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Ventilatory airflow patterns and control of respiratory gas exchange in insects

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Ventilatory airflow patterns and control of respiratory gas exchange in insects

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

In Ecology & Evolutionary Biology

by

Erica Christine Heinrich

Dissertation Committee:
Professor Timothy J. Bradley, Chair
Professor Matthew J. McHenry
Professor Adriana D. Briscoe

2015

DEDICATION

To my family for their love and support,

to Alex,

and to all of my colleagues and friends
who have been instrumental in
my development as a scientist.

"Insects live and feed, move, grow and multiply like other animals; but they are so varied in form, so rich in species, and adapted to such diverse conditions of life that they afford unrivalled opportunities for physiological study."

-V. B. Wigglesworth

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ACKNOWLEDGMENTS

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[3] **Heinrich, E. C.** and Bradley, T. J. (2014) Temperature dependent variation in gas exchange patterns and spiracular control in *Rhodnius prolixus. The Journal of Experimental Biology 217*: 2752-2760.

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[2] **Heinrich, E. C.**, McHenry, M. J., and Bradley, T. J. (2013) Coordinated ventilation and spiracular activity produce unidirectional airflow in the hissing cockroach, *Gromphadorhina portentosa*. *The Journal of Experimental Biology*. **216**: 4473-4482.

[1] **Heinrich, E. C**., Farzin, M., Klok, C.J., McKinley, B., Harrison, J.F. (2011) The effect of developmental stage on the sensitivity of cell and body size to hypoxia in *Drosophila melanogaster*. *The Journal of Experimental Biology* **214**: 1419-1427.

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[1] **Heinrich, E. C.,** Fragata, I. Kezos, J. N., Rose, M. R., Matos, M., Bradley, T. J., Mueller, L.D., and Santos, M. Confounding factors in thermal ramping assays: An empirical test of mechanistic models.

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[3] **Heinrich, E. C.**, German, D. P., and Bradley, T. J. The effect of respiratory pattern on oxidative damage in insects.

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[2] **Heinrich, E.C**., Klok, C.J., Harrison, J.F., Farzin, M., and McKinley, B. (2010) Mechanisms of hypoxia effects on body size in *Drosophila melanogaster*. *Integrative and Comparative Biology*. **50**: e72.

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European Journal of Entomology
Journal of Comparative Physiology - B

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American Association of University Women

ABSTRACT OF THE DISSERTATION

Ventilatory airflow patterns and control of respiratory gas exchange in insects

By

Erica Christine Heinrich

Doctor of Philosophy in Ecology & Evolutionary Biology

University of California, Irvine, 2015

Professor Timothy J. Bradley, Chair

The insect respiratory system is composed of a network of air-filled tracheal tubes that open to the atmosphere via spiracles along the lateral sides of the body. Trachea branch throughout the body and deliver oxygen directly to tissues. Insects control respiratory gas exchange patterns by modulating the opening and closing of the spiracle valves. The function of these respiratory patterns and the complete mechanism by which the spiracle valves are controlled is unknown. The work presented here investigates (1) the airflow patterns through the tracheal system, (2) the physiological contributors to respiratory pattern and possible spiracle control mechanisms, and (3) the adaptive significance of discontinuous gas exchange in insects.

I used flow-through respirometry, video analyses of spiracle and abdominal movements, and hyperoxic tracer gases to determine if ventilating insects utilize tidal or unidirectional airflow in their respiratory system. I found that *Gromphadorhina portentosa* coordinates spiracular valve movements and abdominal contractions to produce unidirectional airflow through the tracheal system, with air entering through the thoracic spiracles and exiting through the posterior abdominal spiracles.

Previous studies have determined that the respiratory pattern of insects is a function of metabolic rate. However, most studies of ectotherms utilize changes in ambient temperature to manipulate metabolic rate. I exposed *Rhodnius prolixus* to two metabolic stimuli, changes in ambient temperature or digestion of a blood meal, and measured components of their gas exchange pattern. I found that the volume of carbon dioxide released during a spiracle opening decreased with temperature, and that *R. prolixus* abandoned discontinuous gas exchange at a lower metabolic rate when metabolism was increased via increased ambient temperature compared to digestion.

Lastly, I tested the longstanding "oxidative damage hypothesis" regarding the adaptive function of discontinuous gas exchange. I exposed *Drosophila melanogaster* to hypercapnic (6% CO_2) gas treatments to prevent spiracular closure, and then measured the amount of oxidative damage (lipid peroxidation, protein carbonyl content, and superoxide dismutase activity) that accumulated in these populations versus a control group. I found that acute exposures (3 hours) of hypercapnia increased lipid peroxidation but had no effect on protein carbonyl formation or antioxidant enzyme activity. While results suggest that the discontinuous gas exchange pattern may not function to prevent excess oxidative damage, additional studies are required to investigate the chronic effects of disrupting this respiratory pattern.

INTRODUCTION

THE INSECT RESPIRATORY SYSTEM

Insects are the most diverse group of animals and comprise a majority of all known animal species. Their evolutionary success is evidenced by low rates of extinction and their dominance of most terrestrial ecosystems (Labandeira & Sepkoski 1993). One contributing factor to this success may be insect's unique gas exchange system, which provides high oxygen diffusion capacity to support a wide range of metabolic demands.

Unlike vertebrates and other arthropods, insects have a completely air-based respiratory system. An extensive network of air-filled tracheal tubes open along the lateral sides of the body, branch throughout the insect, and deliver oxygen directly to tissues (Fig. 1). Tracheal tubes are formed as invaginations of the integument of the cuticle (Edwards 1953). The finest branches of the tracheal system are called tracheoles. These are sometimes found to be filled with fluid, the function of which remains unknown (Wigglesworth 1983). Wigglesworth and Lee (1982) found that nearly every mitochondria in insect flight muscle was in direct contact with a tracheole, sometimes resulting in invaginations of the cellular membrane around the terminal end of the tracheole tube. As a result of the high rate of diffusion of oxygen in air (oxygen diffuses about 10^6 and CO_2 diffuses 10^4 times faster in air than water) and the close proximity of mitochondrial cells to tracheoles, insect flight muscle is the most metabolically active tissue known (Weis-Fogh 1964; Krogh 1920).

The tracheal system allows for efficient oxygen delivery in insects with high metabolic demands and large body sizes. This system is especially important since cutaneous diffusion has become limited in terrestrial insects as their external cuticle has

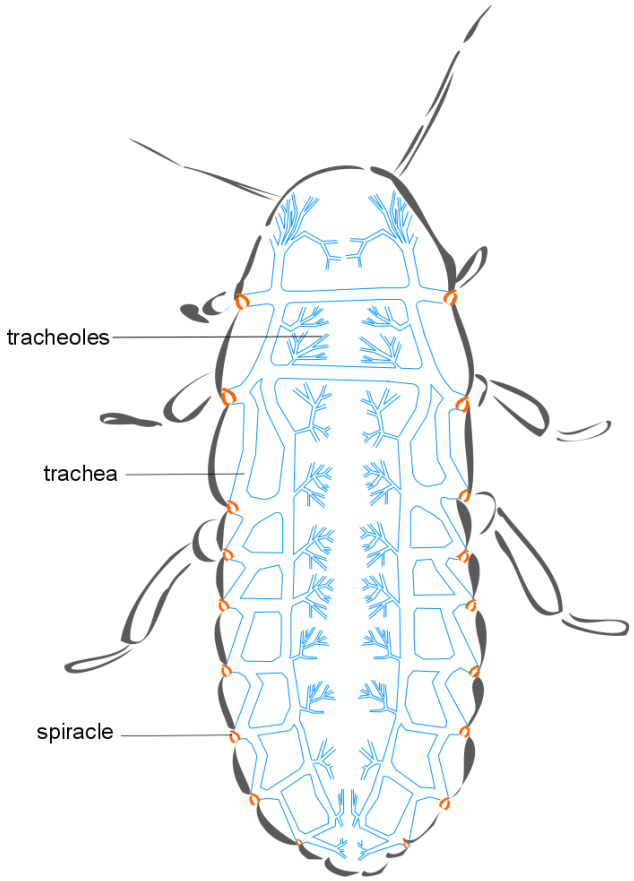

Fig. 1. A schematic diagram of the insect tracheal system.

adapted to minimize water loss (Wigglesworth 1972). However, some primitive insects (Poduridae), and all insect eggs, rely on cutaneous diffusion for gas exchange (Edwards 1953). Similarly, a majority of CO_2 release by aquatic larvae likely occurs via cutaneous diffusion. Mosquito larvae are capable of surviving completely submerged in aerated water without access to air (Wigglesworth 1933). Some portion of gas exchange may occur via cutaneous diffusion in all insects. Thorpe demonstrated cutaneous CO_2 release from the soft cuticle of larvae, and from the intersegmental cuticle of adult Coleoptera (Thorpe

1928). While cutaneous diffusion may play an important role in CO_2 release, especially in aquatic insects, O_2 uptake likely occurs primarily via the tracheal system.

Valves called spiracles occur at tracheal openings along the lateral sides of the body and control the exchange of respiratory gases between the tracheal system and the environment. The morphology of the spiracles varies across species, but generally consists of a valve structure that opens into an internal atrium space which separates the peritreme from the tracheal anastomosis (Mill 1998). Insects vary in their number of spiracles, typically with 2-4 thoracic spiracle pairs and 7-8 abdominal spiracle pairs (Mill 1998). There is also variability in the structure of the closing apparatus (Mill 1998; Wigglesworth 1972). A lever type apparatus may include an opener and/or closer muscle which pull on a cuticular hinge to move a valve plate which closes the spiracle opening (Fig. 2). Sometimes the opening muscle is replaced with an elastic element which holds the spiracle open in the absence of closer muscle constriction. Another apparatus sometimes found on the interior of the spiracle structure involves a closer muscle which pulls on either end of a flexible rod to construct a section of trachea.

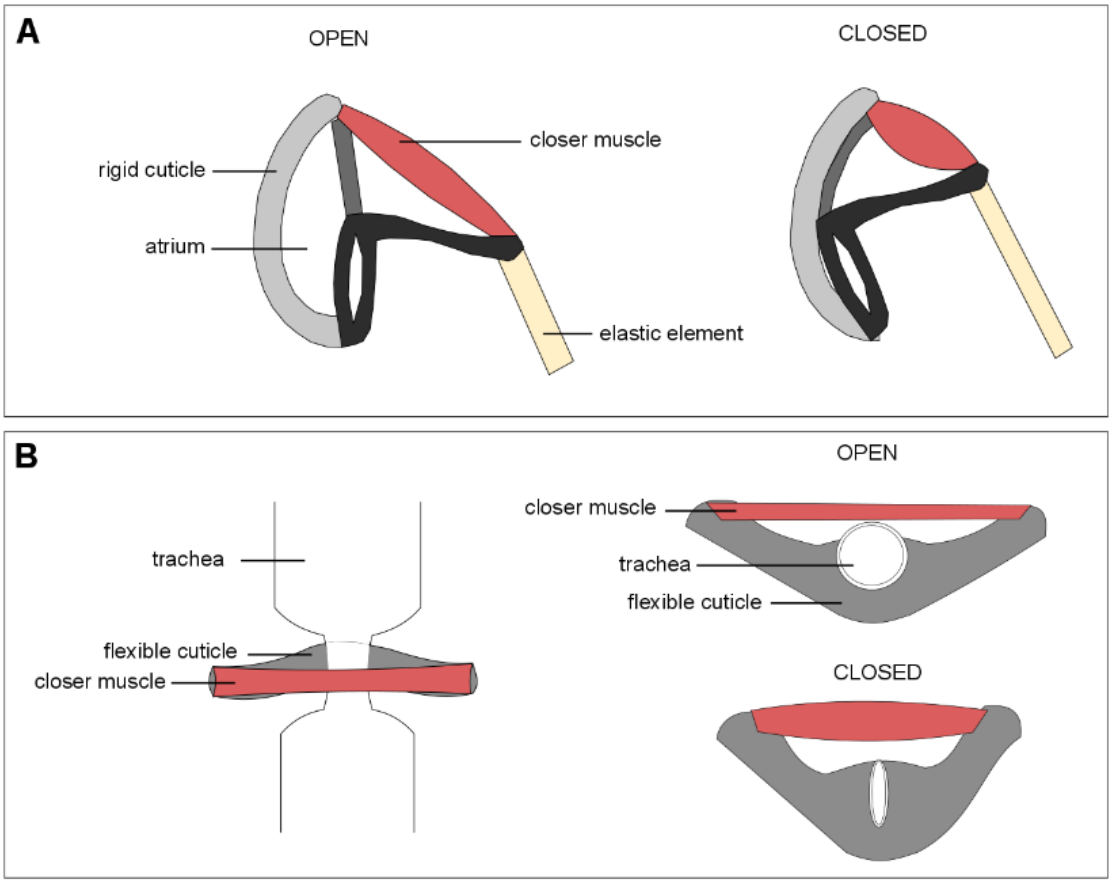

Fig. 2. The structure and function of two different spiracle closing mechanisms. (A) Contraction of the closer muscle pulls on a cuticular lever which moves to cover the atrium. Upon relaxation of the closer muscle, the lever arm is pulled back into the open position by an elastic element. (B) An internal valve mechanism. Flexible cuticle surrounds a section of a tracheal tube medially to the spiracle atrium. Contraction of the closer muscle pulls the ends of the cuticular segment together, causing a pinching of the tube and resulting closure of the spiracle. Figure modified from (Chapman et al. 2013).

VENTILATION

Due to the high rate of oxygen diffusion in air, the role of ventilation in gas exchange has been unclear. Early calculations by Krogh suggest that diffusion alone is sufficient to deliver oxygen to metabolically active tissues (Krogh 1920). This diffusion hypothesis is supported by studies of tracheal system hypertrophy which demonstrate that the mass-specific cross sectional area of the tracheal system increases when insects are reared in

decreased atmospheric oxygen concentrations (Loudon 1989; Henry & Harrison 2004). However, rates of ventilation by abdominal pumping are known to increase with metabolic rate (Miller 1981) and with decreased atmospheric oxygen availability (Harrison et al., 2006). Ventilation therefore appears to be important for oxygen delivery, yet it is unknown how the convective air currents produced by abdominal pumping manifest within the tracheal system and contribute to respiratory gas exchange. The first chapter of this dissertation aims to identify these patterns of airflow.

Ventilatory patterns are controlled by the central nervous system. In locusts, a central pattern generator (CPG) in the metathoracic ganglion produces two distinct patterns of neural output depending on the behavior and metabolic rate of the insect: continuous or discontinuous (Bustami & Hustert 2000). During discontinuous ventilation, ventilation can occur in 5-6 minute periods, followed by 15-20 minute periods of no efferent output from the CPG (Bustami & Hustert 2000). Ventilation frequency has been shown to depend on internal P_{CO2} in locusts (Krolikowski & Harrison 1996) and temperature in honeybees (Lighton & Lovegrove 1990). Ventilatory movements have also been shown to occur in synchrony with the movement of the pterothorax associated with wing beats during flight in locusts. This thoracic pumping is calculated to contribute to a large fraction of the total ventilatory airflow (Weis-Fough 1967).

In addition to abdominal movements resulting from the ventilatory CPG, tracheal and airsac compression may also contribute to airflow in the tracheal system. Phase contrast x-ray imaging of live insects reveal tracheal tube compression in the head and thorax of beetles, crickets, ants, and locusts (Westneat et al. 2003; Socha et al. 2010; Greenlee et al. 2013). Airsacs may serve many functions, both respiratory and non-

respiratory in nature. Non-respiratory functions may include improving flight performance by lowering the insect's specific gravity, reducing the mechanical dampening of wing beats during flight, maintaining external dimensions, or maintaining hydrostatic equilibrium in aquatic insects (Wigglesworth 1963). Compression of the airsacs or tracheal tubes may also function to improve airflow through the major trachea, reducing the volume of stale air in the tracheal system and increasing the diffusion gradient for oxygen from the major trachea to the tissues.

CONTROL OF THE SPIRACLES

The movement of spiracle valves is an important gas exchange regulator in both ventilating and non-ventilating insects. Spiracle valves are known to open and close in coordination with ventilatory movements, perhaps as the result of control by a similar central pattern generator (Heinrich et al., 2013). Spiracles can also open and close as a function of intratracheal oxygen and carbon dioxide partial pressures, in the absence of ventilation (Förster & Hetz 2010; Schneiderman 1960). The mechanism by which spiracles respond to these intratracheal gases remains unknown. Some data suggests that the spiracle closer muscle relaxes in the presence of dissolved CO_2 (Hoyle 1960; Forster 2010), resulting in spiracle opening when internal CO_2 partial pressures reach a given threshold.

If spiracle activity, and therefore the respiratory pattern, is controlled by a mechanism similar to that of vertebrates (a function of pH), then this poses a challenge to insects as a result of their ectothermy. Increased atmospheric temperature may not only increase the metabolic rate, but because increased temperature leads to decreased solution pH as a result of the ionization of water, it may also affect the respiratory pattern. Such a

relationship between temperature and respiratory control would have important implications for studies of metabolism in insects where ambient temperature was used as a tool to manipulate metabolic rate (Contreras & Bradley 2009). The second chapter of this dissertation aims to uncouple the effects of temperature and metabolic rate on respiratory pattern. The results from this study also shed light on possible spiracle control mechanisms.

RESPIRATORY GAS EXCHANGE PATTERNS

As a result of the precise control of the spiracle valves, insects are capable of producing distinct patterns of gas exchange. The spiracles cycle between three states: (1) the open phase, during which the valve opens wide, a majority of the built up CO_2 is released from the tracheal system, and intratracheal oxygen values reach atmospheric levels, (2) the closed phase, during which the spiracle valves close and gas exchange between the tracheal system and the environment can drop to zero, and (3) the flutter phase, during which micro-openings of the spiracles allow oxygen to diffuse into the tracheal system and only a small proportion of the accumulated CO_2 is released. The frequency at which the insect cycles through these phases increases with metabolic rate. Studies of insect respiratory gas exchange describe three possible patterns (Fig. 3): (1) continuous gas exchange, during which CO_2 release is detected at a constant rate, (2) cyclic gas exchange, during which CO_2 release cycles regularly between periods of low and high CO_2 release, and (3) discontinuous gas exchange, during which CO_2 release drops to zero in between bursts of CO_2 release, sometimes accompanied by a flutter phase detectable as

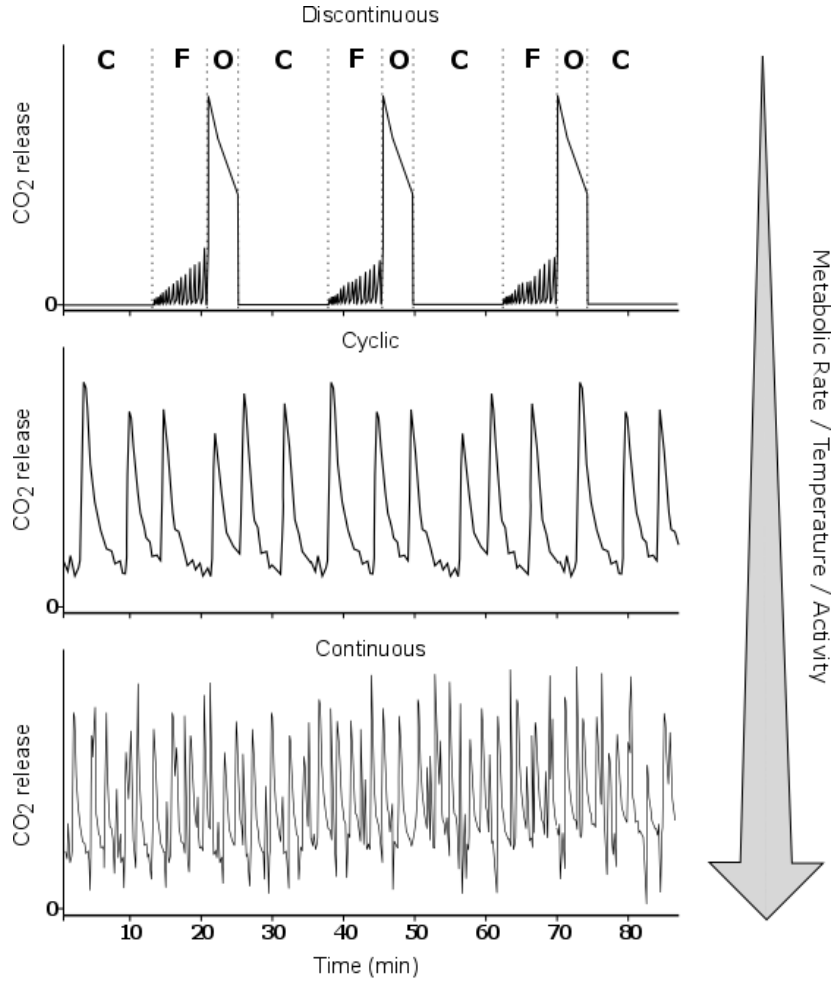

Fig. 3. A schematic of the three defined gas exchange patterns in insects**.** The closed (C), flutter (F), and open (O) phases are all observed in the discontinuous pattern. The cyclic and continuous patterns are observed during flow-through respirometry recordings as a result of increased cycle frequency caused by increased metabolic rate, temperature, or activity. Complete removal of CO_2 in the chamber between respiratory bursts is not observed when the time constant of the respiratory chamber is too low due to insufficient rate of airflow through the system. Modified from (Contreras et al. 2014).

smaller bursts of CO_2 release preceding the larger bursts. While these patterns have been classified separately in the past, it seems clear that these three patterns are a manifestation of the same spiracle control system and differ only in their cycle frequency (Gray & Bradley 2006).

