening, the valve moves inward and pulls away from the membrane, revealing the inside of the spiracular atrium.

apparatus appeared to pulsate in some observations, no complete closures of the thoracic valves were observed.

In contrast, the open and closed positions in the abdominal spiracles were clear (see Fig. 4). A single internal valve was observed to move from a closed position in which the valve rested perpendicular to the wall of the spiracle atrium, to an open position in which the valve was pulled inward and the inside of the spiracle atrium was visible. In many video observations of abdominal spiracles, a membrane was visible to which the valve would connect during closure, presumably to hermetically seal the spiracle. Dissections revealed that medial to the spiracular opening, the trachea branches immediately into three to four major vessels, which extend in multiple directions. These findings are similar to those described by Dailey and Graves (Dailey and Graves, 1976).

Spiracular activity

Our observations indicate that abdominal spiracles 4–8 on both sides of the insect open and close at the same time. Occasionally, variations are observed because of incomplete opening of valves or short lags in opening between spiracles in a pair (supplementary material Fig. S1). A chi-squared test for independence indicates that there is a significant relationship between the position of the left spiracle and the position of the right spiracle across all observations (χ^2_1=10443.79, P<0.01 N=23,042), and that the positions of the left and right spiracles are not independent of one another. A similar relationship is found within all individual spiracle pairs, including same-segment and different-segment pairs (Table 1). Both same-segment and different-segment pairs were found in concordant states more frequently than discordant states. Additionally, all spiracle pairs were found in concordant states more frequently than expected by chance and in discordant states less frequently than expected by chance (Table 1). Both spiracles in any given pair were found closed in 54.9–75.3% of our observations, both open in 12.8–32.5% of our observations, and in discordant states in only 2.6–10.7% of our observations.

Spiracle 3 remained closed throughout all video observations. This was expected as this spiracle is specialized for sound production and has been shown to open only during hissing (Nelson, 1979). Thoracic spiracles sometimes pulsated at frequencies similar to observed abdominal spiracular valve movements, but remained open during all observations. These pulsations were characterized by incomplete closures and slight valve movements while in the open position. For these reasons, spiracles 1 through 3 were not included in the chi-squared analyses. Pulsations are not believed to represent ‘fluttering’ behavior because the valves remained open.

Airflow patterns

Unidirectional airflow was demonstrated when a hyperoxic tracer gas introduced into the thoracic chamber was detected in the abdominal chamber (Fig. 5). Oxygen bursts recorded from the abdominal region coincided with spiracular openings and with bursts

Table 1. Results of chi-squared tests for independence of spiracular positions

Spiracle pair			Right spiracle state						
			Observed		Expected				
Left	Right	Left spiracle state	Closed	Open	Closed	Open	χ^2	d.f.	*P*
4	4	Closed	0.679	0.063	0.524	0.218	1264.174	1	<0.01
		Open	0.028	0.231	0.183	0.076			
5	5	Closed	0.753	0.031	0.660	0.124	807.646	1	<0.01
		Open	0.088	0.128	0.182	0.034			
6	6	Closed	0.707	0.026	0.548	0.185	1432.507	1	<0.01
		Open	0.040	0.226	0.199	0.067			
7	7	Closed	0.672	0.028	0.525	0.176	1124.864	1	<0.01
		Open	0.076	0.223	0.224	0.075			
8	8	Closed	0.563	0.051	0.383	0.231	1215.952	1	<0.01
		Open	0.061	0.325	0.241	0.145			
8	7	Closed	0.549	0.076	0.396	0.229	898.860	1	<0.01
		Open	0.085	0.290	0.238	0.138			
8	6	Closed	0.630	0.068	0.459	0.240	1301.187	1	<0.01
		Open	0.026	0.275	0.198	0.103			
8	5	Closed	0.555	0.105	0.413	0.246	797.214	1	<0.01
		Open	0.072	0.269	0.213	0.127			
8	4	Closed	0.645	0.107	0.498	0.253	1082.530	1	<0.01
		Open	0.018	0.230	0.164	0.084			
8	3	Closed	0.652	0.348	0.652	0.348	193.831	1	<0.01
		Open	0.000	0.000	0.000	0.000			

