The Journal of Experimental Biology 214, 3062-3073

doi:10.1242/jeb.053991

RESEARCH ARTICLE

Regulation of gas exchange and haemolymph pH in the cockroach *Nauphoeta cinerea*

Philip G. D. Matthews* and Craig R. White

School of Biological Sciences, The University of Queensland, St Lucia, Queensland 4072, Australia

*Author for correspondence at present address: School of Earth and Environmental Sciences, The University of Adelaide, Adelaide, South Australia 5005, Australia (phil.matthews@adelaide.edu.au)

Accepted 15 June 2011

SUMMARY

Ventilatory control of internal CO_2 plays an important role in regulating extracellular acid–base balance in terrestrial animals. While this phenomenon is well understood among vertebrates, the role that respiration plays in the acid–base balance of insects is in need of much further study. To measure changes in insect haemolymph pH, we implanted micro pH optodes into the haemocoel of cockroaches (*Nauphoeta cinerea*). They were then exposed to normoxic, hypoxic, hyperoxic and hypercapnic atmospheres while their haemolymph pH, $\dot{V}_{CO_2}$ and abdominal ventilation frequency were measured simultaneously. Intratracheal O_2 levels were also measured in separate experiments. It was found that cockroaches breathing continuously control their ventilation to defend a haemolymph pH of 7.3, except under conditions where hypoxia (<10% O_2) induces hyperventilation, or where ambient hypercapnia is in excess of haemolymph (>1% CO_2). In contrast, intratracheal O_2 levels fluctuated widely, but on average remained above 15% in normoxic (21% O_2) atmospheres. Decapitation caused the cockroaches to display discontinuous gas exchange cycles (DGCs). The alternating periods of ventilation and apnoea during DGCs caused haemolymph pH to fluctuate by 0.11 units. Exposure to hypoxia caused haemolymph pH to increase and initiated brief bouts of spiracular opening prior to the active ventilation phase. The spontaneous occurrence of DGCs in decapitated cockroaches indicates that central pattern generators in the thoracic and abdominal ganglia generate the periodic gas exchange pattern in the absence of control from the cephalic ganglion. This pattern continues to maintain gas exchange, but with less precision.

Key words: gas exchange, insect, haemolymph pH, DGC, hypercapnia, hypoxia.

INTRODUCTION

Animals regulate their ventilation to ensure an adequate supply of O_2 to their tissues while eliminating CO_2. While aquatic animals primarily control their ventilation to extract sufficient O_2 from a milieu with a low capacitance for O_2 and a high capacitance for CO_2, terrestrial animals find atmospheric O_2 readily available and generally control their ventilation to regulate CO_2 elimination (Dejours, 1988). This regulation of internal CO_2 partial pressure (P_{CO_2}) has direct consequences for the acid–base balance of the animal's extracellular body fluids, as CO_2 reacts with water to form carbonic acid. However, most terrestrial animals use this reaction to their advantage, hypoventilating or hyperventilating to adjust the P_{CO_2} of their body fluids, thereby counteracting short-term fluctuations in acid–base balance. Although the trend towards CO_2-sensitive ventilatory control and elevated body fluid P_{CO_2} is apparent in most air-breathing animals [e.g. molluscs (Barnhart, 1992), crustaceans (Burggren, 1992) and vertebrates (Reeves, 1977)], the control of gas exchange among insects, and more particularly its role in acid–base balance, is poorly understood.