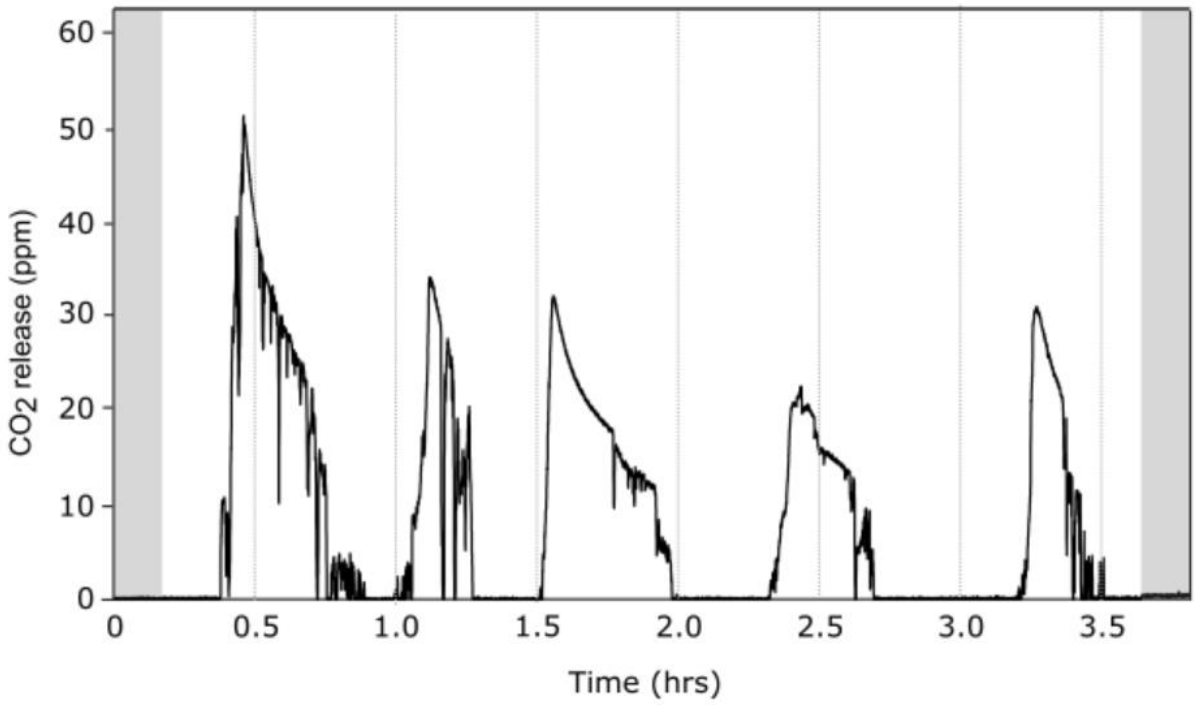

Fig. 4. Discontinuous gas exchange observed in a *Manduca sexta* pupa at 18°C**.** Baseline recordings from an empty chamber are highlighted in grey.

During low metabolic states, the spiracular closed phase becomes prolonged (Fig. 4), sometimes with no gas exchange occurring between the insect and the environment for up to hours in diapausing pupae. Many hypotheses have emerged to explain the adaptive significance of this discontinuous pattern of gas exchange (Table 1). Conflicting evidence has led to the conclusion that this pattern may serve multiple functions in insects, but that it emerged as the result of the interaction between two physiological feedback loops which regulate internal partial pressures of carbon dioxide and oxygen (Contreras et al. 2014). Nonetheless, the benefits of this behavior, such as reduced respiratory water loss in some insects, cannot be overlooked. These secondary benefits can have implications for insect survival (Lighton 1996; Schimpf et al. 2011; Matthews & White 2012).

Table 1. Summary of hypotheses describing the function of the discontinuous gas exchange pattern (DGC). Modified from (Contreras et al., 2014).

Hypothesis	Description	Proposed by
Hygric hypothesis	Spiracular closure reduces respiratory water loss	(Buck et al., 1953)
Chthonic hypothesis	Spiracular closure steepens O_2 and CO_2 gradients, improving gas exchange in subterranean habitats	(Lighton & Berrigan 1995)
Strolling arthropod hypothesis	Prolonged spiracular closure prevents tracheal mite infestation	(Miller 1974)
Oxidative damage hypothesis	Spiracular closure reduces amount of O_2 reaching tissues, therefore decreasing oxidative damage	(Bradley 2000)
Metabolic rate hypothesis	Oxygen demand determines the respiratory pattern	(Contreras & Bradley, 2010)
Respiratory adequacy hypothesis	Oxygen delivery capacity determines the respiratory pattern	(Contreras & Bradley, 2009)
Emergent properties	DGC results from the interaction between two feedback loops that respond to internal CO_2 and O_2	(Chown & Holter 2000)
Neural hypothesis	DGC occurs during periods of low brain activity	(Matthews & White 2011a)

The oxidative damage hypothesis suggests that the discontinuous gas exchange pattern reduces oxidative damage of tissues by allowing the insect to maintain low P_{O2} values in the tracheal system throughout the closed and flutter phases (Bradley 2000; Hetz & Bradley 2005). During the closed phase, tracheal oxygen partial pressures drop steadily from near atmospheric levels to about 4 kPa (varies in different insects), which then triggers the flutter phase to initiate. The micro-openings of the flutter phase allow enough oxygen to diffuse into the tracheal system that the intratracheal P_{O2} remains around 4 kPa until the spiracle open phase is initiated by a high P_{CO2} threshold (Förster & Hetz 2010).

The maintenance of this low P_{O2} near the tissues may prevent excess exposure to damaging oxygen and radical production during periods of low metabolic demand.

Few studies have tested the oxidative damage hypothesis. Matthews and White found that locusts are unable to maintain low intratracheal P_{O2} values when exposed to atmospheric hyperoxia (Matthews et al. 2012). Another study used live cell fluorescence microscopy to observe reactive oxygen species (ROS) production and found that ROS production did not increase when the tracheal system was shocked with hyperoxic air (40% O_2) after a period of anoxia which forced spiracular opening (Boardman et al. 2012). While these results do not support the oxidative damage hypothesis, they have not directly measured tissue damage as a result of prolonged disruption of the discontinuous pattern. The third chapter of this dissertation aims to address this gap in the data.

CHAPTER 1:

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

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 1964; Wigglesworth 1983). However, insects have additional processes for regulating gas exchange, including the use of complex respiratory patterns, active ventilation (Bailey 1954; Levy & Schneiderman 1966; Slama 1988; Duncan & Byrne 2002; Chown & 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_{CO2} and P_{O2}) and metabolic rate (Lighton 1996; Chown & Holter 2000; Hetz & Bradley 2005; Contreras & Bradley 2009; Förster & 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; Slama 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 & Schneiderman 1974; Förster & Hetz 2010). Spiracular valves are controlled by interacting feedback loops that trigger spiracles to open in response to high internal P_{CO2} or low P_{O2}, as well as close in response to

high P_{O2} (Levy & Schneiderman 1966; Förster & 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 & Williams 1953; Punt et al. 1957; Burkett & Schneiderman 1974; Chown et al. 2006; Förster & 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-Fough 1967; Duncan & Byrne 2002; Duncan et al. 2010; Groenewald et al. 2012), as well as lepidopteran and dipteran pupae (Slama 1976; Slama 1984; Slama & Rembold 1987; Wasserthal 2001; Lehmann & Schützner 2010). Despite oxygen's high rate of diffusion in air, insects utilize multiple methods for ventilation. These methods include abdominal pumping (Weis-Fough 1967), thoracic pumping associated with wingbeats, as described in locusts and dragonflies (Weis-Fough 1956; Weis-Fough 1967), and the compression and expansion of airsacs and tracheae by either body wall movements or pressure transmission from the hemolymph (Wigglesworth 1963; Slama 1984; Wasserthal 2001; Socha et al. 2008; Lehmann & 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-Fough 1967). Conversely, airflow from posterior to anterior has been demonstrated in the honey bee (Bailey 1954) and the dung beetle (Duncan & 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 & 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. 1.1) 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.

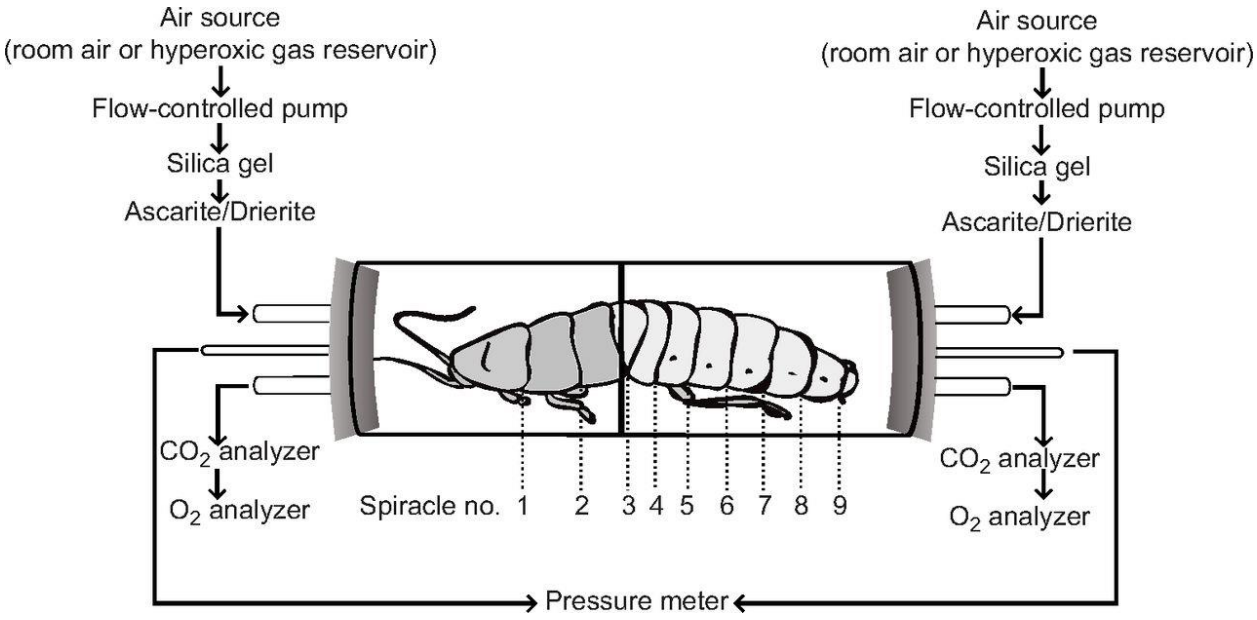

Fig. 1.1. To test for directional airflow, the thorax and abdomen of *Gromphadorhina portentosa* were separated into two chambers with a latex diaphragm. The first two pairs of legs were restrained within the thoracic chamber and the third pair of legs was restrained along the abdomen. The diaphragm 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.

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 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. 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. 1.1 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 & 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 & 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. 1.1). 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_2release 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 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. 1.2). These episodes of rhythmic bursts of CO_2 release (labeled 'O' in Fig. 1.2) are interrupted by 5–10 min spiracular closed phases (labeled 'C' in Fig. 1.2), 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. 1.3).

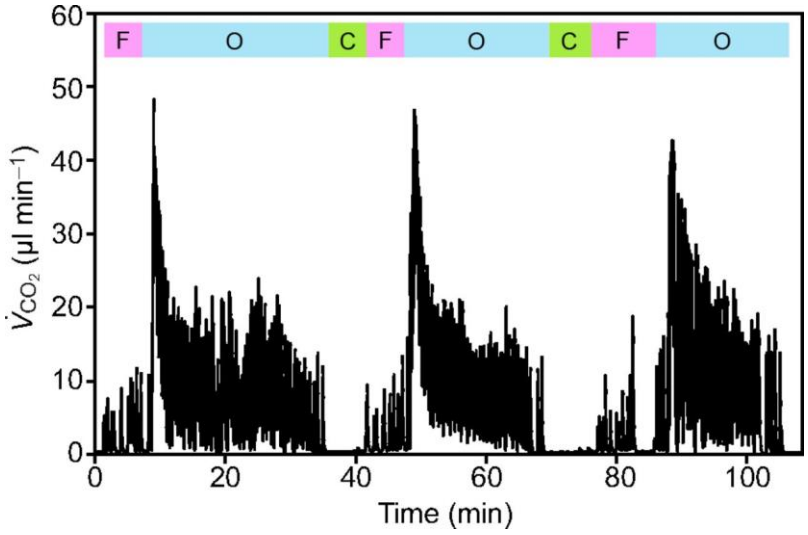

Fig. 1.2. An example of discontinuous CO_2 release from an unrestrained adult male *G. portentosa* at 20°C in normoxia, measured at 600 ml min^{-1}. Periods of ventilation are separated by 5–10 min of spiracular closure. The discontinuous pattern consists of a flutter phase (F), in which micro-openings 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.

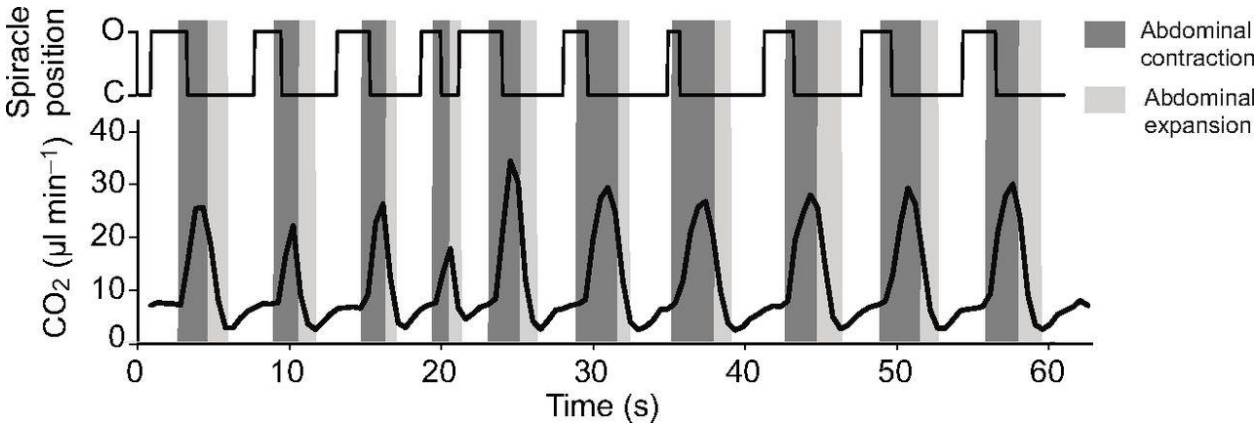

Fig. 1.3. The position of spiracle 7 (right side) over time, recorded as open (O) or closed (C), is associated with ventilatory movements and CO_2 release. Data were recorded from a restrained female *G. portentosa* at 15°C. This pattern was typical in individuals utilizing ventilatory movements. Spiracles 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.

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 described four possible spiracle structures (Snodgrass 1935). 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 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. 1.4). 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 & 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 (Fig. 1.5). 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 1.1). 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 1.1). 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.

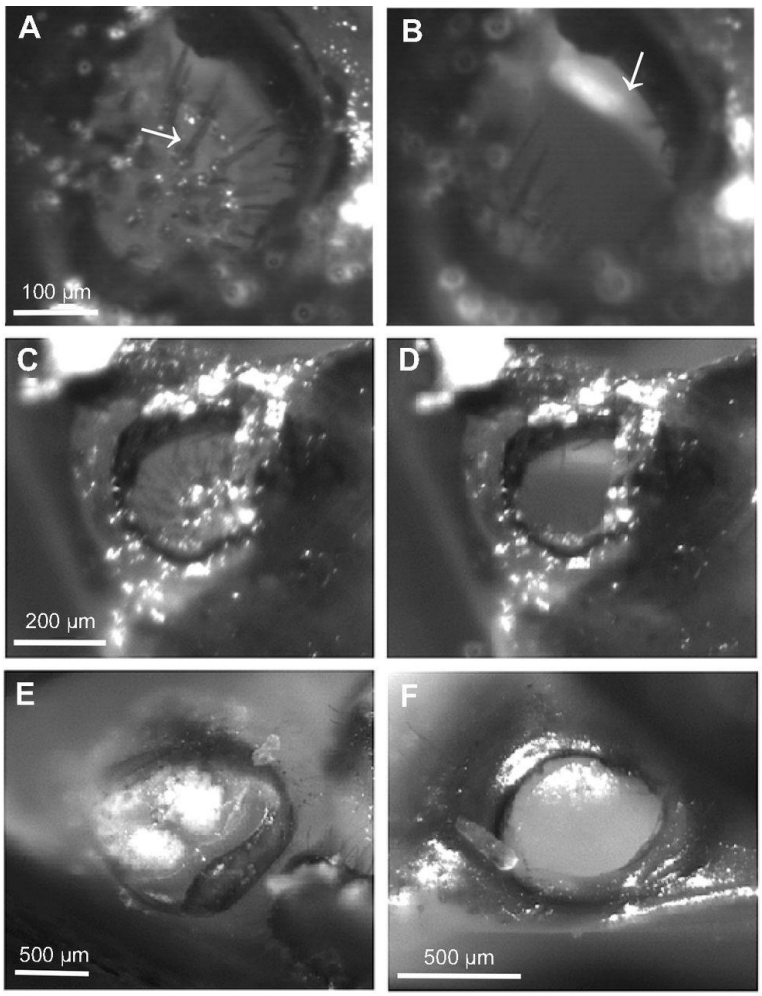

Fig. 1.4. Single frames from video recordings showing closed abdominal spiracles (A,C), open abdominal spiracles (B,D), an open thoracic spiracle (E) and the closed first abdominal spiracle (hissing spiracle) (F). Bristles are visible on the valve structure of abdominal spiracles (labeled with an arrow 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.