Data are proportions of total observations across seven individuals in which a particular state was found.

of CO_2. However, we did not find evidence of airflow from the abdominal to the thoracic region (Fig. 6). When a hyperoxic tracer gas was introduced to the abdominal chamber, no oxygen bursts were detected in the thoracic chamber. In fact, very little CO_2 was detected in the thoracic chamber in any recordings. Thoracic CO_2 release remained below 50 μl min^{-1} compared with bursts of 100–200 μl min^{-1} from the abdomen (Figs 5, 6). Similar results were obtained regardless of the order in which the thoracic and abdominal segments were exposed to the hyperoxic tracer gas.

The absence of leaks was verified by the lack of movement of tracer gas from one chamber to another in the absence of ventilatory behavior. Similarly, possible leaks produced during ventilation were not present, as no anterior–posterior movement of tracer gas was detected. A leak during ventilation would have resulted in oxygen movement in both directions.

In secondary trials, pressure measurements from each chamber were obtained to determine whether the observed airflow patterns were a function of pressure gradients between each chamber driving

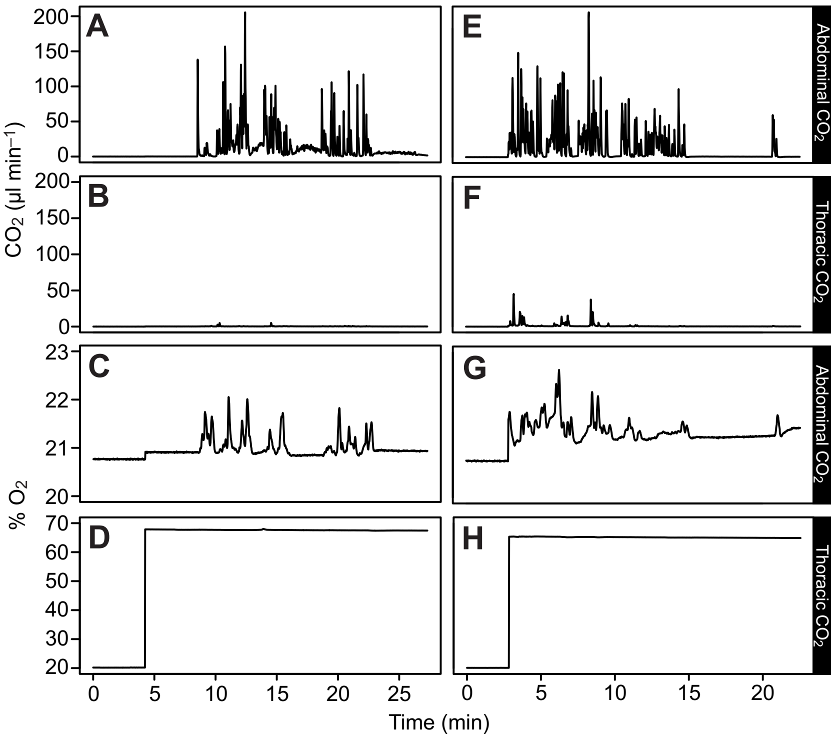

Fig. 5. Results from respirometry trials in which a hyperoxic tracer gas was used to detect airflow direction in two adult cockroaches (individual 1: A–D; individual 2: E–H). When 70% oxygen was profused through the thoracic chamber (D,H), bursts of oxygen were observed in the abdominal chamber (C,G) in association with CO_2 release from the abdomen (A,E). Minimal CO_2 release was observed from the thoracic region in all recordings (B,F).