Insects were among the very first air-breathing animals to invade the terrestrial environment, and by almost any metric they are the most successful. They possess a highly effective respiratory system, which consists of a network of air-filled tubes that branch throughout all parts of their body, opening to the atmosphere through muscular valves called spiracles. Insects display a range of respiratory gas exchange patterns by controlling when they open and close their spiracles and when they use muscular contractions to actively ventilate their tracheal system. Active insects tend to take up O_2 and release CO_2 continuously, but resting insects may display alternating periods of high and low gas exchange, or even protracted periods with no gas exchange (Marais et al., 2005). These patterns are known to change in response to changes in metabolic rate or inspired levels of O_2 and CO_2. Early investigations into the effects of O_2 and CO_2 on insect gas exchange patterns showed that insects in air containing more than 2% CO_2 opened their spiracles continuously and increased ventilation frequency, whereas oxygen levels had to be severely reduced to achieve the same effect (Kitchel and Hoskins, 1935; Miller, 1960a; Miller, 1960b; Wigglesworth, 1935). From this it was concluded that CO_2 rather than O_2 was the more important respiratory stimulus (Miller, 1960a). However, whether insects were controlling their gas exchange in order to maintain the acid–base balance of their haemolymph or to maintain a stable P_{CO_2} irrespective of body-fluid pH remained unknown. It was suggested initially that insects regulate gas exchange in response to changes in body-fluid pH. This view is supported by experiments on fleas, flies and cockroaches that show both ventilation rate and spiracle aperture increase in response to injections of acid solutions into the insect's haemocoel (Case, 1957; Case, 1961; Snyder et al., 1980; Wigglesworth, 1935). Unfortunately, interpreting these results is difficult because acidifying an insect's haemolymph unavoidably shifts its $HCO_3^- \leftrightarrow CO_2$ equilibrium towards CO_2, thereby confounding any independent effect of low haemolymph pH with increased haemolymph and intratracheal P_{CO_2}. Attempts to separate the effects of pH and CO_2 have produced mixed results. Studies on cockroaches have shown that irrigating their ventral nerve

chord with a range of different acids stimulates abdominal pumping in the whole animal and rhythmic action potentials in isolated nerve chord preparations (Case, 1961; Snyder et al., 1980). Low pH solutions were found to produce greater and more consistent increases in ventilation frequency than solutions with high P_{CO_2} buffered to a uniform pH (Snyder et al., 1980). But haemolymph pH does not appear to influence gas exchange the same way in grasshoppers, with several studies concluding that no relationship between haemolymph pH and ventilation frequency exists independent of P_{CO_2} (Gulinson and Harrison, 1996; Harrison, 1989; Krolikowski and Harrison, 1996). These inconsistent results may be due to different chemoreceptor responses in the different insect groups (Harrison, 2001), although this possibility has yet to be investigated. Thus, there remains a gap in our understanding of this fundamental aspect of insect physiology.

In recent years, research on insect respiration has become increasingly concerned with a curious pattern of gas exchange called the discontinuous gas exchange cycle (DGC). It consists typically of three sequentially repeating phases (open, closed and flutter phases) characterised by the activity of the insect's spiracles. Depending on the metabolic rate of the insect, the closed and flutter phases can last for many hours, during which time CO_2 release is minimal. The accumulation of respiratory CO_2 within the insect's haemolymph during the closed and flutter phases lowers its pH (Buck and Keister, 1958; Levy and Schneiderman, 1966). Many researchers have hypothesised that the DGC must confer some adaptive benefit, as it appears to directly conflict with both the insect's acid–base balance and respiratory demands by periodically impeding gas exchange, and yet has evolved independently among many different insect orders (Chown et al., 2006; Marais et al., 2005). Fluctuations in haemolymph pH of ~0.06 units have been measured in butterfly pupae during DGCs (Hetz and Wasserthal, 1993) and fluctuations of ~0.05 units were recorded from the grasshopper *Taeniopoda eques* during periodic ventilation (Harrison et al., 1995). Of the five insect orders known to display typical closed/flutter/open DGC patterns, measurements of intratracheal O_2 levels and haemolymph pH exist only for two: lepidopteran pupae and adult grasshoppers (Harrison et al., 1995; Hetz and Wasserthal, 1993; Hetz et al., 1993; Krolikowski and Harrison, 1996). Comprehensive measurements of haemolymph pH, CO_2 release and intratracheal O_2 levels are currently lacking for any species of insect. In order to elucidate the mechanisms underlying insect gas exchange patterns we measured *in vivo* haemolymph pH, intratracheal O_2, CO_2 release and ventilation frequency in speckled feeder roaches (*Nauphoeta cinerea*) during continuous and discontinuous ventilation while exposed to normoxia, hypoxia, hyperoxia and hypercapnia. This study also compares the relative magnitude of the insect's response to inspired levels of O_2 and CO_2, as well as how these responses affect haemolymph pH.