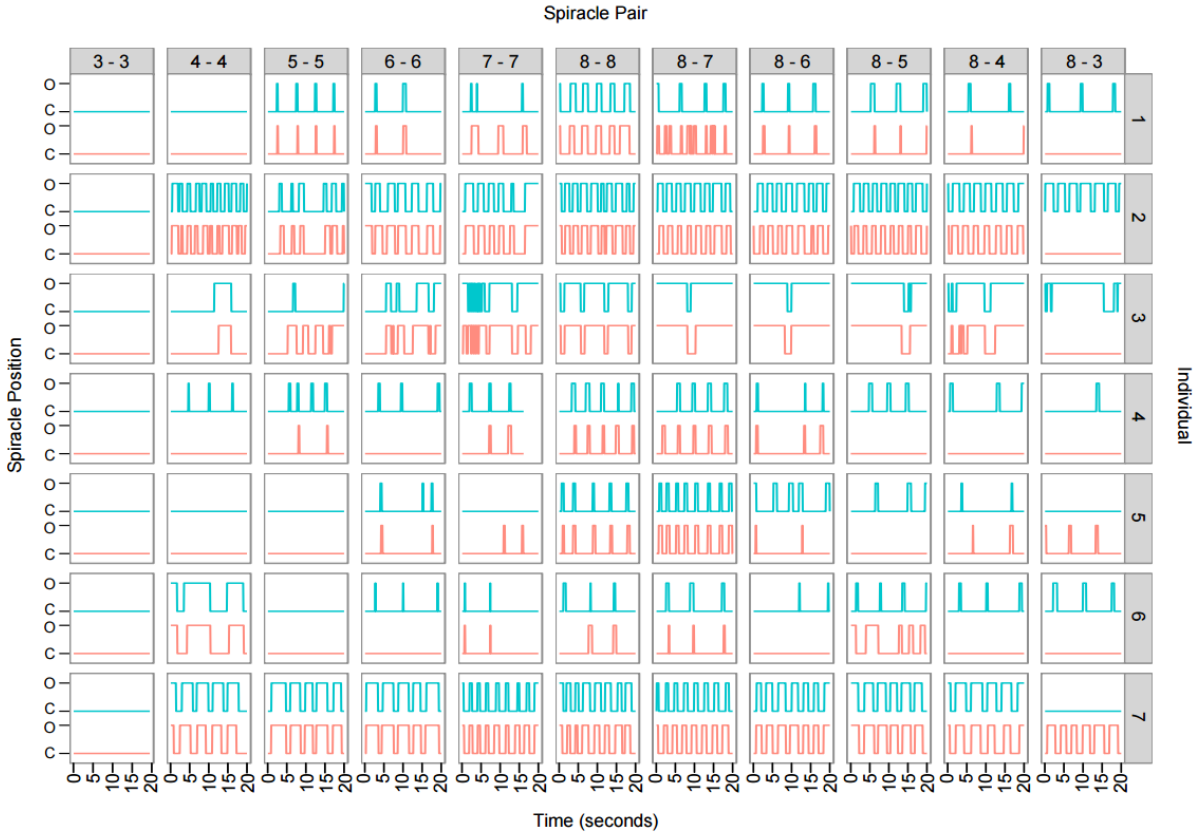

Figure 1.5. Data utilized in spiracular synchrony analyses. Random 20 second video samples from 7 individual cockroaches showing abdominal spiracular activity of two spiracles on right (blue) and left (red) sides of the body. Each column includes data for one spiracle pair across 7 individuals. Spiracle pair numbers (labeled in gray boxes above each column) refer to spiracles located at the positions described in Fig. 1.1 of the manuscript. Each row includes data from 1 individual. Open and closed spiracle positions are referred to on the y-axis of each row as "O" and "C" respectively, such that an upward deflection of a line graph indicates a spiracle opening event. All videos were recorded at different times. As described in our results, some observations of discordant spiracle states resulted from short lag periods between opening events across spiracles in a pair, or from incomplete opening events (i.e. incomplete detachment from membranous structure in spiracle atrium despite small inward deflections of the valve). In most observations, spiracles across the entire abdomen appear to open and close together, with the exception of spiracle 3 which only opens during sound production.

Table 1.1. 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.

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. 1.6). Oxygen bursts recorded from the abdominal region coincided with spiracular openings and with bursts of

CO_2. However, we did not find evidence of airflow from the abdominal to the thoracic region (Fig. 1.7). 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 1.6, 1.7). 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 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. 1.8).

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

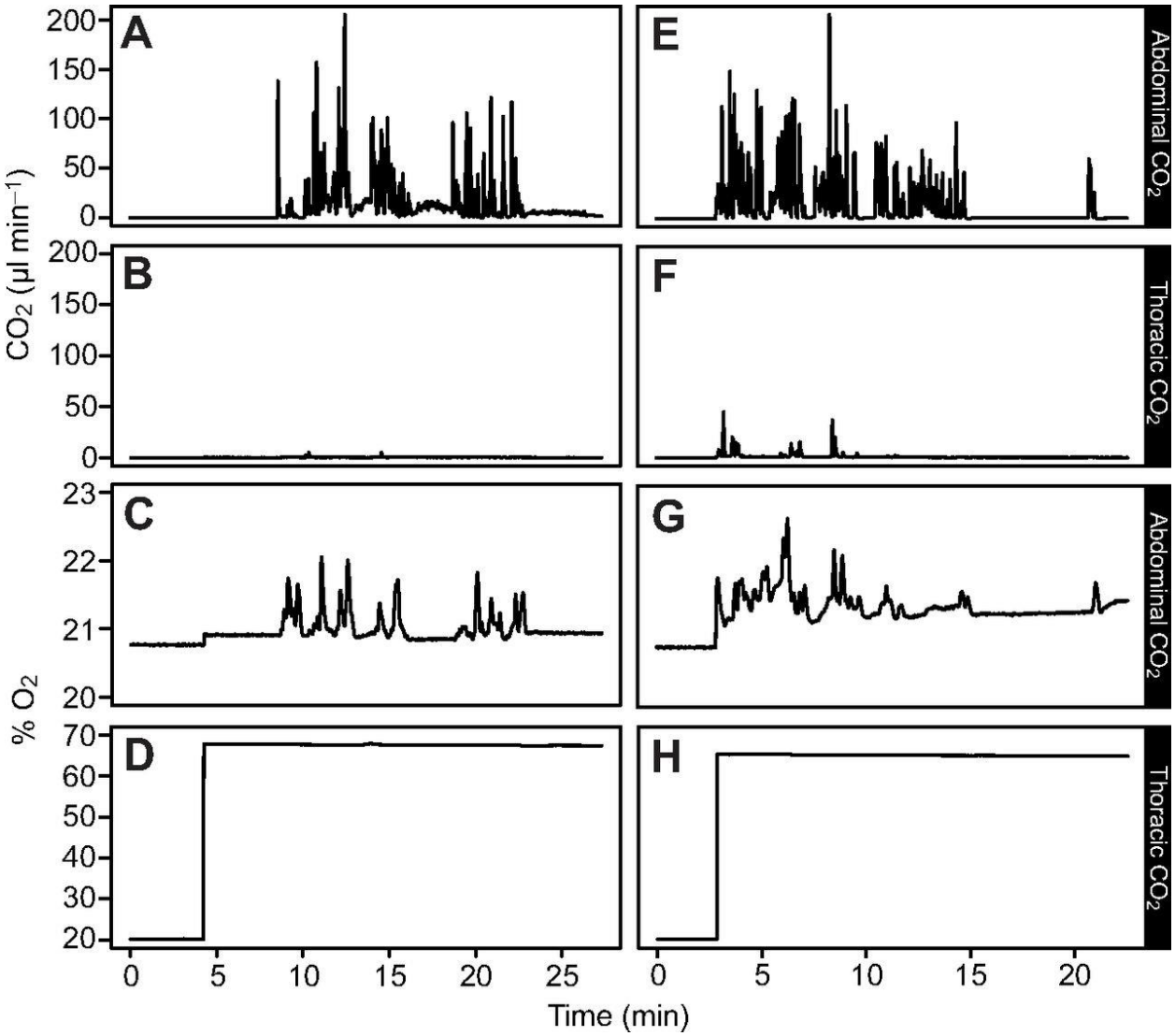

Fig. 1.6. Results from respirometry trials in which a hyperoxic tracer gas was used to detect airflow direction in two adult cockroaches (individual 1: A–D; individual 2: E–H). When 70% oxygen was profused through the thoracic chamber (D,H), bursts of oxygen were observed in the abdominal chamber (C,G) in association with CO_2 release from the abdomen (A,E). Minimal CO_2release was observed from the thoracic region in all recordings (B,F).

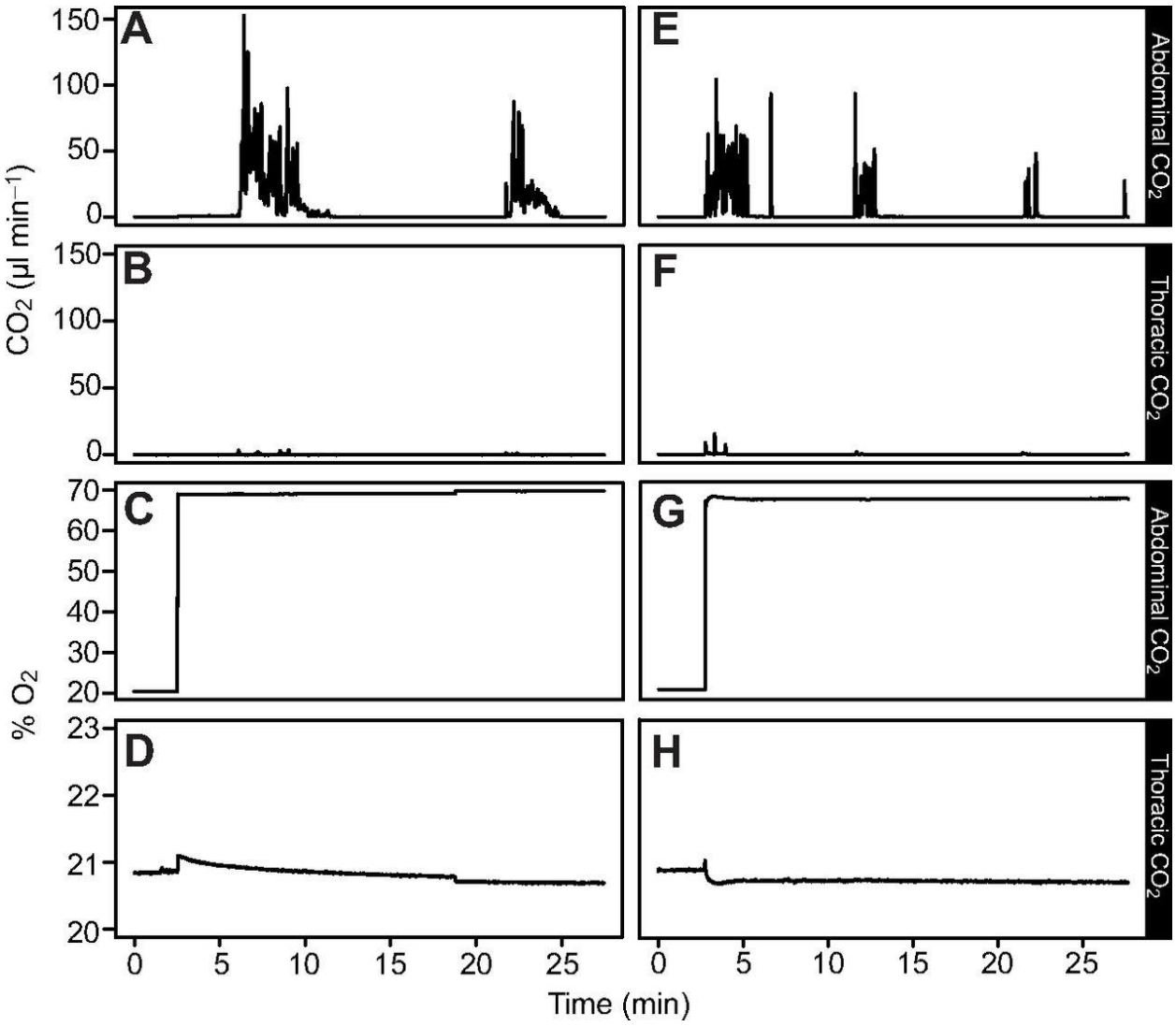

Fig. 1.7. Results from respirometry trials in which a hyperoxic tracer gas was used to detect airflow direction in two adult cockroaches (individual 1: A–D; individual 2: E–H). When 70% oxygen was profused through the abdominal chamber (C,G), bursts of oxygen were not detected in the thoracic chamber (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).

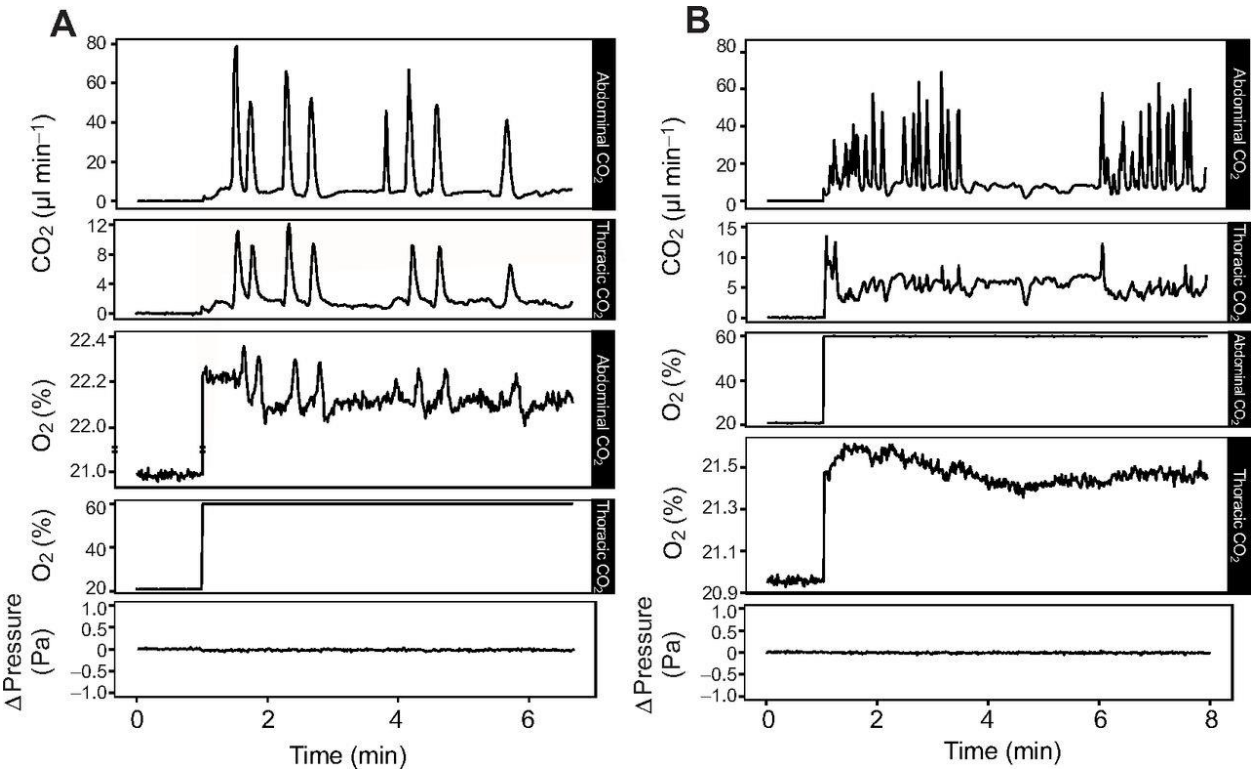

Fig. 1.8. Recordings of CO_2 release and oxygen content in the chambers containing the thorax and abdomen of *G. portentosa*. (A) When hyperoxic air was introduced to the thoracic chamber, bursts of oxygen were detected in the abdominal chamber. These oxygen bursts are associated with CO_2 release from 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).

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 contraction and closed during abdominal expansion (Fig. 1.9). Between abdominal contractions, the spiracles remained closed in 58% of observations (spiracles 4–8), and open in 42% of observations (spiracles 4–8).

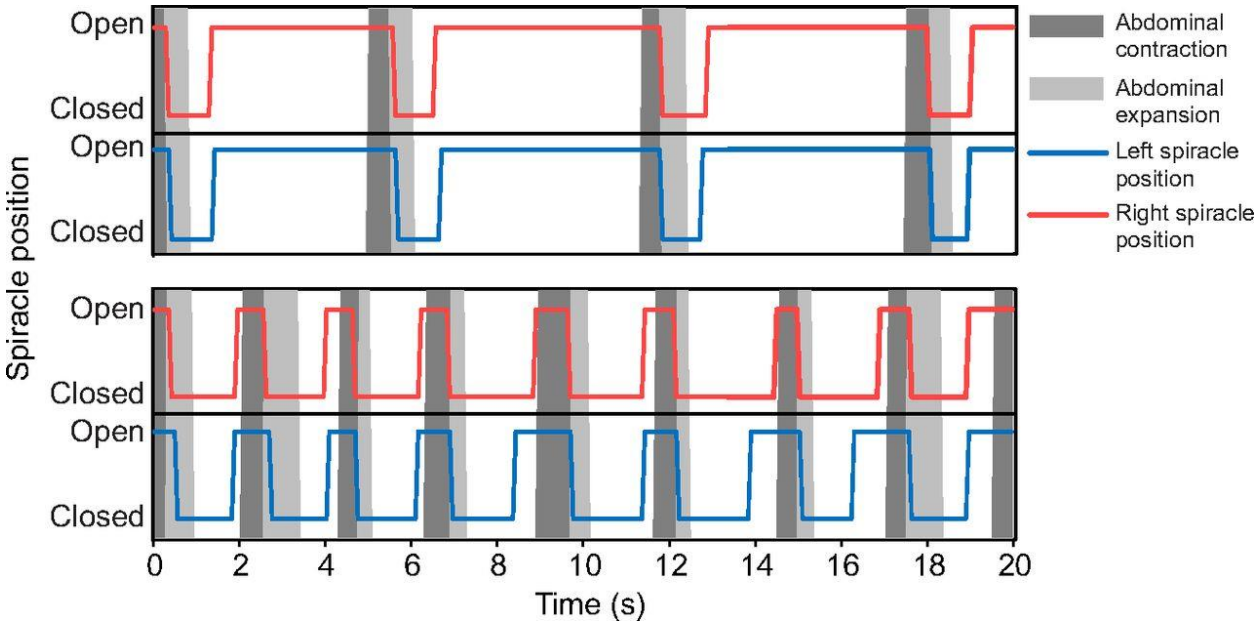

Fig. 1.9. Spiracle activity is associated with abdominal ventilatory movements. Spiracle positions from two different cockroaches are displayed as line graphs fluctuating between the open and closed state, and abdominal ventilatory movements as bars separated into periods of abdominal contraction and 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.

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 1.3, 1.9). 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 1.5, 1.6, 1.7, 1.8 and 1.9,

1.10, Table 1.1). 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 & 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 1.1). 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 & 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 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 (Slama 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. 1.10). 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

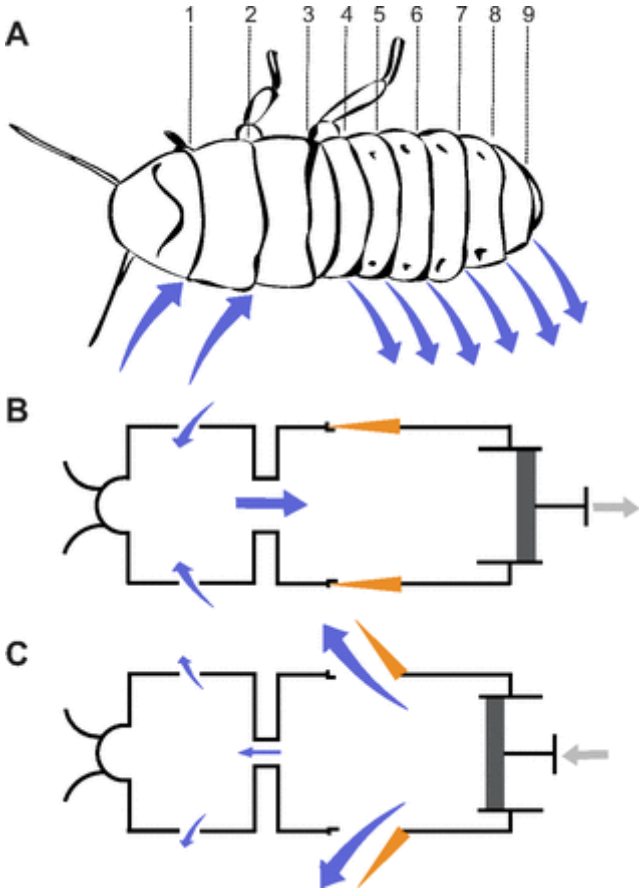

Figure 1.10. A proposed model of ventilatory airflow in G. portentosa based on video analyses of spiracle activity, flow-through respirometry observations, and tracer gas movement. (A) Airflow directions through all nine spiracles during active ventilation. Spiracle 3 opens only during hissing and otherwise 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.

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 1.6, 1.7 and 1.8). Therefore, the mechanism by which *G. portentosa* prevents thoracic CO_2release 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 & 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 & Bradley 2006). Because our current understanding of

spiracular control explains tightly regulated spiracular activity associated with intratracheal P_{CO2} and P_{O2}, 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 & Schneiderman 1966; Burkett & Schneiderman 1974; Contreras & Bradley 2009; Förster & Hetz 2010). The active ventilatory pattern exhibited by many insects and illustrated in *G. portentosa* in Figs 1.3 and 1.9 is apparently controlled by a central pattern generator that may itself be sensitive to P_{O2} inside the insect.