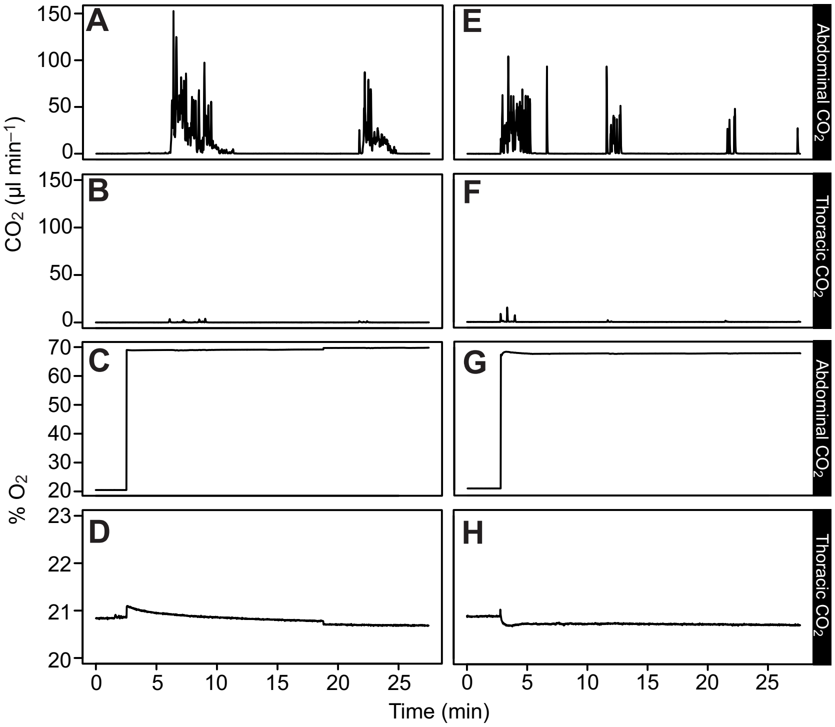

Fig. 6. Results from respirometry trials in which a hyperoxic tracer gas was used to detect airflow direction in two adult cockroaches (individual 1: A–D; individual 2: E–H). When 70% oxygen was profused through the abdominal chamber (C,G), bursts of oxygen were not detected in the thoracic chamber (D,H). CO_2 release was observed in the abdominal chamber (A,E) but very little CO_2 was released from the thorax (B,F).

the tracer gas movement. During these tests, we observed unidirectional movement of the hyperoxic tracer gas from the thoracic to abdominal chamber only. No variation in pressure was detected between these two chambers (Fig. 7).

On some occasions, particularly after prolonged exposure to the hyperoxic gas, unidirectional flow was not detected. These events were characterized by a lack of tracer gas movement in any direction. Because we were only able to confirm unidirectional flow in the presence of a diaphragm, we also cannot rule out that the absence of unidirectional flow in these cases was not due to the diaphragm constricting the cockroach in some manner, obstructing the movement of air from thorax to abdomen.

Spiracle activity was coordinated with abdominal ventilatory movements. Abdominal spiracles opened during abdominal