MATERIALS AND METHODS

Animals

Cockroaches [*Nauphoeta cinerea* (Olivier 1789)] were purchased from The Herp Shop (Ardeer, VIC, Australia) as final instar nymphs and adults. They were kept in a 60 l plastic tub and fed *ad libitum* on a diet of carrot and dry cat food. Only adult males were used in experiments. Animals were weighed immediately prior to experimentation. Mean cockroach weight was 432.5±25 mg.

Respirometry

Gas mixtures of O_2 and CO_2 in N_2 were produced using three mass flow controllers (Aalborg, Orangeburg, NY, USA) calibrated for N_2 (0–500 ml min^{-1}), O_2 (0–500 ml min^{-1}) and CO_2 (0–50 ml min^{-1}) connected to high-purity N_2 (>99.99% pure) and standard O_2 (>99.5% pure) pressurised gas cylinders. The mass flow controllers were regulated using the voltage outputs of a DT9853 analogue output module (Data Translation Inc., Marlboro, MA, USA) controlled by a desktop PC. All mass flow controllers were calibrated using an NIST traceable 1–10–500 ml bubble flow meter (Bubble-O-Meter, Dublin, OH, USA). The CO_2/O_2 composition of the gas mixtures was accurate to within <1% of the set value. Flow rates were monitored using the flowmeter of an SS3 subsampler (Sable Systems, Las Vegas, NV, USA) located upstream of the respirometry chamber. The flow rate for all experiments was 500 ml min^{-1}.

Rates of CO_2 release in the CO_2-free gas mixtures were measured using a Li-7000 CO_2/H_2O infrared gas analyzer (LI-COR, Lincoln, NB, USA) in differential mode. In this configuration, the gas stream from the mass flow controllers first passed through cell A of the infrared gas analyzer, then through the respirometry chamber, before passing through cell B. The difference in CO_2 between the air entering and leaving the respirometry chamber was given as μmol mol^{-1} CO_2 (ΔCO_2). This was converted to the rate of CO_2 release ($\dot{V}_{CO_2}$; ml h^{-1} g^{-1}) using the equation:

$$\dot{V}_{CO_2} = \frac{(\Delta CO_2 / 1{,}000{,}000) \times \dot{V}_I}{M}, \quad (1)$$

where $\dot{V}_I$ is the rate of flow of CO_2-free air into the chamber (ml h^{-1}) and M is the mass of the insect (g).

Ventilation frequency

Cockroaches had their wings clipped by 1 cm and were then fixed by their pronotum to a 35×35 mm square of card with melted Investo dental modelling wax (Ainsworth Dental Company Pty Ltd, Marrickville, NSW, Australia). The cockroach was placed on its back within a 36×36×25 mm respirometry chamber. A 5 mm^2 window cut into the card beneath the cockroach's abdomen was placed above a 3.4×4.2 mm infrared photodiode/LED chip (SFH 9202, Osram Opto Semiconductors, Regensburg, Germany) to detect abdominal pumping movements associated with ventilation. The photodiode was connected to a custom-built circuit that produced a voltage change proportional to the abdomen's distance from the photodiode. This allowed abdominal ventilation frequency (f_V, cycles min^{-1}) to be measured, but only allowed for a qualitative measure of abdominal amplitude. Cockroaches were exposed to stepwise changes in O_2 (40, 30, 21, 15, 10 and 5% O_2) and CO_2 (0.3, 0.6, 1, 2, 3 and 4% CO_2) with each exposure lasting 15 min and a minimum 1 h between exposure to oxygen or carbon dioxide treatments.

Haemolymph pH measurement

Haemolymph pH was measured using implantable fibre-optic pH optodes (140 μm diameter) connected to a PreSens pH-1 micro optical pH meter (PreSens GmbH, Regensburg, Germany). The pH probes were calibrated at 25°C using seven sodium phosphate pH buffers that varied between pH 5 and 8 in 0.5 pH increments. Optical pH probes show slight cross-sensitivity to the ionic strength of the sample, with high ionic strength mimicking lower pH. Therefore, the pH buffers were made up with an ionic strength of 0.35 using NaCl to approximate the ionic strength of cockroach Ringer's solution and ensure correct operation of the probes. All sodium phosphate buffers were checked using a HI9025C pH meter with combined pH electrode (Hanna Instruments, Woonsocket, RI, USA) calibrated at 25°C with standard pH 4 and 7 buffer solutions.