Our data suggest that coordinated patterns of spiracular activity and ventilatory movements facilitate unidirectional airflow (Bailey 1954; Duncan & Byrne 2002; Woodman et al. 2008) (see Figs 1.6, 1.7). 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.

CHAPTER 2:

Temperature-dependent variation in gas exchange patterns and spiracular control in *Rhodnius prolixus*

INTRODUCTION

The insect respiratory system provides tightly controlled and highly efficient mechanisms for oxygen uptake and carbon dioxide release. It is composed of a network of air-filled tracheal tubes that open to the atmosphere along the lateral sides of the body and branch throughout the insect to deliver oxygen directly to metabolically active tissues. Spiracular valves, located at each tracheal opening, control the exchange of gases between the atmosphere and the tracheal system. Although much is known about the responses of the spiracles to different stimuli, it remains unclear how and where respiratory gases are sensed. Understanding these aspects of spiracular control is a challenging problem because gas exchange in insects is uncoupled from hemolymph circulation. Insects face additional challenges in that they must control their respiratory behavior to account for changes in metabolic rate due to activity as well as temperature.

Insects utilize different patterns of respiratory gas exchange depending on their metabolic demands (Contreras and Bradley, 2009). These demands may vary as a result of fluctuations in ambient temperature, activity, or other costly processes such as digestion and reproduction. The most extensively studied gas exchange pattern is the discontinuous gas exchange cycle, which has been described in at least seven different insect orders (Lighton 1996; Marais et al. 2005; Gray & Bradley 2006; Chown et al. 2006; Contreras & Bradley 2009). This behavior consists of three phases: an open phase in which the spiracles open to allow gas exchange to occur, a closed phase in which the spiracular valves remain

tightly sealed for a prolonged period of time, and a flutter phase in which micro-openings of the spiracles allow oxygen to enter the body while a low oxygen partial pressure is maintained within the insect.

In the present study we used *Rhodnius prolixus* (Stål 1859) to test two current models of insect respiratory control, one concerning the effects of metabolic rate and one addressing spiracular control. The first is the model proposed by Contreras and Bradley (Contreras & Bradley 2009), which suggests that insect gas exchange patterns are determined largely by metabolic rate. They propose that the pattern changes from discontinuous to continuous as metabolic rate is increased. The second model we have tested is Förster and Hetz's dual feedback model of spiracular control. This model indicates that spiracle activity is regulated by internal partial pressures of carbon dioxide and oxygen (Burkett & Schneiderman 1974; Förster & Hetz 2010). This model proposes that at the end of a spiracular closed phase, critically low oxygen content within the insect triggers the spiracles to begin fluttering, allowing oxygen to enter the tracheal system. As very little CO_2 is released by the insect during the flutter period, eventually the increasing internal CO_2 content triggers a second feedback mechanism to initiate spiracular opening when a critically high P_{CO2} is reached. Chown and Holter (Chown & Holter 2000) referred to this mechanism via their emergent properties hypothesis and argue that discontinuous gas exchange emerged as a function of this physiological feedback rather than serving any adaptive function (Chown et al. 2006). This control system consists of two interacting feedback loops that monitor internal P_{O2} and P_{CO2} and trigger spiracular opening events when critical thresholds are reached.

The precise mechanism by which the partial pressures of these gases are detected is unknown. It is also unclear what triggers the start of the closed phase. It is conceivable that spiracular closure may be initiated when sufficient CO_2 has been released to reach a critically low value, or when diffusion of oxygen to the tracheal system produces a critically high oxygen value. Conversely, spiracular openings may be of a constant duration resulting from the timing of neural inputs or spiracle-opening muscle contractile properties.

Based on the Förster and Hetz dual feedback loop model of the discontinuous gas exchange pattern, an increase in metabolic rate should lead to an increase in the frequency of open phases, as at higher metabolic rates CO_2 accumulates in the insect faster during the closed and flutter phases. Conversely, the volume of CO_2 released per respiratory burst should remain unchanged as it reflects the amount of accumulated CO_2 required to reach the threshold partial pressure that triggers spiracular opening. In contrast to this expectation, it has been demonstrated that increasing the metabolic rate by increasing temperature leads to a decreased volume of CO_2 released by both a silkworm and an ant during an open phase (Schneiderman & Williams 1955; Lighton 1988). A decreased volume of CO_2 release during a burst might indicate a reduction in critical P_{CO2} required to open the spiracles. Consequently, these results might indicate that either the CO_2 sensor is itself sensitive to temperature or there is an effect of temperature on the P_{CO2} detected by the sensor.

Rhodnius prolixus provides certain valuable features as a model insect for studying respiratory control models. *Rhodnius prolixus* is a blood-feeding insect. Before each molt, the insect ingests and processes a blood meal, which leads to an increase in metabolic rate up to 10 times that of the resting individual over 15 days (Bradley et al. 2003; Zwicky &

Wigglesworth 1956). However, in the absence of an appropriate host in the vicinity, the insects remain quite still. Therefore, after a period of acclimation, the insects remain very still in a respirometer. Additionally, although *Rhodnius* breathe discontinuously, they show no external signs of active ventilation. Finally, the metabolic rate of *Rhodnius* can be modified in two distinct ways, namely through changes in temperature or by feeding. This allows us to test models of respiratory control using two means of adjusting metabolic rate, and thus respiratory demand.

Here, we tested the Contreras and Bradley model (Contreras & Bradley 2009) by exposing *R. prolixus* to two different metabolic stimuli: feeding and temperature manipulation. We then characterized and measured different aspects of the gas exchange pattern to determine whether metabolic rate alone predicts the gas exchange pattern utilized by the insect or whether there are additional factors that contribute to respiratory behavior. The Förster and Hetz spiracular control model was tested by comparing measurements of burst frequency and volume at different temperatures and metabolic rates. This model, as described above, indicates that the volume of CO_2 released in a respiratory burst should remain constant regardless of metabolic rate as an internal P_{CO2} threshold controls spiracular opening. Elevated metabolic rates should therefore be compensated for exclusively by increased burst frequency.

To further examine the spiracular control model, we recorded CO_2 release patterns from insects exposed to hyperoxia to determine whether elevated atmospheric oxygen affected respiratory control. While the trigger for spiracular opening events appears to be tightly linked to intratracheal P_{CO2}, the mechanism responsible for triggering the end of the open phase remains unknown. We hypothesized that if a critically high oxygen threshold

triggers the end of the spiracular open phase, then insects exposed to atmospheric hyperoxia would produce decreased CO_2 burst volumes compared with insects in normoxic environments. An increased partial pressure gradient from the atmosphere to an internal oxygen sensor would decrease the time required to reach the oxygen threshold and trigger spiracular closure. Combined, these experiments test two models of spiracular control, and may provide insight into the molecular mechanism by which insects detect metabolic gases.

MATERIALS AND METHODS

Animals

Fifth instar *R. prolixus* were obtained from a colony at the University of California, Irvine. The colony was maintained at 27°C and 80% relative humidity (RH) with a 12 h:12 h day:night cycle. Individuals were separated into two treatment groups of six individuals each. The mean (±s.d.) mass of individuals was 41.53±13.07 mg in the first group and 43.08±6.69 mg in the second group. The first group was fed a blood meal to increase the metabolic rate, and the second group remained unfed and experienced a range of temperatures to induce changes in metabolic rate. Insects in both treatment groups were fasted for 3 weeks prior to the start of the experiment. Resting metabolic rates for insects in the fed treatment group were recorded 1 day prior to feeding. Insects in the fed treatment group then took a blood meal from a rabbit immediately prior to the first recordings.

After the completion of the above experiments, two additional groups of individuals were collected. One group of six individuals was used in a second trial of temperature

experiments and another group of seven individuals was used for oxygen manipulation tests (mass 43.15±5.97 mg).

Procedure for temperature and feeding trials

CO_2 release patterns of individuals in the temperature treatment group were recorded at temperatures ranging from 18 to 38°C. To minimize the effects of acclimation or heat stress, the order of temperature exposure was determined by creating a semi-random sequence in which most temperature transitions did not exceed a change of more than 6°C and the highest temperature was experienced either first or last. Temperatures were encountered in the following order for the first temperature treatment group, and in the reverse order for the second temperature treatment group: 38, 24, 30, 34, 36, 32, 28, 24, 20, 18, 22, 26°C. The insects experienced each temperature for a total of 48 h, which included a 24 h equilibration period followed by another 24 h period in which two 30 min recordings were taken from each individual. Experiments were conducted in a temperature-controlled room that maintained a programmed temperature within ±0.5°C. The insects in the fed treatment group were maintained at 26±0.5°C for the duration of the study. Two 30 min recordings were taken daily from each individual for 17 days after feeding.

In both the feeding and temperature treatment groups, insects remained in their chambers throughout the duration of the study to reduce handling stress. Each insect was placed into a 2 ml cylindrical plastic chamber and provided with a 1 cm^2 piece of filter paper. Insects were perfused with a slow flow of hydrated room air (80% RH, ~25 ml min^{-1}) during periods when measurements were not being taken to prevent desiccation

and CO_2 build-up. Room air was humidified by bubbling through a saturated ammonium sulfate solution.

During flow-through respirometry, a mass flow controller pushed air at a constant flow rate of 200 ml min^{-1} (Sierra Instruments Inc., Monterey, CA, USA) through columns of Drierite (W. A. Hammond Drierite Co. Ltd, Xenia, OH, USA) and Ascarite (Thomas Scientific, Swedesboro, NJ, USA) to remove water and CO_2. Air then passed through an 8-channel multiplexer (Sable Systems International, Las Vegas, NV, USA) which was programmed to direct the airstream through six channels leading to individual insects for 30 min intervals. Between each recording from an insect, the multiplexer directed air through a seventh channel containing an empty chamber to obtain a baseline recording. A 90 s pause in data recording was issued at the beginning of each channel switch while dry air began flowing through the new channel. After the chamber, air passed through a CO_2 gas analyzer (model 6262, Li-Cor Inc., Lincoln, NE, USA).

CO_2 readings were converted from analog to digital signals with a Sable Systems Universal Interface II and recorded with Expedata data acquisition software (Sable Systems). Data were zeroed in Expedata to account for any baseline drift and values for average metabolic rate, burst volume, burst duration and cycle period were recorded. These values were obtained from baseline-corrected recordings in Expedata and tabulated in Excel, where CO_2release rates were converted from ppm to µl h^{-1} (Microsoft, Redmond, WA, USA).

As indicated in the Introduction, *Rhodnius* remain quiescent between molts unless a blood host is available. Our data collection occurred after 24 h of undisturbed acclimation at each temperature. Visual observation of the insects in the chambers during the

discontinuous gas exchange cycle gave no indication of ventilatory movements of the abdomen or locomotor activity. To further verify these observations, we conducted simultaneous measurements of CO_2 release and activity, and video recordings under low (25°C) and high (35°C) temperature conditions to determine whether activity affected the gas exchange pattern. The insect was placed in a clear, cylindrical glass chamber housed inside a temperature-controlled cabinet (Pelt-5 Temperature Controller, Sable Systems). Respirometry plumbing was arranged as previously described. Supplementary material Fig. S1 shows data from an infrared AD2 activity detector (Sable Systems) recording movement of the insect. The results from these trials indicate that the insects breathe discontinuously while quiescent and that no visual evidence of ventilatory movements can be seen. Locomotory movements, such as those that occurred immediately after a change in temperature, clearly disturb the respiratory rhythms (supplementary material Fig. S1). The absence of such disturbed periods in our experimental recordings is evidence that activity was not a confounding variable during our measurement of gas exchange patterns.

Oxygen manipulation trials

Rhodnius prolixus were tested for changes in gas exchange patterns in 21% and 40% oxygen environments before and after feeding to determine whether a critically high oxygen threshold played a role in triggering spiracular closure. The temperature was maintained at 26°C for all oxygen tests. CO_2 release patterns were recorded from seven, fifth instar individuals using the same flow-through respirometry setup previously described excluding the multiplexer, as individuals were tested one at a time. Recordings

were first taken in 21% oxygen for 1 h, followed by 1 h of recordings at 40% oxygen. A reservoir of hyperoxic air was produced by mixing dry room air and 100% oxygen in a large Mylar balloon. During the 40% oxygen trials, air was pulled from this reservoir by the mass flow meter and re-dried and scrubbed of CO_2prior to entering the experimental chamber. Oxygen concentrations were verified by flowing air from the balloon through an Oxzilla Differential Oxygen Analyzer (Sable Systems). Oxygen percentages for all hyperoxic trials were maintained at 40±1%.

Data collection and analysis

One goal of the present study was to analyze the volume of CO_2 released per burst, or spiracular open phase, as a function of metabolic rate. The burst volume in unfed *R. prolixus* is fairly straightforward to measure from a flow-through respirometry recording. It represents the amount of CO_2 released during an open phase relative to the zero baseline (see Fig. 6). We used Expedata to measure these volumes (volume/s × s=volume). However, the burst volume in fed *R. prolixus* can be considered in two different ways. As in the unfed insects, one can consider the volume of a burst to be the amount of CO_2 released during the open phase over and above a zero rate of release. Alternatively, as fed individuals display an elevated rate of release in the interburst periods as a result of greater spiracular activity during the flutter phase, the volume of a burst can also be interpreted as the CO_2 release volume over and above the interburst release rate. CO_2 accumulates during the interburst periods until it reaches the critical partial pressure required to initiate a burst. Following the burst, the closed phase is very short in fed individuals as a result of the elevated metabolic rate, and therefore the rate of CO_2 release

rapidly returns to the elevated flutter rate. The burst volume consequently can be viewed as the volume of CO_2 released at intervals, over and above this steady-state release rate (Fig. 4). We quantified burst volume in fed *R. prolixus* using both approaches, i.e. by measuring the volume released relative to zero rates of release, and relative to the rates observed during the interburst flutter phase.

To determine the average burst volume for an individual, a maximum of six individual bursts were measured per 30 min recording at each temperature or each day post-feeding. At the lowest metabolic rates, at least two bursts per recording were available for measurement. The beginning of a peak was defined as the point at which the CO_2 recording became greater than the average CO_2 value in the interburst period. The end of a burst was determined as the point at which the CO_2 recording returned to the average interburst period level. The rate of CO_2 release during a respiratory burst was calculated as the burst volume divided by the burst duration. Cycle periods were defined as being from the start of one open phase to the start of the next open phase. An average cycle period for each 30 min recording was found by measuring the time between the start of the first peak and the start of the last peak in the recording, and dividing this time by the number of peaks that occurred during that time period.

Statistical tests were performed in RStudio (rstudio.com). Averages were taken of all measurements for one individual at a given temperature, or day after feeding, for use in analyses. Data from temperature trials were combined to produce a sample size of 12 individuals per temperature. Data from feeding trials were evaluated via regression analyses with metabolic rate as a continuous variable. Temperature trial data were

analyzed via one-way ANOVA. Oxygen manipulation trials were analyzed using two-way ANOVA with feeding status and oxygen treatment as grouping variables.

RESULTS

Metabolic variation in feeding and temperature manipulation trials

Fed individuals displayed a progressive increase in metabolic rate after feeding until about day 10, when metabolic rate began to decrease (Fig. 2.1A). The mean (±s.d.) peak metabolic rate for fed individuals (37.938±5.098 µl h^{-1}) was 13.9 times higher than the mean resting metabolic rate (2.732±0.597 µl CO_2 h^{-1}) for these insects at 26°C, the normal rearing temperature. When unfed individuals were exposed to a range of temperatures from 18 to 38°C, mean metabolic rate increased with temperature (ANOVA, $F_{10,108}$=24.05, P<0.001;Fig. 2.1B) and peak metabolic rates at 38°C were on average 3.5 times higher than the resting rate. There was no significant difference between the mean metabolic rate at 26°C across the two temperature trials ($t_{9.841}$=−0.5574,P=0.5897). Near the rearing temperature for these individuals, metabolic rate remained fairly constant, but decreased at temperatures below 26°C and increased at temperatures greater than 32°C. Metabolic rates of individuals in the temperature treatment and feeding treatment overlapped only during the first measurements made immediately after feeding, after which, metabolic rates of fed individuals surpassed the highest metabolic rates observed in temperature trials.

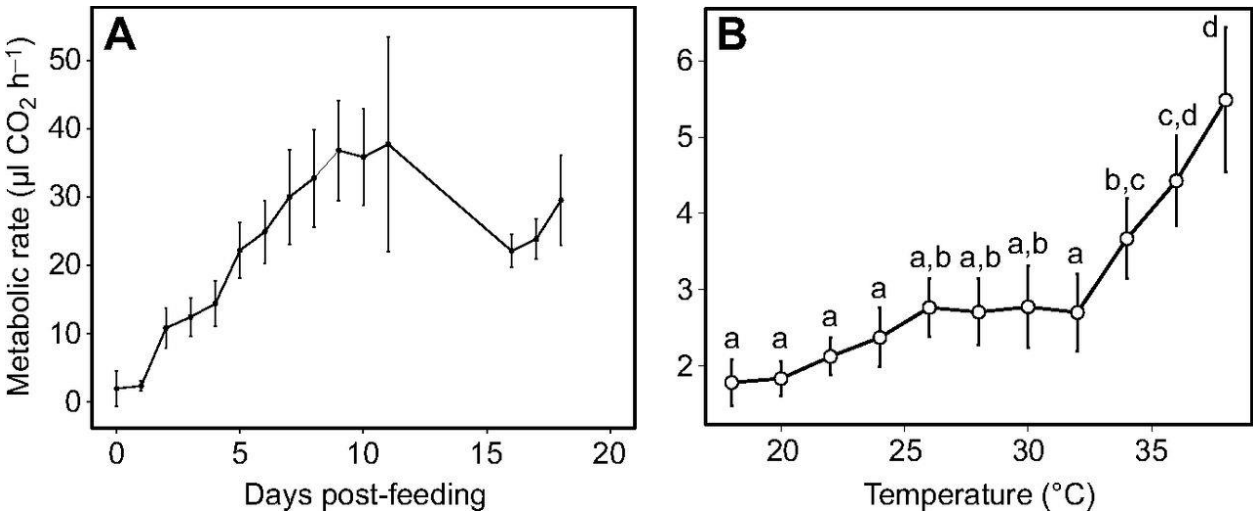

Fig. 2.1 Metabolic variation in temperature and feeding trials. (A) The rate of CO_2release over time (days post-feeding) in seven, fifth instar *Rhodnius prolixus*. (B) The rate of CO_2 release as a function of ambient temperature in 14 unfed, fifth instar *R. prolixus*. Error bars represent 95% confidence intervals. Different letters indicate a significant difference between values.