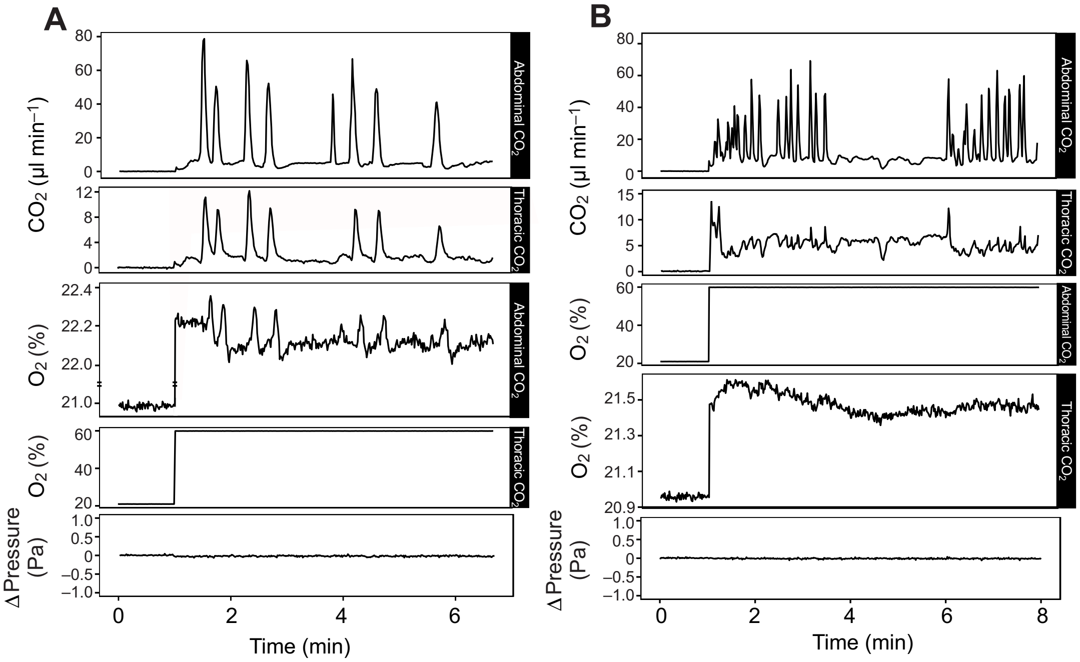

Fig. 7. Recordings of CO_2 release and oxygen content in the chambers containing the thorax and abdomen of *G. portentosa*. (A) When hyperoxic air was introduced to the thoracic chamber, bursts of oxygen were detected in the abdominal chamber. These oxygen bursts are associated with CO_2 release from the abdominal segment. (B) When hyperoxic air was introduced to the abdominal chamber, there were no distinguishable oxygen bursts detected in the thoracic chamber. In each test, there was no difference between the pressures in each chamber. The first minute of each recording indicates baseline values measured from empty chambers (CO_2 and O_2) or from the atmosphere (pressure).

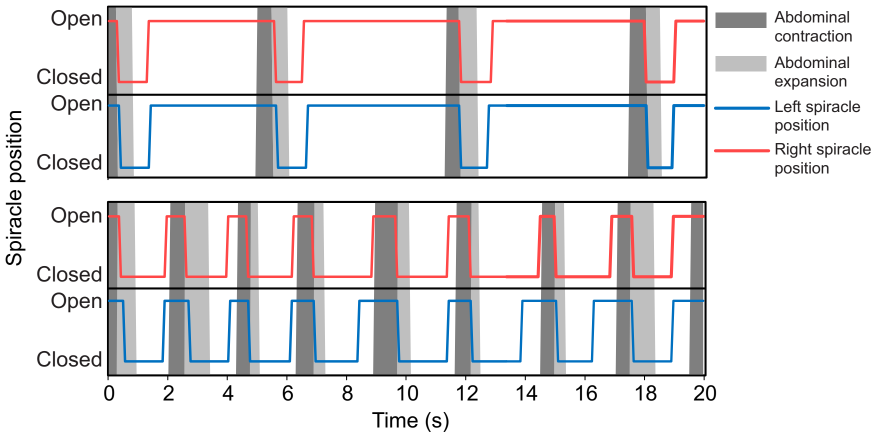

Fig. 8. Spiracle activity is associated with abdominal ventilatory movements. Spiracle positions from two different cockroaches are displayed as line graphs fluctuating between the open and closed state, and abdominal ventilatory movements as bars separated into periods of abdominal contraction and expansion. Top: data obtained from the same-segment pair number 8 on individual 1; bottom: data obtained from same-segment pair number 7 on individual 2. Spiracles are open during abdominal contraction and closed during abdominal expansion.

contraction and closed during abdominal expansion (Fig. 8). Between abdominal contractions, the spiracles remained closed in 58% of observations (spiracles 4–8), and open in 42% of observations (spiracles 4–8). However, regardless of the spiracular position between contractions, the pattern of opening during contraction and closing during expansion was observed in most cases (88% of observation of spiracles 4–8).