Unfed *Rhodnius* exhibit discontinuous gas exchange with discrete bursts of CO_2 release and intervening periods of very low CO_2 release, often indistinguishable from zero. Traditionally, the discontinuous gas exchange cycle has been characterized by the presence of three periods, the closed period, followed by the flutter period, followed by the open period. During flutter, the spiracles open briefly to allow oxygen into the tracheal system and to regulate the tracheal P_{O2}. During this phase, the volume of CO_2 released is much smaller than the volume of oxygen taken up because of the larger partial pressure gradient for oxygen. As a result, only a very limited release of CO_2occurs during spiracular flutter. CO_2 release during flutter is further limited in unfed *Rhodnius*, as a result of the notably low oxygen demand (Bradley et al. 2003). The difficulty in distinguishing the closed from the flutter phase on the basis of the amount of CO_2 released has caused many investigators to treat these two phases as a single interburst phase (Chappell & Rogowitz 2000; Shelton & Appel 2001; Duncan et al. 2010). However, in our experiments,

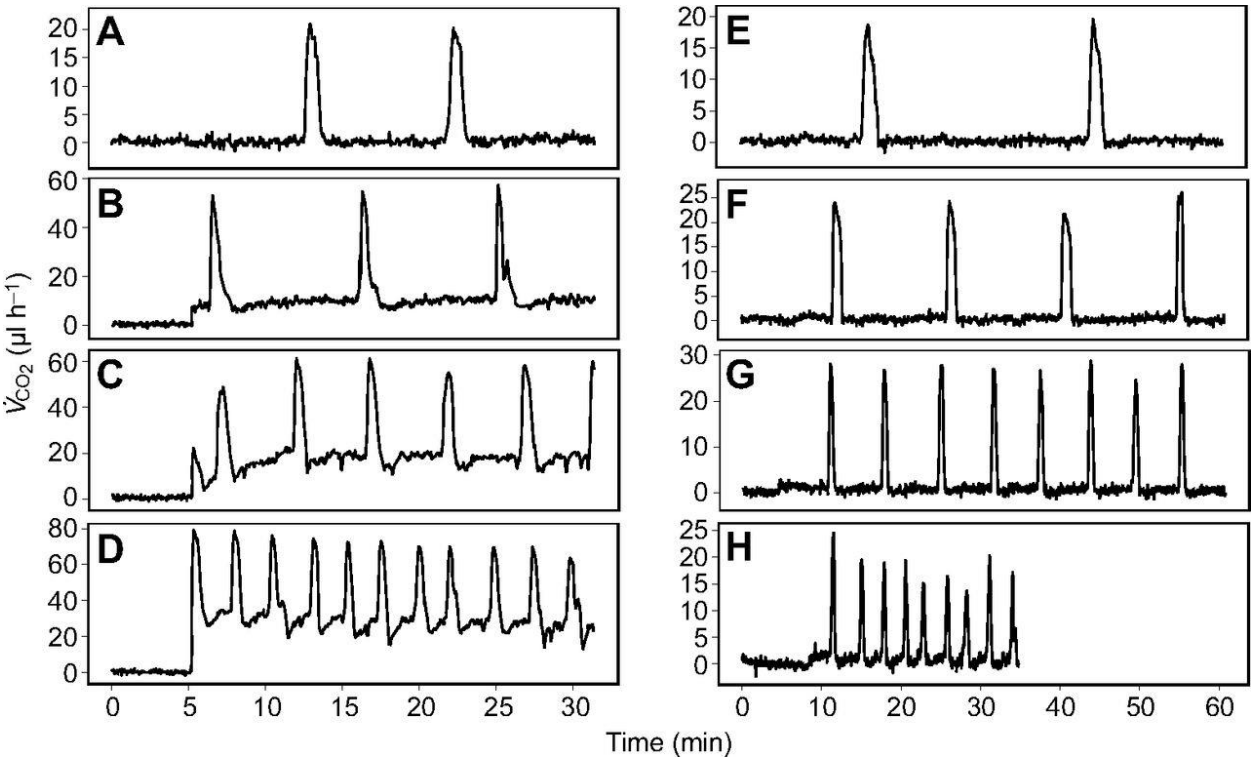

Fig. 2.2. Examples of respiratory patterns observed in fed and unfed *R. prolixus*. Each set of graphs displays the rate of CO_2 release over time from a single individual from each treatment. Shaded regions indicate baseline recordings. (A–D) Sample respiratory patterns for fed *R. prolixus* as metabolic rate increases at 0, 2, 6 and 10 days post-feeding, respectively. (E–H) Sample respiratory patterns for unfed *R. prolixus* at 18, 24, 30 and 36°C, respectively. Recordings were shortened at very high temperatures (H) as the high burst frequency allowed the appropriate measurements to be made over a shorter time period.

particularly at higher temperatures, a flutter phase is discernible, in that the rate of CO_2 release in the closed phase was distinctly lower than that in the flutter phase (Fig. 2.2H; Fig. 2.3).

When insects were equilibrated to higher temperatures, the time interval between bursts was shortened, reflecting the higher metabolic rate of the insects. When the time between bursts becomes shorter than the time constant of the chamber, observed CO_2 release appears continuous (Gray & Bradley 2006). As such, one out of six individuals

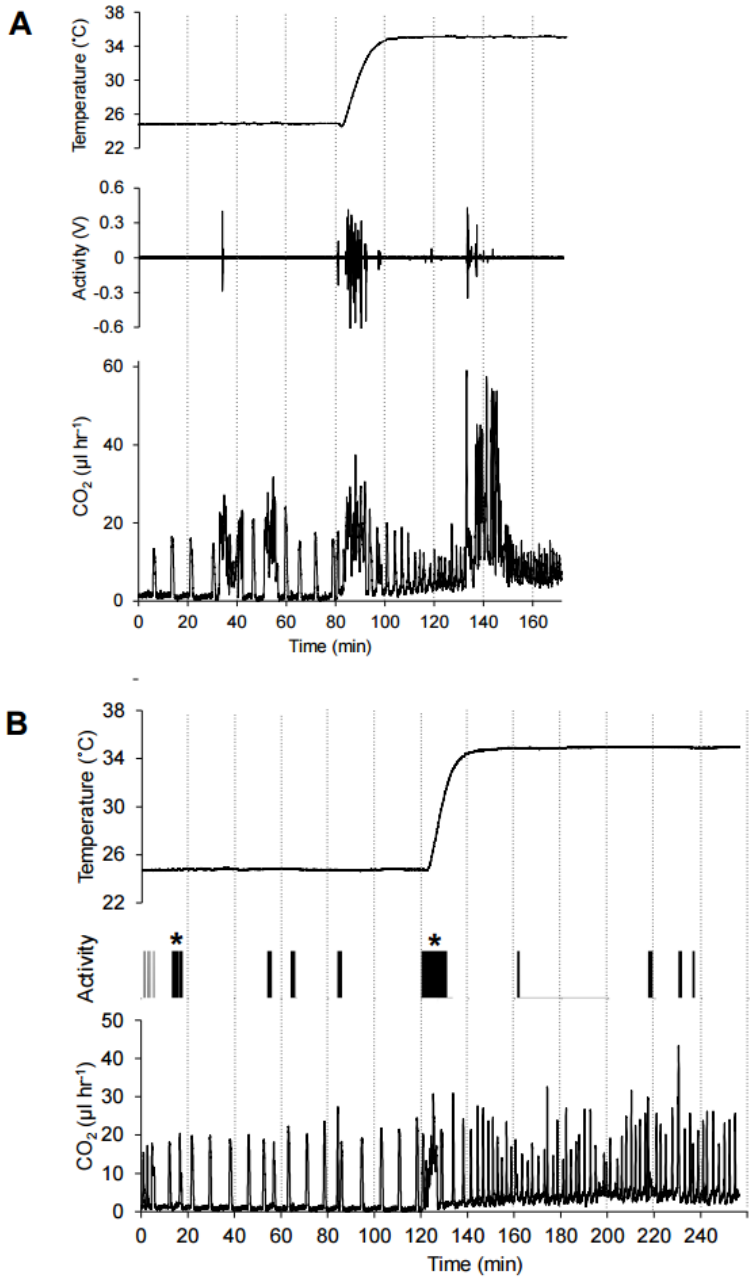

Fig. 2.3. Activity and CO_2 release in an unacclimated *Rhodnius* over time at a low (25˚C) and high (35˚C) temperature. Overall activity patterns did not differ across the two temperatures. (A) Periods of activity detected by an infrared activity detector result in a disruption of the discontinuous gas exchange pattern and elevated rate of CO_2 release at both temperatures. One disruption of the pattern was not associated with activity. (B) periods of intense movement observed visually from video recordings (e.g. escape behavior, indicated with asterisks) resulted in a disruption of the discontinuous pattern in one case, but the pattern was maintained in another. Other periods of minimal movement (grooming, adjusting body position) had similar effects in that they only occasionally disrupted the discontinuous pattern. After the 24 h acclimation period utilized in our study, lengthy periods of discontinuous gas exchange were observed with no apparent effects of locomotion.

in the first temperature treatment group and three out of six individuals in the second treatment group ceased to produce detectable discontinuous bursting patterns at 38°C with a flow rate of 200 ml min^{-1}. As a result, the recorded CO_2 release was continuous and characteristics of the gas exchange pattern were no longer examined at this temperature and above.

When *Rhodnius* were allowed to take blood meals to repletion, metabolic rate increased substantially over a period of several days (Fig. 2.1A), resulting in a CO_2 release pattern with more frequent bursts and an elevated rate of interburst CO_2 release (Fig. 2.2A–D). More O_2 is required between bursts in order to support the higher aerobic metabolic rate. This oxygen is supplied by increasing the duration and/or frequency of spiracular micro-openings during the interburst period. The increased uptake of oxygen therefore results in an increased rate of CO_2 release during flutter (Fig. 2.2D). In some traces, the rate of CO_2 release immediately after an open phase is lower than later in the interburst period (Fig. 2.2C,D). We interpret this to mean that there is a short closed phase after the open phase, but the high metabolic rate dictates a rapid transition to the flutter phase in fed individuals.

Respiratory characteristics in feeding trials

When fed a bloodmeal, *R. prolixus* maintained a discontinuous pattern of CO_2 release even as the metabolic rate increased. However, variations in the characteristics of the discontinuous pattern were observed. When burst volume (BV) was measured as the increase over the interburst release rate, the volume increased with an increase in

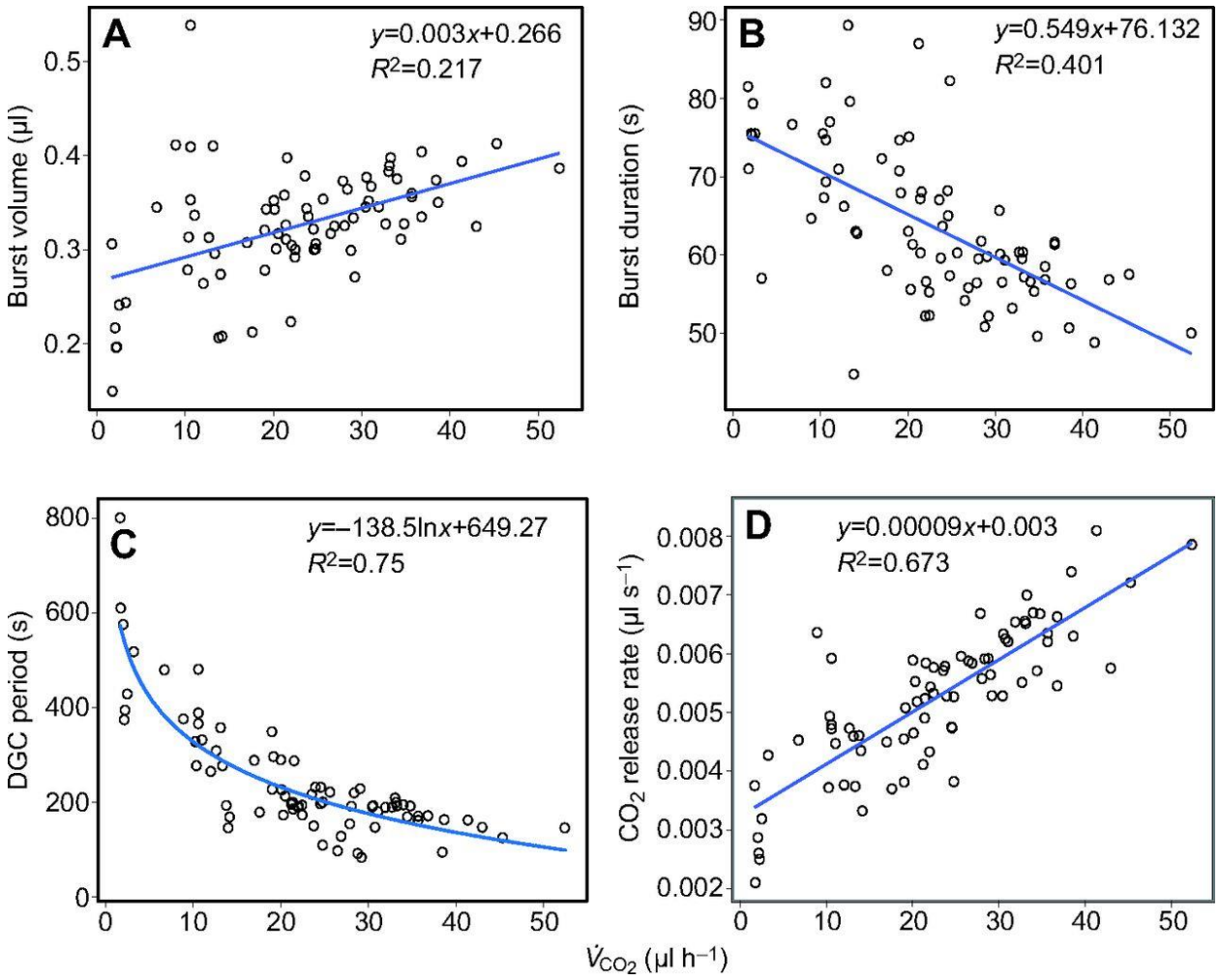

Fig. 2.4. Effects of feeding-induced changes in metabolic rate on measured respiratory characteristics. The change in burst volume (A), burst duration (B), discontinuous gas exchange cycle (DGC) period (C) and open phase CO_2 release rate (D) as a function of metabolic rate in fed *R. prolixus*. A slight increase in burst volume was observed as metabolic rate increased. This increase was more pronounced if burst volume measurements included the steady-state CO_2 release rate during interburst periods. An increase in burst volume and a decrease in burst duration resulted in a significant increase in the rate of CO_2 release during a spiracular opening period. The cycle period decreased initially and leveled off near 150 s per cycle. Blue lines are linear regressions in A, B and D and a logarithmic regression in C. For A–D, $P<0.001$.

metabolic rate (MR) (Fig. 2.4A; BV=0.003MR+0.266,R^2=0.217, $F_{1,75}$=22.03, P<0.001). When the burst volume was measured as the increase relative to a zero baseline (see Fig. 2.5, volume measurement 2), this relationship became more pronounced (BV=0.015MR+0.261, R2=0.838). Respiratory bursts became shorter in duration as metabolic rate increased

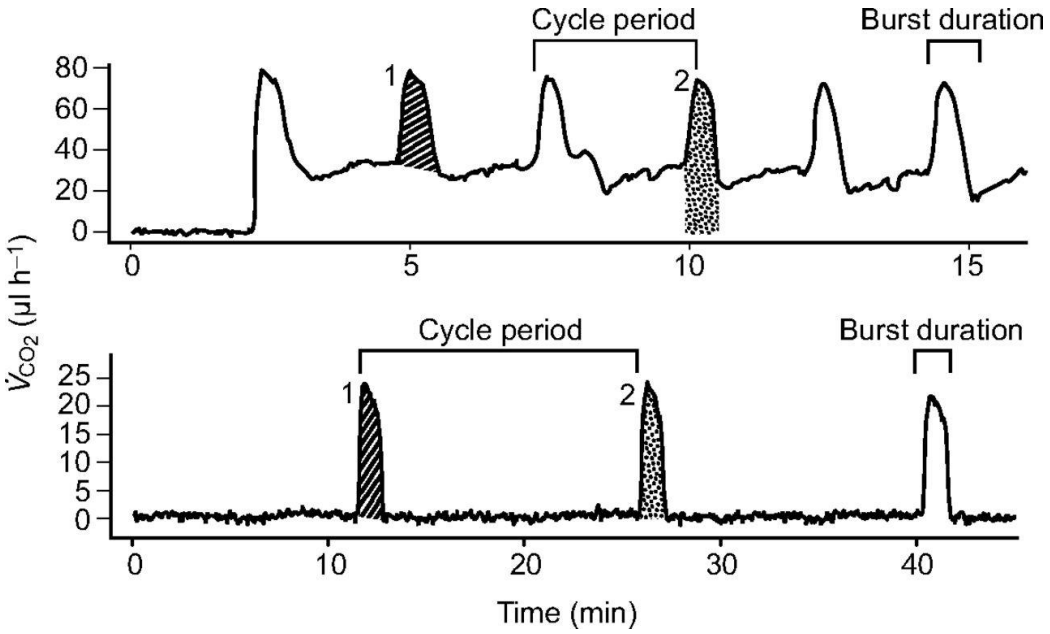

Fig. 2.5. Methods of data collection during different metabolic states. Two examples are shown. Burst volumes were collected as the volume of CO_2released above the steady-state release during flutter (1), or as the total CO_2 release during an opening event (2). Twelve bursts were sampled from each individual to find an average burst volume for each treatment. Cycle periods were defined as the time between the start of one burst and the start of the next consecutive burst. Burst durations were defined as the time from the start of the burst to the point at which CO_2 release returns to the steady-state value.

(Fig.2.4B; duration=−0.549MR+76.132, R^2=0.401, $F_{1,75}$=51.82, P<0.001). The average cycle period displayed an initial decrease with increasing metabolic rate but began to reach a plateau at an intermediate metabolic state (Fig. 2.4C; period=−138.5lnMR+649.27, R^2=0.75, $F_{1,75}$=225.27, P<0.001). A decrease in burst duration and an increase in the volume of CO_2 released in a burst resulted in a significant increase in the calculated CO_2 release rate (burst volume/burst duration) (Fig. 2.4D; rate=0.00009MR+0.003, R^2=0.673,$F_{1,75}$=157.2, P<0.001).

Respiratory characteristics in temperature trials

Unlike the increase in burst volume that occurred with increased metabolic rate following feeding, burst volume decreased with an increase in temperature (T) (Fig. 2.6A, ANOVA, $F_{10,108}$=24.51, P<0.001; BV=−0.024T+0.980 above 27°C). Burst duration and cycle period also decreased with increasing temperature (Fig. 2.6B, ANOVA, $F_{10,108}$=122.4, P<0.001; Fig. 2.6C, $F_{10,108}$=51.63, P<0.001). A one-way ANOVA also indicated a significant relationship between the burst phase CO_2 release rate and temperature (Fig. 2.6D, $F_{10,108}$=4.694, P<0.001), with a Tukey HSD test demonstrating that all rates were similar with the exception of the rate measured at 38°C, which was significantly lower than rates at all temperatures except for 18 and 22°C.

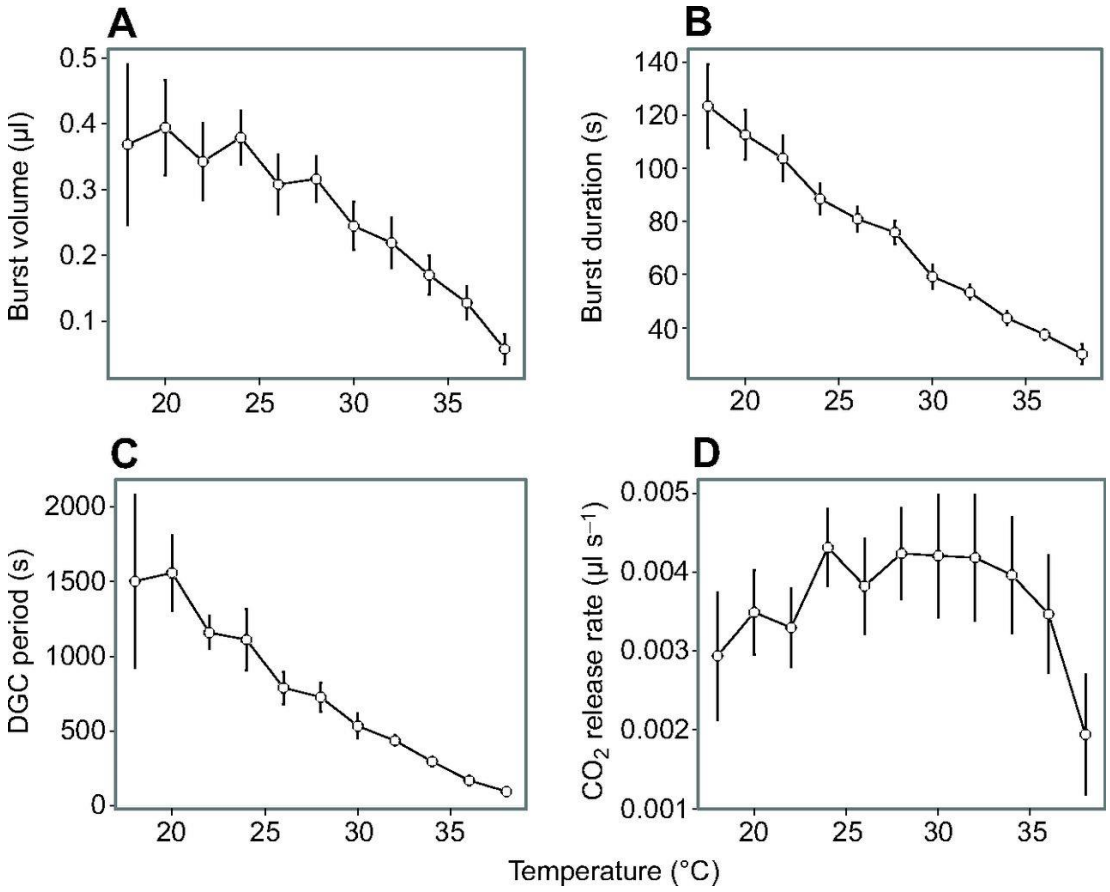

Fig. 2.6. Effects of temperature-induced changes in metabolic rate on measured respiratory characteristics. The change in burst volume (A), burst duration (B), discontinuous gas exchange cycle (DGC) period (C) and open phase CO_2 release rate (D) as a function of metabolic rate in unfed *R. prolixus* at temperatures ranging from 18 to 38°C. Increases in temperature led to decreases in burst volume, burst duration and cycle period. There was no significant change in the rate of CO_2release during a burst over the range of 18 to 34°C. Error bars represent 95% confidence intervals.