DISCUSSION

We analyzed spiracular activity across the entire body of *G. portentosa* using video recordings of spiracles. Openings of the abdominal spiracles coincide with individual CO_2 bursts and abdominal pumping movements (Figs 3, 8). By following the movement of a hyperoxic tracer gas from the thoracic to abdominal regions, we found evidence for unidirectional airflow during ventilation. Our results suggest that synchronized abdominal pumping and spiracular movements produce airflow into the thoracic spiracles and out of the abdominal spiracles during periods of active ventilation (Figs 5–9, Table 1). This is the first study to use simultaneous recordings of CO_2 release, abdominal contractions and observations of the activity of multiple spiracular valves to examine the mechanism by which unidirectional airflow patterns are produced in a ventilating insect.

Spiracle morphology

Contrary to Nelson (Nelson, 1979), who reports three pairs of thoracic spiracles, we identified only two pairs of thoracic spiracles and seven pairs of abdominal spiracles in *G. portentosa*. Our observations support the findings of Dailey and Graves (Dailey and Graves, 1976) that the thorax includes only two pairs of spiracles, which is consistent with the tracheal anatomy described by Snodgrass (Snodgrass, 1935). This may be a result of the reduction or loss of the metathoracic spiracles, which is a common occurrence in the thoracic tracheal anatomy of insects (Snodgrass, 1935). However, although external structures may be reduced or missing, the neural structures associated with innervation of the third thoracic spiracle may still be present.

Spiracle coordination in the abdominal segment

The abdominal spiracles open and close nearly in unison (Table 1). The observed discordant states were primarily due to very short lags between the opening events of spiracles in a pair or a result of incomplete valve movements, which did not meet our criteria for an opening event. We suspect that these lags and inadequate valve movements were caused by variation in opener muscle tensions of the two spiracles. Coordination of same-segment pairs is not surprising because spiracles on the same segment are stimulated by transverse nerves that originate from central ganglia (Case, 1957; Nelson, 1979). The soma for opener and inhibitory motor neurons are found in the ganglion containing the spiracle pair, and closer motor neurons have soma in the ganglion in the next anterior segment (Nelson, 1979). In *P. americana*, the spiracles on the posteriormost abdominal segments are the only spiracles that are innervated only by a nerve from the same segment (Case, 1957). This central control would allow same-segment spiracles to remain coordinated during ventilation. Case (Case, 1957) reports a closing reflex, in which stimulation of the mechanoreceptory hairs on one spiracle stimulate both spiracles on a segment to close, which provides further evidence for central control of spiracular closure.

The activity of spiracle valves and ventilatory movements may be controlled by a pattern generator in the central nervous system (CNS). Such a control system would explain the high degree of coordination observed in the abdominal spiracle pairs. However, it remains possible that this central control could be overridden by local effects of CO_2 on the closer muscle (Hoyle, 1960). In particular, CO_2 may interfere with the transmission of action potentials across the neuromuscular junction (Hoyle, 1960). However, if local CO_2 accumulation has a significant effect on normal spiracular function, it is likely to have similar effects on same-segment spiracles because they are in the same anatomical region.

We found that spiracles on different abdominal segments were also highly coordinated with one another. It is possible that a central pattern generator similar to the one that controls ventilation in locusts (Bustami and Hustert, 2000) or American cockroaches (Woodman et al., 2008) may be involved in sending signals to the abdominal ganglia that control spiracle pairs on each segment. The ventilatory central pattern generator is located in the metathoracic ganglion in locusts, and has been shown to increase its cycle frequency as temperature increases, causing an increase in ventilatory frequency and a decrease in respiratory burst duration (Newman et al., 2003). Our observations of coordination among all abdominal spiracles may be explained by a rhythmic nervous output originating in the metathoracic ganglion and traveling posteriorly. As discussed below, we found that spiracle activity is correlated with ventilatory movements, suggesting that these activities may be controlled by the same central pattern generator.

Because all of our observations were made in actively ventilating cockroaches, it is possible that different patterns of spiracular activity are utilized during non-ventilating states, or in insects incapable of active ventilation. Snodgrass (Snodgrass, 1935) described variation in spiracle functionality, including cases in which insects were capable of utilizing all spiracle pairs (holopneustic), only anterior

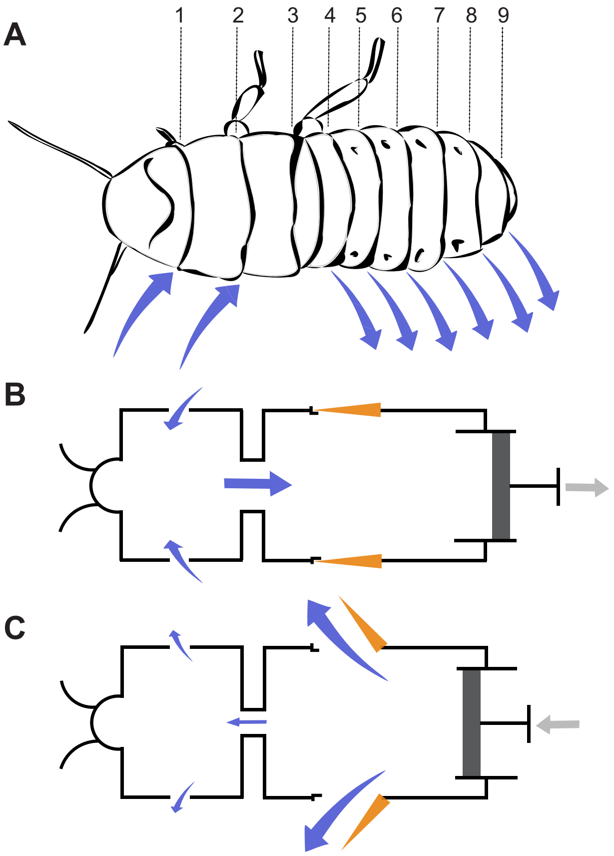

Fig. 9. A proposed model of ventilatory airflow in *G. portentosa* based on video analyses of spiracle activity, flow-through respirometry observations, and tracer gas movement. (A) Airflow directions through all nine spiracles during active ventilation. Spiracle 3 opens only during hissing and otherwise remains closed. (B) During abdominal expansion, the abdominal spiracles close and air is drawn into the thoracic spiracles. (C) The abdominal spiracles open during abdominal contraction and air is pushed out primarily through abdominal spiracles. In *G. portentosa*, the thoracic spiracles remained open, allowing some CO_2 release from the thorax. Because thoracic CO_2 release is minimal, backflow from the abdomen to the thorax may be limited by the resistance of the tracheal system or by active constriction of the trachea.

and posterior spiracles (amphipneustic), only anterior spiracles (propneustic) or only posterior spiracles (metapneustic). Lepidopteran larvae and pupae, for instance, are thought to utilize only one or two of the prothoracic spiracles for periodic gas exchange (Sláma, 1988).

Unidirectional airflow

Our results indicate that abdominal spiracles function in concert with abdominal ventilatory movements to produce unidirectional airflow from the thoracic to the abdominal regions (Fig. 9). According to this model, air enters through the thoracic spiracles during abdominal expansion, when the abdominal spiracles (4 to 9) are closed. Air is then expelled from the abdomen through the opening of abdominal spiracles while the abdomen compresses. This model is consistent with the results of Kaars (Kaars, 1979) and Nelson (Nelson, 1979), who inferred anterior to posterior airflow from measurements of spiracle motor neuron activity, and post-synaptic action potentials in spiracle opener and closer muscle.