Effects of hyperoxia on discontinuous gas exchange pattern characteristics

Table 2.1 provides results of two-way ANOVA tests performed on fed and unfed *R. prolixus* in 21% and 40% oxygen treatments. There was no significant effect of oxygen concentration on the metabolic rates of the unfed or fed groups. The volume of CO_2 released in a respiratory burst was not affected by feeding, but increased significantly in fed individuals exposed to hyperoxia (Fig. 2.7A). There was no effect of oxygen concentration on the burst duration (Fig. 2.7B). However, feeding had a significant effect on

burst duration, with fed individuals displaying significantly longer bursts. The cycle period was significantly increased in fed compared with unfed individuals at 21% and 40% oxygen (Table 2.1). There was a significant effect of oxygen concentration on the rate of CO_2 release during a burst in fed individuals (Table 2.1), with the rate being higher in 40% oxygen. There was also a higher rate of CO_2 release during a burst in unfed individuals compared with fed individuals at 21% oxygen.

Table 2.1 Two-way ANOVA results for all respiratory variables in oxygen treatment trials

Measurement	Source	SS	d.f.	MS	*F*	*P*
Metabolic rate (μl CO_2 h^{-1})	Feeding	3277	1	3277	289.992	**<0.001**
	Oxygen	1	1	1	0.058	0.812
	Feeding:oxygen	3	1	3	0.231	0.635
	Error	271	24	11		
Burst volume (μl)	Feeding	0.002	1	0.002	1.067	0.312
	Oxygen	0.057	1	0.057	24.861	**<0.001**
	Feeding:oxygen	0.014	1	0.014	6.096	**0.021**
	Error	0.055	24	0.002		
Cycle period (s)	Feeding	2.3×10^{6}	1	2.3×10^{6}	181.584	**<0.001**
	Oxygen	43,785	1	43,785	3.445	0.0758
	Feeding:oxygen	58,103	1	58,103	4.572	**0.043**
	Error	3.1×10^{5}	24	12,709		
Burst duration (s)	Feeding	3412	1	3412	90.13	**<0.001**
	Oxygen	94	1	94	2.491	0.128
	Feeding:oxygen	90	1	90	2.384	0.136
	Error	909	24	38		
Open phase CO_2 release rate (μl s^{-1})	Feeding	1.1×10^{-5}	1	1.1×10^{-5}	17.264	**<0.001**
	Oxygen	5.5×10^{-6}	1	5.5×10^{-6}	8.942	**0.006**
	Feeding:oxygen	3.9×10^{-6}	1	3.9×10^{-6}	6.276	**0.019**
	Error	1.5×10^{-5}	24	6.2×10^{-7}		

Bold indicates significance.

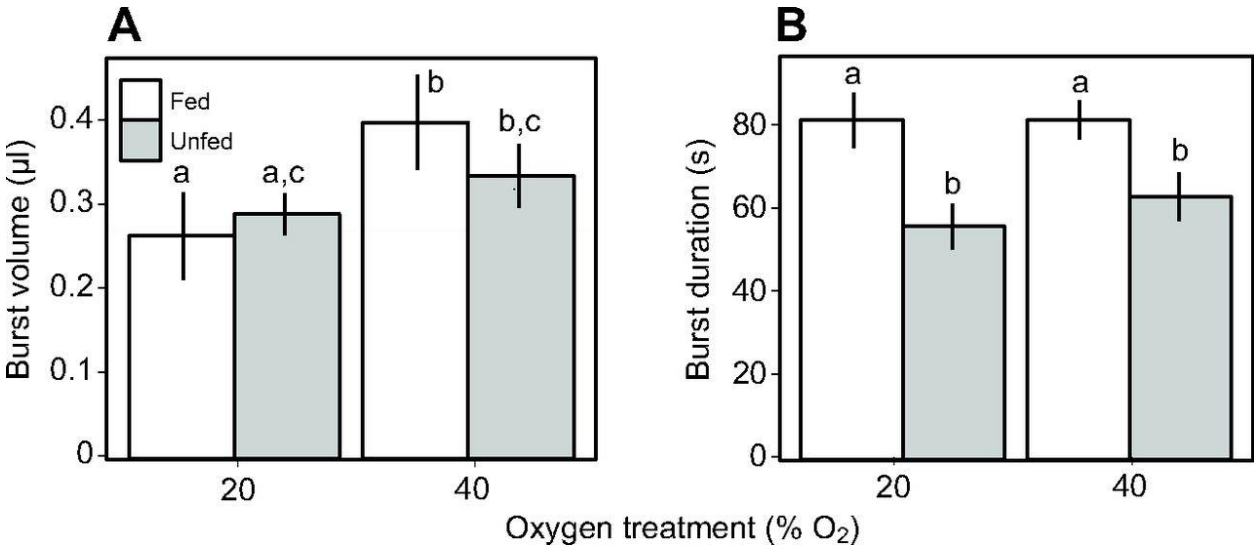

Fig. 2.7. Effects of oxygen content and feeding status on respiratory pattern. Respiratory burst volumes (A) and burst durations (B) for fifth instar *R. prolixus* under two treatments (unfed or fed a complete bloodmeal) following exposure to normoxic air (21% O_2) or acute exposure to hyperoxic (40% O_2) air. Error bars indicate 95% confidence intervals. See Table 2.1 for ANOVA results on all measured variables.

DISCUSSION

The dual feedback model of insect respiratory control (Levy & Schneiderman 1966; Chown & Holter 2000; Förster & Hetz 2010) proposes that spiracular activity is controlled via two feedback mechanisms that regulate internal partial pressures of oxygen and CO_2. Spiracles initiate a fluttering response when tracheal P_{O2} reaches a critically low threshold and spiracles open when tracheal P_{CO2} reaches a critically high threshold. The interaction between these two sensory systems produces a pattern of respiratory CO_2release in which large bursts of CO_2 are separated by prolonged periods of spiracular closure or low CO_2 release rates.

The presence of a critical P_{CO2} threshold dictates that the amount of CO_2released in a single respiratory burst, or spiracular open period, would remain constant as long as the threshold value does not change. However, many studies have demonstrated that the

respiratory burst volume decreases with increased temperature in ants (Lighton 1988; Quinlan & Lighton 1999; Vogt & Appel 2000), beetles (Duncan & Dickman 2001) and the cecropia silkworm (Schneiderman & Williams 1955). Others have found no change in burst volume with increasing temperature (Davis et al. 1999; Klok & Chown 2005), or even an increase in burst volume (Shelton & Appel 2001; Basson & Terblanche 2011). Our results from temperature manipulation experiments in *R. prolixus* show a decrease in burst volume with increasing temperature (Fig. 2.6). Above the rearing temperature of 27°C, burst volume decreased linearly with temperature ($BV=-0.024T+0.980$). These results are inconsistent with expectations based on the dual feedback model, unless the critical CO_2 threshold changes with temperature.

To determine whether these changes in gas exchange pattern were due to a direct effect of temperature or an indirect effect on metabolic rate, we also measured these characteristics in gas exchange patterns obtained from *R. prolixus* during digestion of a blood meal. Throughout the duration of meal processing, *R. prolixus* experiences a range of metabolic rates up to 13 times their resting metabolic rate. In contrast to our observations from temperature trials, we found that burst volumes within the population did not decrease with increased metabolic rate but actually increased slightly (Fig. 2.4), regardless of whether burst volume was measured as the increase over flutter release rates or baseline zero values. However, these volume increases are much smaller in scale than the decreases observed in temperature trials. As a result, we conclude that the dual feedback model of spiracular control adequately describes respiratory behavior and can accommodate metabolic variation at a single temperature. The conundrum remains, however, that some aspect of the respiratory control mechanism is temperature sensitive

and that the respiratory control model becomes more complex if changes in temperature are considered.

Effects of temperature on gas exchange patterns

There was a 3.5-fold increase in the average metabolic rate of *R. prolixus* over the temperature range of 18 to 38°C (Fig. 2.1). In addition to these changes in metabolism, the increase in temperature also induced changes in the gas exchange pattern. As temperature increased there was a significant decrease in the volume of CO_2 released during a burst as well as a decrease in burst duration (Fig. 2.6). We found no change in the rate of release of CO_2 during a burst with the exception of a decrease in rate at 38°C (Fig. 2.6D). Cycle period decreased with increased temperature. In some insects, bursts became so frequent that they were no longer distinguishable via flow-through respirometry, and the pattern therefore appeared as continuous gas exchange. Overall, the results of the temperature experiments are consistent with the findings of Contreras and Bradley (Contreras & Bradley 2009), in that CO_2release patterns transitioned from discontinuous to continuous as metabolic rate increased with temperature.

Effects of feeding on gas exchange patterns

Fed insects experienced a much larger range of metabolic rates compared with temperature trial rates. Throughout the duration of meal processing, average peak metabolic rates increased up to 13.9 times the average resting value. However, despite the larger increase in metabolism, fed insects continued to produce discontinuous patterns of CO_2 release throughout the experiment. In contrast to the gas exchange pattern changes

observed in temperature trials, burst volumes increased slightly and burst durations decreased with metabolic rate in fed insects (Fig. 2.4). As a result, CO_2 release rates (volume/duration) during a burst increased with metabolic rate. Fed insects produced more frequent bursts as their metabolic rate increased, but cycle period leveled off at about 150 s per cycle, allowing the maintenance of discontinuous CO_2 release patterns.

Contreras and Bradley (Contreras & Bradley 2009) proposed that an insect's metabolic rate determines its gas exchange pattern. In the present study, the differences in gas exchange patterns between insects exposed to temperature or feeding treatments indicate that metabolic rate is not the only factor determining respiratory behavior in insects. At a constant temperature, gas exchange patterns can be predicted by metabolic rate and are explained by the dual feedback loop model of spiracular control (Levy & Schneiderman 1966; Förster & Hetz 2010). We found that temperature also has strong effects on gas exchange pattern independent of its effect on metabolic rate. These effects are not accommodated by the model put forward by Contreras and Bradley (Contreras & Bradley 2009) or by the dual feedback loop model in its simplest form.

Analysis of temperature effects on gas exchange pattern characteristics

We found in *R. prolixus* that increased temperature led to decreased burst volume during spiracular openings. As the presence of a constant CO_2 trigger threshold for spiracular opening at a given temperature has been well described (Levy & Schneiderman 1966; Harrison et al. 1995; Förster & Hetz 2010), and seems to be verified in this insect (present study), a decrease in the amount of CO_2 released in a respiratory burst could be

the result of premature initiation of the open phase or premature initiation of the closed phase.

It may be informative to consider the results presented here in the context of the process of CO_2 sensing. In vertebrates, CO_2 is sensed as pH in the brain, a site separated from the blood, which is highly buffered, by the blood–brain barrier (Guyton & Hall 2006). The sensors in the brain are sensitive to pH and in this location the pH of the solution is directly proportional to P_{CO2}in the blood. Premature spiracular opening in insects might also be explained by the physical properties of CO_2 or an effect of temperature on the respiratory control system. As temperature increases from 18 to 38°C, the solubility coefficient of CO_2 in water decreases from 4.149×10^{-2} to 2.474×10^{-2} mol $l^{-1}atm^{-1}$ (Weiss 1974). As a result, for any given quantity of CO_2 produced, less will become dissolved in the aqueous portions of the body, resulting in a higher P_{CO2} in the tracheal system. As P_{CO2} is in equilibrium throughout the body, this results in a higher P_{CO2} at the sensor location, wherever it may be. As such, a smaller quantity of CO_2 is required to initiate spiracular opening, resulting in smaller volumes of CO_2 released per burst. Congruently, Levy and Schneiderman (Levy & Schneiderman 1966) and Förster and Hetz (Förster & Hetz 2010) note a positive relationship between temperature and the tracheal P_{CO2} at the threshold for spiracle opening.

However, the observation that burst volume decreases with increasing temperature is also compatible with an alternative explanation. P_{CO2} may be detected via pH in an isolated sensory organ, as in vertebrates. If so, then as temperature increases, an additional pH reduction is experienced in the sensory organ as a result of increased water ionization, causing the pH triggering threshold to be reached prematurely and the open phase to be

initiated at a lower P_{CO2} than expected. Hemolymph pH in grasshoppers decreases above 25°C (Harrison 1988), but does not change significantly throughout the respiratory cycle in grasshoppers (Harrison et al. 1990; Harrison et al. 1995) or cockroaches (Matthews & White 2011b). However, in vertebrates the CO_2-sensing neurons are separated from the blood by the blood–brain barrier. This allows the neurons to respond to P_{CO2} through a change in pH of the cerebrospinal fluid while being insensitive to blood pH. Perhaps in insects, neurons are also sensing the pH of the perineural fluid, which is similarly isolated from the hemolymph (Treherne & Pichon 1972). A two-layer barrier protects the brain, ganglia and major nerves from direct contact with the hemolymph, which is well buffered (Nation 2002). This barrier consists of an outer acellular layer, which functions as a barrier to ions and osmolites in the hemolymph, and a second layer composed of perineural cells. The perineural cell layer is largely impermeable as a result of an abundance of tight and gap junctions (Lane & Skaer 1980).

The effects of CO_2 on spiracular function have been described (Hazelhoff 1926; Hoyle 1960; Wigglesworth 1935), but the mechanism by which this signal is detected remains unknown. Case (Case 1957) argued that spiracles open either in response to CO_2-induced acidity or as a result of the release of bound CO_2 by other acids. In other studies, Case found that hypoxia increased the sensitivity of the spiracles to CO_2 (Case 1954; Case 1956). He (Case 1957) concluded that spiracular opening is therefore produced directly through the action of CO_2 on the spiracle muscle or another receptor cell that is permeable to CO_2 but not to hydrogen ions. Similarly, Hoyle (Hoyle 1960) found that CO_2 plays a direct role in the relaxation of the spiracular closure muscle, even under continued nervous excitation, through interference with the transmission of action potentials across the

neuromuscular junction. Badre et al. (Badre et al. 2005) provide evidence for direct inhibition of glutamate receptors of insect skeletal muscle by CO_2.

Based on this evidence, Förster (Forster 2010) proposed two possible mechanisms by which this direct action of CO_2 may take place: (1) direct inhibition of glutamate receptors by CO_2 or (2) CO_2 diffusion into the post-synaptic cell causing a pH disturbance that inactivates the receptor. Our results support the second of these proposed models. Increased temperatures would reduce intracellular pH in addition to the pH disturbance caused by accumulated dissolved CO_2, causing premature receptor inactivation and spiracular opening events, which occur before the CO_2 threshold would have been reached based on CO_2-induced pH changes alone. These premature openings would explain the reduced CO_2 emission volumes during the spiracular open phase detected at high temperatures.

Effects of hyperoxia

The dual feedback model of insect respiratory control attributes the initiation of the flutter phase to a critically low P_{O2} threshold, and the initiation of the open phase to a threshold high CO_2 concentration. It is silent with regard to the factors that cause the cessation of the open phase, i.e. the initiation of the closed phase.

One can hypothesize that the cessation of the open phase could be controlled by the oxygen concentration in the insects reaching a threshold high level, or by the CO_2 concentration in the insect reaching a threshold low level. Alternatively, cessation of the open phase could be simply a timing event such that the open phase had a specific temporal duration. We can rule out this last explanation based on the data in

Figs 2.4 and 2.6. Regardless of how metabolic rate is increased, the duration of the open phase decreases with increasing metabolic rate.

We chose to test the oxygen threshold hypothesis by examining the duration of the open phase in 21% and 40% oxygen environments. We found that the duration of the open phase did not change significantly under these two oxygen regimes. We are left with the conclusion that the rate at which oxygen enters the insect during the open phase does not control the temporal length of the open phase.

The experiments we have described here are unable to either confirm or falsify the hypothesis that the open phase is terminated when P_{CO2} reaches a threshold low level. It is clear that the amount of CO_2 released in a burst declines with increasing temperature. However, we are unable to determine on the basis of our results whether this is due to decreased solubility of CO_2 in water at elevated temperatures or to a shift in pH in cells responsible for spiracular control. Either process could explain the observed changes in burst volume.

Physiological and ecological implications

As small ectotherms, insects are subject to substantial temperature fluctuations on both a daily and a seasonal basis. The respiratory control system of *R. prolixus* is sensitive to temperature, resulting in different gas exchange patterns in response to both metabolic rate and ambient temperature. Increased temperature results in reduced CO_2 release during the open phase of the discontinuous gas exchange cycle and premature abandonment of the discontinuous pattern at metabolic rates lower than those reached by fed individuals who maintained this pattern.

Many researchers have argued that the discontinuous gas exchange cycle provides adaptive advantages to insects. Unmatched metabolic rate and oxygen supply may lead to excess oxidative damage (Hetz & Bradley 2005), and an inability to produce prolonged spiracular closures may have detrimental effects on water balance (Chown & Davis 2003). Oxidative damage and water loss would both increase with increasing temperature. The maintenance of the discontinuous respiratory cycle even at higher metabolic rates may have important physiological implications for the fed insects, which are experiencing high metabolic rates and rapid tissue growth. Alternatively, perhaps the discontinuous gas exchange cycle has no adaptive value and is simply a result of cycling partial pressures of respiratory gases (Chown & Holter 2000).

Furthermore, researchers have often utilized temperature as a means of inducing changes in metabolic rate to examine the effects of different metabolic states on various physiological parameters. We have shown that the effects of temperature on respiratory behavior are significant and can overshadow the effects of metabolic rate alone. As a result, caution should be exercised when investigating physiological responses to metabolic rates induced via temperature. Interactions between effects of temperature and metabolism must be considered. When possible, researchers should attempt to include multiple sources of metabolic manipulation, such as temperature, feeding or activity, to gain a better idea of the variation in physiological responses to metabolic changes.

The fact that respiratory controls are temperature sensitive may provide new techniques for determining the sites and mechanisms by which insects sense the partial pressures of respiratory gases. Localized temperature manipulation, for example of the head or of a specific spiracle, could allow us to make predictions about the molecular

mechanisms and specific tissues with which an insect senses respiratory gases. Additionally, localized tests of pH sensitivity, for instance in spiracular motor neurons, may provide supporting evidence for Hoyle (Hoyle 1960) and Case's (Case 1957) ideas about CO_2 sensing via pH changes at the neuromuscular junction. Future studies may also look more closely at the intratracheal $P_{O2}$and P_{CO2} threshold values for spiracular activity across a range of different temperatures to verify how tracheal gas partial pressure thresholds change with temperature.

Understanding the details of this control system will provide insight into how animals with vastly different respiratory physiologies – that is, vertebrates and insects – evolved strategies to solve the same basic problem of controlling the exchange of respiratory gases. Furthermore, these results display the importance of understanding the interactive effects of different metabolic stressors in ectothermic animals, and their effects on respiration.

CHAPTER 3:

The effect of respiratory pattern on oxidative damage in insects

INTRODUCTION

Insects are capable of precisely controlling the movement of respiratory valves, called spiracles, which modulate gas exchange between the tracheal system and the environment. Spiracles typically cycle between three phases, the "open phase" during which the valves are open and gas exchange occurs freely between the tracheal system and the environment, the "flutter phase" during which micro-openings of the spiracles allow oxygen to diffuse into the tracheal system while internal P_{CO2} continues to rise, and the "closed phase" during which the valves remain shut and no gas exchange occurs (see figures 3, 4, and 1.2). During periods of low metabolic demand, the closed phases can become prolonged and the resulting respiratory pattern is referred to as discontinuous gas exchange.

The adaptive significance of the discontinuous gas exchange pattern has been debated extensively (see Table 1; Contreras et al. 2014; Chown et al. 2006). It has been proposed, based on a literature analysis of studies using flow-through respirometry to measure respiratory patterns, that the behavior independently evolved at least 5 times in insects (Marais et al. 2005). However, the ability to detect discontinuous gas exchange with flow through spirometry depends on certain aspects of the study's methodology. In flow-through respirometry, a constant stream of air is pushed through a chamber housing an insect and the CO_2 released by the insect is detected downstream of the chamber. Therefore, a sufficiently high air flow rate must be used to produce a high enough time constant within the chamber housing the insect to detect spiracle closed phases (Gray &

Bradley 2006). While faster air flow rates allow you to detect shorter closed phases, they also result in lower signals and increased risk of desiccation stress on the insect. Therefore the absence of recorded closed phases under most experimental conditions does not serve as sufficient evidence to suggest that an insect is incapable of producing a discontinuous gas exchange pattern.

Studies of spiracle control indicate that the spiracles will cycle through the close, flutter, and open phases with increased frequency as metabolic rate increases as a result of two interacting feedback loops involved in the detection and response to tracheal P_{O2} and P_{CO2} (Förster & Hetz 2010; Schneiderman 1960). Fluttering is initiated when a low intratracheal O_2 threshold is reached, and opening is initiated when a high CO_2 threshold is reached. While the longstanding debate about the function of the discontinuous gas exchange pattern was focused on finding the one adaptive benefit that supports the evolution of this behavior, it seems clear that this pattern is an emergent property of this respiratory control system, which must maintain internal P_{CO2} and P_{O2} values at homeostatic levels. Furthermore, it is likely that the resulting prolonged spiracle closure provides multiple benefits to insects, including the reduction of respiratory water loss (Schimpf et al. 2009; Matthews & White 2012).

Hetz and Bradley (2005) proposed that the discontinuous gas exchange pattern prevents excess oxidative damage in insects. This hypothesis is based on the observation that intratracheal P_{O2} values are maintained at low levels (~4 kPa, depending on the insect) during the closed and flutter phases. Without this control of internal oxygen content, the insect may be exposed to excessive oxidative damage as a result of the high rate of diffusion of oxygen through the air-based gas exchange system (O_2 diffuses 10^6 times, and CO_2 10^4

times, faster in air than water) (Krogh 1920). High tissue P_{O2} values may result in high levels of oxidative damage as a result of increased production of reactive oxygen species (ROS). These ROS can damage lipids, proteins, DNA, and biomembranes within cells.

To date, evidence supporting this hypothesis includes the maintenance of low internal P_{O2} values during the flutter phase of the gas exchange cycle (Hetz & Bradley 2005; Förster & Hetz 2010) and the observation that the duration of spiracle closure will often increase when insects are exposed to atmospheric hyperoxia (Harrison et al. 2006; Lighton et al. 2004). Conversely, Matthews et al. (2012) used fiber optic oxygen probes to measure intratracheal oxygen content and reported that internal P_{O2} values were not maintained at sufficiently low values when insects were exposed to atmospheric hyperoxia. These results suggest that the primary function of the discontinuous pattern is to maintain the internal P_{O2} above a minimal level and not to defend the tissues from oxidative damage. Another study used live cell fluorescence microscopy to observe reactive oxygen species (ROS) production and found that ROS production did not increase when the tracheal system was shocked with hyperoxic air (40% O_2) after a period of anoxia which forced spiracular opening (Boardman et al. 2012). While these results do not support the oxidative damage hypothesis, they have not directly measured tissue damage as a result of prolonged disruption of the discontinuous pattern.

Our study provides a different direct test of the oxidative damage hypothesis based on 3 hour acute exposure to varied O_2 and CO_2 gas treatments. Insects open their spiracles in hypercapnia (CO_2 > ~6%) as a result of the relaxation of the valve closer muscle (Hoyle 1960; Förster & Hetz 2010). We can therefore use hypercapnia to prevent prolonged spiracle closure and fluttering and as a result induce a more continuous pattern of gas

exchange. In addition to a hypercapnic treatment, we also exposed insects to hyperoxia, and a combination of hypercapnia and hyperoxia to increase the diffusion gradient for oxygen into the insect during forced spiracle opening. We then quantified lipid and protein oxidative damage (lipid peroxidation and protein carbonyl formation) and the amount of antioxidant enzyme activity (superoxide dismutase) in insects exposed to these treatments. We hypothesized that exposure to hypercapnia would increase the amount of oxidative damage across the whole body as a result of the inability to maintain low internal P_{O2} values. We also hypothesized that exposure to hypercapnic hyperoxia would result in increased oxidative damage above that of the hypercapnic treatment as a result of increased atmospheric oxygen availability during forced spiracle opening.

METHODS

Animals

Drosophila melanogaster were obtained from the B line in the Rose lab at the University of California, Irvine. Flies were reared at room temperature on a 24 hour light cycle in plexiglass rearing chambers with a cloth lid apparatus. Populations were maintained at a density of about 1000 flies per chamber. Eggs were collected weekly by placing petri dishes with banana-based medium and yeast paste into rearing boxes. Eggs were collected in groups of 60 by hand and placed into cylindrical plastic vials with 5 mL of banana-based medium to develop. Prior to the start of the gas treatments, 5-7 day old adult flies were transferred to new plastic vials (approximately 120 adults per vial) containing 5 mL of a new cornmeal-based medium. Flies remained in these new vials for 24 hours before the onset of the gas treatments. During this time, a subgroup of flies in a

positive control treatment were also transferred to new glass vials (approximately 120 adults per vial) containing 5 mL of cornmeal medium containing 10 mM Paraquat dehydrate (see Paraquat treatment" below).

Due to the large number of flies required for the biochemistry assays, replicate populations from the same *D. melanogaster* line were tested each week for 16 weeks, resulting in a final maximum sample size of 16 for each treatment. A second round of flies were reared under the same conditions and exposed to the same treatments approximately 6 months after the completion of the first trials in order to conduct an additional protein carbonyl ELISA assay and determine the effect of handling and transport on oxidative damage (comparing a "no-treatment" group to the standard "control" group). For this second protein carbonyl assay, 7 samples of 15 flies were taken from each treatment group, resulting in a final sample size of 7 per treatment.

Paraquat treatment

Flies were fed the insecticide Paraquat (1,1' dimethyl-4-4'-bipyridynium dichloride) as a positive control for our biochemistry assays. Paraquat is known to induce oxidative damage by producing superoxide anions which results in the synthesis of ROS (Bonilla et al., 2006). Flies were kept in the vials with a cornmeal-based medium containing 10 mM Paraquat dichloride hydrate (Fluka Analytical, Sigma-Aldrich, St. Louis, MO, USA) for 24 hours. The 10 mM concentration was chosen because previous studies have demonstrated that this is the minimum concentration of Paraquat required to produce maximal superoxide dismutase antioxidant enzyme activity (Rzezniczak et al. 2011).

Surviving flies were flash frozen in liquid nitrogen and stored at -80˚C. Dead flies become trapped in the medium and are therefore lost from the sample during transfer from the vial to liquid nitrogen by inversion of the vial. Paraquat exposure began about 1 day prior to the onset of the gas treatments. This ensured that the Paraquat-fed flies were exposed to the cornmeal medium for approximately the same duration as the gas-treated flies and could be flash frozen at the same age as the gas-treated flies.

Gas treatments

Acute (3 hour) gas exposure treatments took place over two days. Two treatments were administered on day one and two treatments were administered on day two. The order of administration was chosen at random each week. Flies were exposed to 3 hours of each gas treatment because reactive oxygen species have been shown to accumulate over timescales of seconds (Boardman et al. 2012). Furthermore, preliminary studies indicated that there was no long term difference in the death rate between flies in normal air vs. hypercapnia (Helenius et al. 2009), suggesting no advantage to using a longer exposure time and risking interactive effects of desiccation stress.

The gas treatments included: (1) 21% O_2 and 0.4% CO_2 (control); (2) 21% O_2 and 6% CO_2 (hypercapnic); (3) 40% O_2 and 0.4% CO_2 (hyperoxic); and (4) 40% O_2 and 6% CO_2 (mixed, hypercapnic hyperoxia). The hypercapnic treatment results in spiracle opening and was predicted to increase oxidative damage of lipids and proteins. The hyperoxic treatment was predicted to result in no increase in oxidative damage since the flies are still able to utilize the discontinuous gas exchange pattern. The mixed treatment was predicted

to result in the highest rates of oxidative damage since hypercapnia triggers spiracle opening while atmospheric oxygen content is increased.

Tests were conducted at sea level at the University of California, Irvine (~101.325 kPa). Gas mixtures were created by combining O_2, CO_2, and dry room air inside of a mylar balloon. Mixtures were accurate within ± 0.3%.

The purpose of the hypercapnic gas treatments was to stimulate spiracular opening and prevent the insects from properly utilizing the discontinuous gas exchange pattern. To verify that hypercapnic exposure induced spiracular opening, water release measurements were made from populations of *D. melanogaster* exposed to the hypercapnic and mixed gas treatments. For these tests, air was pulled from the mylar balloon at 100 ml min^{-1} by a hydrostatic pump set (Sable Systems, Las Vegas, NV, USA). The pump then pushed the air past a column of silica gel and a column of Drierite (W. A. Hammond Drierite Co. Ltd., Xenia, OH, USA) to remove any traces of water vapor. Air then flowed through a vial containing a population of flies (approximately 250-300 flies per population). The flies had no access to food or water during these tests. Air then passed through a CO_2/H_2O analyzer (LiCor Inc., Lincoln, NE, USA). Voltage outputs from this device were transformed to a digital signal by a UI-2 Universal Interface (Sable Systems) and sent to a laptop for data collection (Acer, San Jose, CA, USA). Expedata software was used to collect and analyze water release measurements (Sable Systems). Data was collected at 1 sample sec^{-1} intervals. Two populations of flies were tested, with each population exposed to either the hypercapnic (1) or mixed gas treatment (2). The average steady-state water release for an approximately 30 minute period was determined after a 15 minute equilibration time in each treatment using Expedata (Sable Systems). The water analyzer was calibrated

manually by correlating untransformed voltage measurements with known volumes of deionized water introduced into a small glass chamber downstream from the analyzer via a micropipette.

During the gas mixture treatments, fly populations from eight vials were combined in four vials (about 240 flies per vial) with 5 mL of cornmeal medium each to prevent effects of desiccation and starvation on oxidative damage measurements. Vials were then capped with rubber stoppers containing glass tubes for the entry and exit of air. The hydrostatic pump (Sable Systems) pulled air from the mylar balloon and pushed it at 200 mL per minute through the four vials which were connected in sequence by rubber tubing. Air exited the last vial and entered a carbon dioxide analyzer followed by an oxygen analyzer (Oxzilla, Sable Systems) for secondary verification of the gas concentrations which the flies were exposed to. After exposure to the gas treatment for 3 hours, the flies were flash frozen in liquid nitrogen and stored at -80˚C until assayed.

Quantification of oxidative damage and stress

Lipid peroxidation:

Tissue samples for the lipid peroxidation assay were prepared by measuring out 25 mg of flies (about 20-25 flies, both male and female) and sonicating in 250 µl RIPA buffer 4 times for 15 seconds at 40V with 30 second intervals between pulses. The sample was then centrifuged at 1600 x g for 10 minutes at 4˚C. The supernatant was removed and either tested immediately or frozen at -80˚C until the next day. The eye pigment of *D. melanogaster* is known to absorb light at the same wavelength as the compound of interest

for this assay, however the red pigment precipitated out of the sample upon centrifugation and the remaining sample was extremely diluted throughout the assay procedure.

Lipid peroxidation in the samples was measured using a Cayman Chemical TBARS Assay Kit (Ann Arbor, MI, USA; Item No. 10009055). This assay quantifies malondialdehydes (MDA), a product of the decomposition of lipid hydroperoxides. During the assay, MDA reacts calorimetrically with thiobarbituric acid at high temperature to produce a color change in the sample. After the reaction was completed, the absorbance of each sample was measured in a microplate at 535 nm. The concentration of MDA in each sample was calculated from a standard curve.

Protein carbonylation:

(1) Calorimetric Assay

Two assays were performed to measure protein oxidative damage (protein carbonyl formation) in the flies during each treatment. The first is a protein carbonyl calorimetric assay (Cayman Chemical) in which protein carbonyls in the sample react with 2,4-dinitrophenylhydrazine (DNPH) to produce a Schiff base and corresponding hydrazone. This product is then measured spectrophotometrically. To prepare the samples, 100 mg of flies were homogenized in 500 µl of cold buffer (50 mM phosphate buffer, pH 6.7, 1 mM EDTA). The sample was then centrifuged at 10,000 x g for 15 minutes at 4°C and the supernatant was removed and stored at -80°C. The 280/260 nm absorbance ratio of the supernatant was measured to verify that there were no contaminating nucleic acids.

The assay was run according to the instructions provided in the Cayman Chemical kit (Item No. 10005020). Samples were separated into two parts, one part was reacted

with DNPH and 2.5 M HCl was added to the other as a control. Each tube was incubated in the dark at room temperature for 1 hour and vortexed every 15 minutes. After incubation, 20% TCA was added to each tube and the samples were placed on ice for 5 minutes the centrifuged at 10,000 x g for 10 minutes at 4°C. The pellet was resuspended in 10% TCA, left on ice for 5 minutes, and re-centrifuged. Each sample then went through three washes with a 1:1 mixture of Ethanol:Ethyl Acetate and the final pellet was resuspended in guanidine hydrochloride and absorbance measured in a microplate at a spectrum of 360-385 nm wavelengths. The absorbance of the control sample was subtracted from the absorbance of the DNPH-reacted sample to calculate total reacted protein carbonyls. The following equation was used to find the protein carbonyl content from the corrected absorbance:

$$Protein\ Carbonyl\ \left(\frac{nmol}{ml}\right) = [\frac{Corrected\ absorbance}{0.011\ \mu M^{-1}}] \times \frac{500\ \mu l}{200\ \mu l}$$

(2) ELISA Assay

An additional test for protein carbonyl content was conducted because no difference in protein carbonyl content was observed in any of the treatments in the calorimetric assay. To verify that the experimental manipulations themselves were not causing large baseline oxidative damage values and preventing the detection of variation across treatments, a new experiment was conducted in which all parameters were the same but a new control was added. A subset of flies was flash frozen in liquid nitrogen directly from their original vials containing banana-based medium on day 6 after eclosion. This is referred to as the "No Treatment" group.

An enzyme-linked immunosorbent assay (ELISA) (BioCell Corp., Papatoetoe, New Zealand) was used to measure protein carbonyl content of these new fly populations. This assay has been used successfully in past studies to determine protein carbonyl formation in flies exposed to hyperoxic stress throughout their whole lifetime (Rascón & Harrison 2010). 15 male flies were homogenized in 800 µL of cold buffer (50 mmol sodium phosphate buffer, pH 7.4, 1% Triton X-100 and protease inhibitors) (Sigma Aldrich, St. Louis, MO, USA) . This resulted in samples with approximately 1.443 ± 0.08 mg ml^{-1} (*M* ± *SD*) protein. The samples were then centrifuged at 10,000 x g for 15 minutes and the supernatant was stored at -80°C until assayed. The assay was conducted in accordance with the kit instructions for the standard procedure (BioCell, batch P117). Samples were thawed and reacted with DNPH. Protein was then bound nonspecifically to a 96-well ELISA plate and a biotinylated anti-DNP antibody and streptavidin-linked horseradish peroxidase are added and bind to DNP-reacted proteins. Finally, a chromatin reagent containing peroxide is added to catalyze the oxidation of TMB, causing a color change in the well, and the absorbance is measured at 450 nm.

A standard absorbance curve was produced from samples containing known concentrations of protein carbonyls. The protein carbonyl content of each sample was calculated from this standard curve.

Superoxide dismutase activity:

Since the accumulation of oxidative damage is dependent on the activity of antioxidant enzymes, we also quantified superoxide dismutase activity to determine if the treatments caused oxidative stress and upregulation of antioxidant enzymes. 25 mg of flies

were homogenized in 125 µL of cold buffer (20 mM HEPES, pH 7.2, 1 mM EGTA, 210 mM Mannitol, 70 mM Sucrose) and stored at -80°C until assayed. The assay was conducted in accordance with the kit instructions (Cayman Chemical, Item No. 706002). 200 µL of sample or standard per well was added to a 96-well plate along with a tetrazolium salt-based superoxide radical detector. Xanthine oxidase was added to each well to initiate superoxide radical production. The plate was incubated on a shaker at room temperature for 30 minutes and the absorbance was read at 450 nm. A standard curve of the linearized rate (LR) was produced from the standard measurements, and the SOD activity was determined from the following equation:

$$SOD\ \left(\frac{U}{ml}\right) = \left[\left(\frac{sample\ LR - y\ intercept}{slope}\right) \times \frac{0.23\ ml}{0.01\ ml}\right] \times sample\ dilution$$

Data analysis and statistics

All respirometry data were collected in Expedata (Sable Systems). Data analysis from biochemical assays were conducted in Excel (Microsoft, Redmond, WA, USA). T-tests were used to compare each treatment group to the control. For each such comparison, a Bonferroni correction was made to account for multiple hypothesis testing. For the first protein carbonyl assay (calorimetric), there was a significant effect of replicate on the measured protein carbonyl content. Therefore, for this assay I ran an analysis of covariance in Statistica 7 (StatSoft, Tulsa, OK, USA) with replicate as a covariate. An ANOVA was run in Statistica 7 on the second protein carbonyl dataset (ELISA) because an additional control was added to the experimental design.

RESULTS

Disruption of spiracular control

Two populations (250-300 individuals each) were exposed to hypercapnic or mixed gas treatments to determine the effect of the treatment on the extent of spiracle opening as observed by the water release rate. Population 1 was exposed to a control and hypercapnic treatment (21% O_2, 6% CO_2) and population 2 was exposed to the control and mixed gas treatment (40% O_2, 6% CO_2). The average water release rate over a 30 minute period increased in hypercapnia and decreased in the mixed gas treatment compared to their controls (Fig. 3.1). Absolute volumes of water release cannot be compared across trials using different populations because population size was large and could not be precisely controlled. Since the respiratory system is the primary source of water release, these results indicate that hypercapnia induced an increased overall duration of spiracle opening and therefore a successful disruption of spiracular control. Similarly, the reduced water release rate in the mixed gas treatment indicates that the duration of spiracle closure increased.

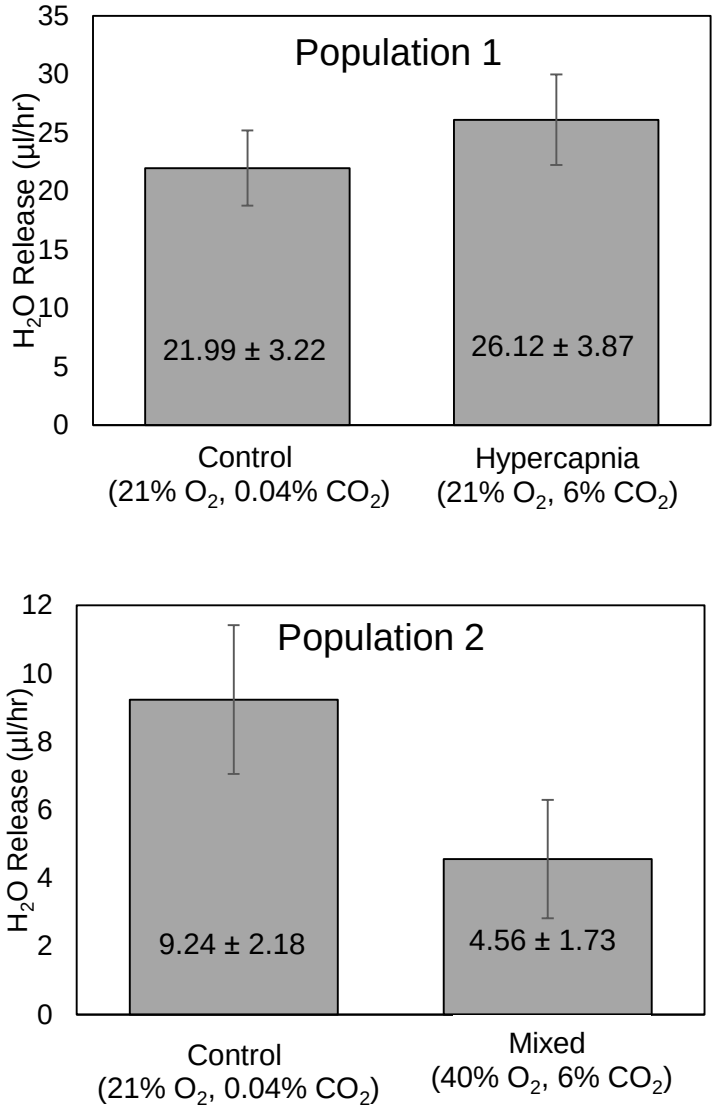

Fig. 3.1. Water release rates from populations of flies exposed to normal, hypercapnic, and mixed gas treatments. Bars represent mean ± standard deviation of the total water release by the whole population over a 30 minute period following 15 minutes of acclimation at each treatment.