Though we were unable to distinguish closure in videos of thoracic spiracles, very little CO_2 release was detected from the thoracic region of these insects during dual-chamber flow-through respirometry experiments (Figs 5–7). Therefore, the mechanism by which *G. portentosa* prevents thoracic CO_2 release during abdominal compression remains unclear. It is possible that the trachea and air sacs in the head and thorax prevent posterior to anterior airflow by compressing under the hydrostatic pressure generated during abdominal compression. This is consistent with studies that have shown compression of tracheal vessels in many insects, including ants and crickets (Westneat et al., 2003), beetles (Westneat et al., 2003; Socha et al., 2008; Waters et al., 2013) and *Manduca sexta* caterpillars (Greenlee et al., 2013). This mechanism to prevent backflow would also be consistent with the abdominal air sac compression that is correlated with abdominal pumping in grasshoppers (Greenlee et al., 2009).

The pattern of ventilation was observed to change after an insect was exposed to the hyperoxic tracer gas for a prolonged period of time. Periods of spiracular closure increased in duration, ventilator periods decreased in duration, and volume of CO_2 released in each burst decreased during prolonged exposure to 70% oxygen. These observations are consistent with other studies that indicate that exposure to hyperoxia triggers prolonged spiracular closure and leads to an overall reduction in the rate of CO_2 uptake ($\dot{V}_{CO_2}$) (Gulinson and Harrison, 1996; Lighton et al., 2004).

Ventilation at rest

Gromphadorhina portentosa exhibits discontinuous gas exchange patterns at low metabolic rates with prolonged periods of active, potentially unidirectional, ventilation during the open phase. The prevalence of active ventilation across insect species during discontinuous respiration is unclear as many studies have utilized flow rates insufficient to differentiate the individual CO_2 peaks produced at a high frequency during the open phase in ventilating insects (Gray and Bradley, 2006). Because our current understanding of spiracular control explains tightly regulated spiracular activity associated with intratracheal P_{CO_2} and P_{O_2}, it seems that there must be an additional overriding control mechanism that allows the spiracles to open and close in association with abdominal movements during unidirectional airflow when intratracheal gas values would reach atmospheric levels. Woodman et al. (Woodman et al., 2008) report combined measurements of CO_2 bursts, CNS output from the metathoracic ganglion, and abdominal pumping in the American cockroach. They argued for the existence of a centralized oxygen chemoreceptor, because CNS output and ventilatory activity responded to changes in ambient oxygen tension.

It is important to differentiate between the DGC and the ventilatory pattern produced by active, pressure-driven bulk air flow. The patterns of closed, flutter and open phases characteristic of DGC are controlled by the partial pressures of O_2 and CO_2 inside the insect (Levy and Schneiderman, 1966; Burkett and Schneiderman, 1974; Contreras and Bradley, 2009; Forster and Hetz, 2010). The active ventilatory pattern exhibited by many insects and illustrated in *G. portentosa* in Figs 3 and 8 is apparently controlled by a central pattern generator that may itself be sensitive to P_{O_2} inside the insect.

Our data suggest that coordinated patterns of spiracular activity and ventilatory movements facilitate unidirectional airflow (Bailey, 1954; Duncan and Byrne, 2002; Woodman et al., 2008) (see Figs 5, 6). It is clear, at least in insects that actively ventilate, that spiracular control is quite complex and provides respiratory control far in excess of simply opening and closing connections between the tracheal space and the external atmosphere.

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AUTHOR CONTRIBUTIONS

All authors contributed to writing the paper and designing the study. E.C.H. carried out the experiments, collected video data and conducted the analyses. M.J.M. assisted with the production and analysis of the video data.

COMPETING INTERESTS

No competing interests declared.

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