Quantification of oxidative damage

Whole body levels of lipid hydroperoxides were quantified by determining the concentration of malondialdehyde (MDA), a product of lipid hydroperoxide decomposition. Flies fed a diet containing 10 mM Paraquat dichloride hydrate and flies exposed to the hypercapnic gas treatment displayed significantly higher levels of MDA compared to

control groups (paraquat: $t(30) = -2.87$, $p = 0.007$; hypercapnia: $t(30) = -2.42$, $p = 0.022$; Fig. 3.2). No other treatment groups demonstrated significant changes in whole body lipid peroxidation levels.

Protein carbonyl formation was quantified via a calorimetric reaction with DNPH. There were no differences detected between any of the treatment groups using this method (ANOVA: $F(4, 38) = 0.342$, $p = 0.848$; Fig. 3.3). An additional ELISA assay was conducted to verify this result. In the second assay, there was still no differences in protein carbonyl content between any of the original treatment groups. However, there was significantly higher protein carbonyl content in flies that did not experience any of the treatments compared to the flies in hypercapnic and hyperoxic treatments (Fig. 3.4).

Increased activity by superoxide dismutase was observed in flies exposed to media containing 10 nM Paraquat dihydrate for 24 hours ($t(29) = -2.19$, $p = 0.037$; Fig. 3.5). One control sample was removed from this analysis due to well contamination causing significantly high absorbance readings outside the normal range of the assay. There was no effect of any gas treatment on superoxide dismutase activity levels in whole body samples.

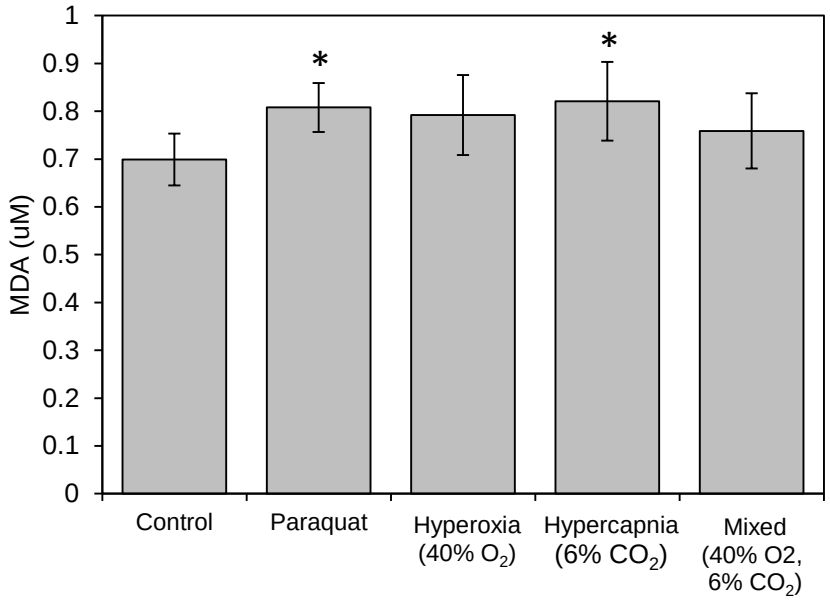

Fig. 3.2. Malondialdehyde (MDA) content in whole-body tissue samples of *D. melanogaster*. Bars represent mean ± 95% confidence intervals. Asterisks indicate significant differences from the control group based on a Bonferroni-corrected t-test.

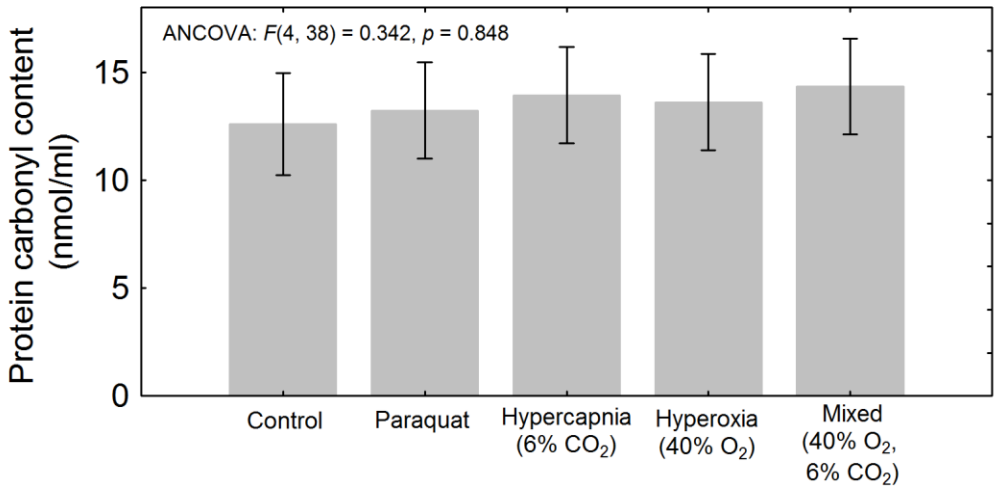

Fig. 3.3. Protein carbonyl content in whole-body tissue samples of *D. melanogaster*. Bars represent mean ± 95% confidence intervals. ANCOVA results include replicate as a covariate.

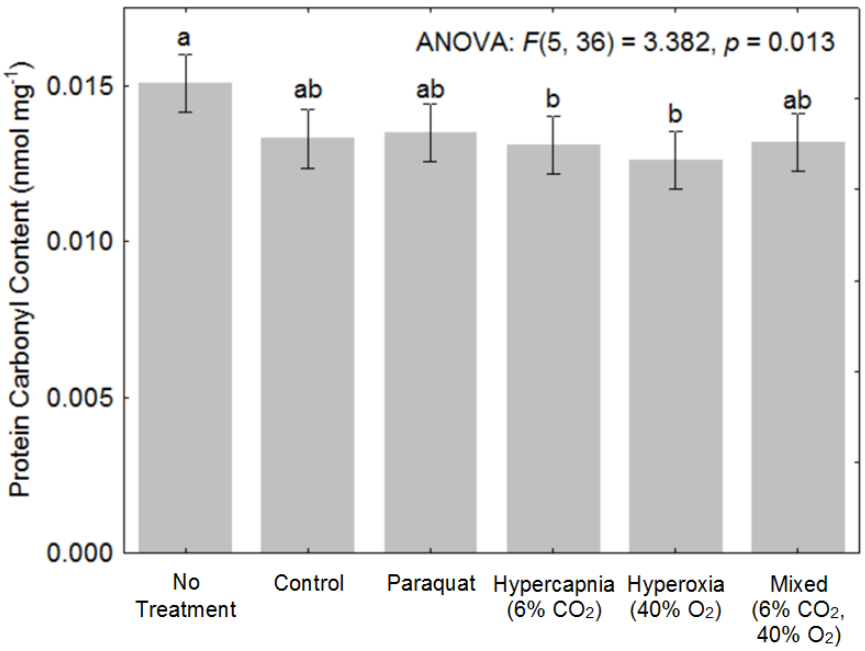

Fig. 3.4. Protein carbonyl content in whole-body tissue samples of *D. melanogaster* measured with the ELISA method. Tissue samples were more dilute than those used in the previous protein carbonyl assay. Bars represent 95% confidence intervals.

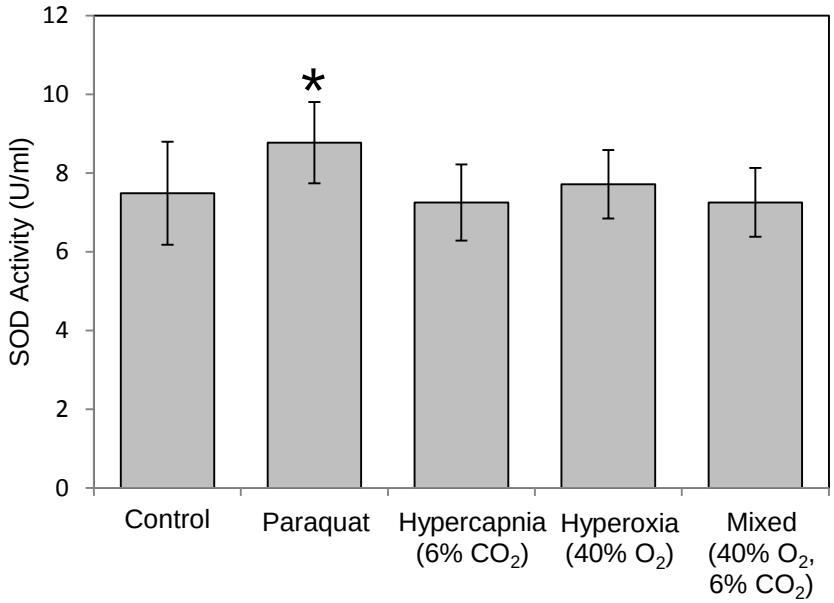

Fig. 3.5. Superoxide dismutase activity in whole-body tissue samples of *D. melanogaster*. Bars represent mean ± 95% confidence intervals. Asterisks indicate significant differences from the control group based on a Bonferroni-corrected t-test.

DISCUSSION

Discontinuous gas exchange may provide a number of benefits to insects. These may include reduced respiratory water loss, prevention of tracheal parasite infections, or the ability to colonize hypercapnic subterranean environments (all existing hypotheses regarding the function of discontinuous gas exchange are provided in Table 1). To date, few studies have examined the hypothesis that discontinuous gas exchange reduces tissue oxidative damage. We tested this hypothesis by measuring whole-body lipid peroxidation and protein carbonyl levels, as well as superoxide dismutase activity in populations of adult *D. melanogaster* exposed to various acute atmospheric gas treatments which disrupt patterns of spiracular activity and prevent the use of discontinuous gas exchange.

We found that acute exposure to mild hypercapnia disrupts the discontinuous gas exchange pattern by preventing prolonged spiracle closure. This is evidenced by an increase in the population-level water release rate in flies exposed to dry air with 6% CO_2 compared to their release rate in normal dry air (Fig. 3.1). We also found that exposure to a mixed gas treatment of hypercapnic hyperoxia (6% CO_2, 40% O_2) decreased water release rates in *D. melanogaster*. This result is in accordance with previous findings that increased atmospheric oxygen levels lead to: (1) decreased ventilation in grasshoppers (Harrison et al. 2006; Bustami et al. 2002; Greenlee & Harrison 1998) and (2) a transient reduction in CO_2 and water release rates (up to a 90% reduction from the continuous rate) in *D. melanogaster* and two ant species during exposure to 100% O_2 (Lighton et al. 2004). Interestingly, the increased oxygen content in the mixed gas treatment appeared to override any signal to open the spiracles in response to hypercapnia. The duration of this

effect is unclear since these values represent average water release rates during a 30 minute period after exposure.

However, despite verifying that prolonged spiracle closure was reduced in hypercapnia, we did not find strong evidence that this acute disruption of the respiratory pattern resulted in increased oxidative damage in *D. melanogaster*. While lipid peroxidation increased moderately after exposure to hypercapnia (Fig. 3.2), there was no indication that protein oxidation increased in hypercapnia (Fig. 3.3; Fig. 3.4) or that the antioxidant response was increased in any of the treatment groups during a three hour exposure (Fig. 3.5). The slight increase in protein carbonyl content in flies which didn't experience any of the original treatment regimens may have resulted from increased antioxidant activity in all treatment groups (Fig. 3.4). It is possible that the extra handling and stress associated with the original experimental treatments (e.g. vial transfer, new media, increased population density) resulted in acute upregulation of antioxidant enzyme activity and therefore a reduced accumulation of oxidative damage in all treatments.

There are multiple factors that may have contributed to these results. Firstly, a 3 hour exposure time may have been insufficient for the accumulation of significant tissue oxidative damage to take place. Some studies in vertebrates give us an idea of the timescale of oxidative damage. In vertebrate muscle, acute bouts of exercise are known to cause increased lipid peroxidation and protein carbonylation via increased radical production by the electron transport chain of the mitochondria, as a result of muscle damage, or as the result of catecholamine production (Reznick et al. 1992). Protein oxidation has also been shown to increase significantly in muscle tissues 8 hours after cecal ligation (Fagan et al. 1996), or up to 1 hour after injection of *Escherichia coli*

lipopolysaccharides in mice (Barreiro et al. 2005), as a result of sepsis. However, the timescales of oxidative damage accumulation are presumably highly variable across systems since damage accumulation is a function of the rate of formation, the breakdown of damaged molecules, and the production and availability of antioxidants, which all vary at the tissue and individual level. As a result, it is difficult to determine if the levels of damage we detected in whole insects after 3 hours of exposure to each treatment are representative of the possible treatment effects over a longer period of time or in a specific tissue.

A second possibility is that damage accumulated at different rates in different tissues. If so, we may not have been able to detect these different responses using measurements from whole-body homogenates. Previous studies have found significant variation in whole-body protein carbonyl content after much longer periods of exposure to hyperoxia than utilized in our experiments (Rascón & Harrison 2010). In Rascón and Harrison's work, adult flies were reared in 1, 10, 21, and 100 kPa O_2 for 7-28 days depending on the population's median lifespan in each treatment. Future studies should therefore utilize longer treatment regimens or measure damage in isolated tissue samples. Flight muscle samples may be particularly useful since they have a high density of tracheoles and mitochondria (Wigglesworth & Lee 1982; Snelling et al. 2012).

Longer treatment regimens can be considered since there is no effect of constant mild hypercapnia (7 or 13%) on the lifespan of *D. melanogaster* (Helenius et al. 2009). Interestingly, in the Helenius et al. (2009) study, the mild hypercapnic treatments did have a significant effect on the ability of flies to survive bacterial infections, suggesting that there are other physiological effects of mildly elevated CO_2 not related to longevity. While it is

possible that larger amounts of oxidative damage were not detected in populations of flies exposed to 3 hour gas treatment because the duration of treatment was too short, it is reasonable to predict reduced survival times in fly populations exposed to hypercapnia throughout adulthood if discontinuous gas exchange safeguards against oxidative damage. This prediction is based on the oxidative damage hypothesis for aging which postulates that oxidative damage, by various metabolic or environmental sources, is a universal contributor to aging (Martin et al. 1996; Nilanjana et al. 2001). Previous studies have demonstrated that both atmospheric hyperoxia and severe hypoxia reduce the adult lifespan of *D. melanogaster* and increase levels of oxidative damage (Rascón & Harrison 2010; Nilanjana et al. 2001). If tissue P_{O2} values are increased significantly in insects unable to utilize normal discontinuous gas exchange, then according to the oxidative theory of aging, we should see reduced lifespans in insects exposed to hypercapnia. However, since we did not find evidence to support this prediction, we cannot say with confidence that discontinuous gas exchange functions to reduce oxidative damage.

Similarly, if hyperoxia has been shown to increase internal tracheal P_{O2} values (Matthews et al. 2012) as well as reduce insect lifespans (Rascón & Harrison 2010), then it is more likely that insects utilize their respiratory control system to safeguard against low tissue P_{O2} and lack the ability to protect against increased oxygen tension. However, this would not explain the increased duration of spiracle closure observed when insects are exposed to hyperoxia. It is possible that these prolonged closed periods are an acute reflex induced by transient increases in ROS prior to upregulation of antioxidant enzymes. ROS are known to interact with a number of cellular processes and molecular targets, including membrane lipid bilayers and ion channels (Dean et al. 2004). These interactions can result

in changes in neuron excitability and synaptic transmission. In humans, exposure to hyperoxia at normobaric pressure often results in a short period of reduced ventilation followed by "hyperoxic hyperventilation", or rates of ventilation that exceed baseline levels (Georgopoulos et al. 1989). Clearly, the relationship between oxygen, ROS, and respiratory control systems are complex. While the intricacies of vertebrate respiratory control have been investigated in detail, we are just scratching the surface in terms of understanding the molecular physiology of insects respiratory control systems.

CONCLUSION

In recent decades, much progress has been made to our understanding of insect respiratory physiology. Today, we know that insects control their spiracle valves through multiple physiological feedback loops which regulate internal partial pressures of CO_2 and O_2. We also know that insects have a high capacity for oxygen delivery as a result of bypassing the circulatory system and delivering oxygen directly to tissues in the gas phase. While diffusion alone may be sufficient to deliver oxygen through the tracheal system to some insects, many other insects utilize abdominal pumping, or other mechanisms to ventilate the tracheal system. These ventilatory movements are controlled by a central pattern generator which is sensitive to temperature and P_{O2}.

However, despite these advancements in our understanding, many aspects of insect respiratory control and function remain unexplored. I have verified that coordinated movement of spiracle valves and abdominal pumping can result in unidirectional airflow through the tracheal system. Possible benefits of this strategy should be explored. This behavior may result in reduced oxygen diffusion distances in the smaller tracheoles and therefore provide increased oxygen delivery capacities to insects with larger body sizes or metabolic rates. Furthermore, the mechanism by which spiracle function and abdominal muscle contraction remain synchronized to produce this pattern is unknown. While spiracles may be controlled locally by internal gas partial pressures, there may be a higher level control mechanism which integrates spiracle movement with abdominal contractions triggered by the central pattern generator.

I have also demonstrated that respiratory patterns and spiracle movements are differentially sensitive to temperature and metabolic rate. Previously, it was thought that

metabolic rate alone determined an insect's respiratory pattern. However, since studies of insect metabolism typically use atmospheric temperature variation as a tool to manipulate metabolic rate, it has not been possible to tease apart the effects of metabolism versus temperature on respiratory patterns or other physiological function. *Rhodnius prolixus* was used as a model in this study to uncouple these factors because their metabolic rate can be increased up to 7 times resting levels during digestion of a large blood meal. In these experiments, we found that increases in temperature lead to increases in discontinuous gas exchange cycle frequency and decreases in open phase CO_2 release volumes that were much greater than the changes observed at the same metabolic rate in fed insects at room temperature. These results indicate that respiratory patterns are not determined by metabolic rate alone and that the mechanism which controls spiracle activity is temperature sensitive. We should be careful to consider the interaction between temperature and physiological processes in ectotherms when using temperature as a tool to manipulate metabolic rate. As observed in this study, different metabolic stimuli may produce very different responses as a result of interacting physiological processes. The same metabolic rate may result in a very different physiological state depending on if the metabolism is stimulated by digestion, body temperature, pregnancy, or activity. In studies where the link between metabolism and another physiological process are being considered, it would benefit physiologists to manipulate metabolic rate in multiple ways to observe these interactive effects.

The data from my experiments with *Rhodnius* also support one possible hypothesis about the control mechanism used to couple P_{O2} and P_{CO2} sensing and spiracle muscle contraction. Like vertebrates, insects may use changes in pH to control patterns of

ventilation and spiracle movement. Both increased P_{CO2} and temperature lead to decreased pH in solutions, including insect hemolymph above 25˚C. The precise location and mechanism used to sense these pH changes remains unknown. Future studies may examine the role of perineural fluid and glial cells in generating pH sensitivity in respiratory neurons. Insect respiratory physiologists should collaborate with neurobiologists to investigate this hypothesis and determine how spiracle activity is linked to ventilatory control.

While the precise spiracle control mechanism is unknown, we know that these valves respond to internal CO_2 and O_2 partial pressures through two interacting feedback loops which create tri-phasic patterns of valve movement. This discontinuous gas exchange pattern has been hypothesized to serve many functions, including reducing oxidative damage of tissues. Some of the results obtained in this study support an alternative hypothesis – that this gas exchange pattern functions not to defend against high oxygen concentrations, but to maintain internal P_{O2} above a low threshold level while prioritizing the reduction of respiratory water loss. However, the insect respiratory system and the molecular cascade of oxidative damage are complex and specific tissue level effects may not have been detected in our studies at the whole body level. Future studies may examine the oxidative damage hypothesis in further detail by disrupting the gas exchange cycle for longer periods of time or measuring oxidative damage in isolated flight muscle.

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