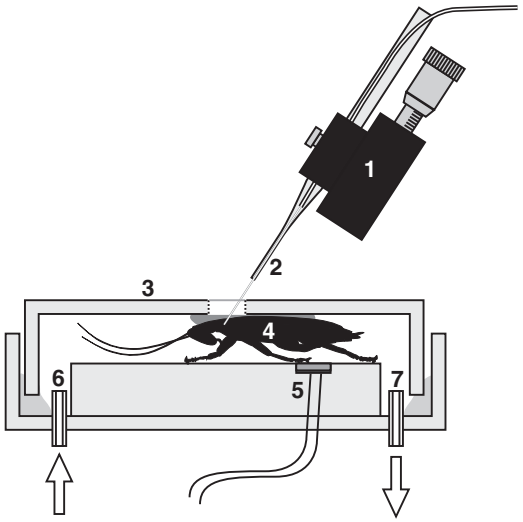

Fig. 1. Respirometry chamber for simultaneously measuring CO_2 release, haemolymph pH and abdominal ventilation frequency. 1, Micromanipulator; 2, pH optode; 3, Petri dish; 4, cockroach; 5, infrared activity detector; 6, air inlet; 7, air outlet.

Simultaneous measurement of haemolymph pH, CO_2 release and f_V was performed in a respirometry chamber constructed from a 50 mm i.d. × 12.5 mm deep polystyrene Petri dish (Fig. 1). The lid of the Petri dish was mounted upside down on an *x*/*y* rotational stage (XYR1/M, Thorlabs, Newton, NJ, USA) beneath a S216 dissecting microscope (Olympus Australia, Mt Waverley, VIC, Australia). A 2 mm i.d. tubing inlet and outlet were also mounted through the lid, allowing gas mixes to be flushed through the chamber to the CO_2 analyser. A 34×34×6 mm acrylic platform containing the activity detector was affixed within the Petri dish lid. To secure the cockroach within the chamber, a 6 mm hole was drilled through the base of the Petri dish, slightly off-centre. Investo dental modelling wax (Ainsworth Dental Company Pty Ltd) was melted around the hole. Cockroaches were cold-knocked-out by being placed within a –3°C freezer for 5 min. They were then affixed within the Petri dish using the melted dental wax around the hole, such that the left side of their pronotum was sealed over the hole. The Petri dish containing the cockroach was then inverted and pressed into a ring of Blu-Tack® modelling putty (Bostik, Thomastown, VIC, Australia) running around the inside edge of the Petri dish lid, thus sealing the chamber while orienting the cockroach's abdomen directly above the activity detector. To insert the pH optode into the cockroach's haemocoel, a 25 gauge needle was first used to cut a <0.3 mm diameter hole in the pronotum, slightly to the left of the insect's mid-line. As the main tracheae are visible through the insect's cuticle, they were carefully avoided during this procedure. A micromanipulator (World Precision Instruments, Sarasota, FL, USA) was then used to insert the pH optode through this opening ~1 mm into the cockroach's haemocoel. A small droplet of haemolymph would occasionally well up through the freshly made incision in the pronotum, but once the pH probe was inserted a clot formed rapidly between the optode and pronotum, preventing further bleeding. Haemolymph pH was measured every 10 s. Following implantation of the pH optode, the cockroach was given a minimum of 2 h to recover while the respirometry chamber was flushed with 21% O_2. After the 2 h recovery period, the insect was then exposed to hypoxia (5% O_2 for 10 min) and then returned to 21% O_2 for a further 1 h. Cockroaches were exposed to stepwise changes in O_2 (40, 30, 21, 15, 10 and 5% O_2 with CO_2 kept constant at 0%) and CO_2 (0.3, 0.6, 1, 2, 3 and 4% CO_2 with O_2 kept constant at 21%), with each exposure lasting 10 min and a minimum of 1 h between exposure to O_2 or CO_2 treatments.

The dissecting microscope, *x*/*y* rotational stage and micromanipulator were all mounted on a 300×450×12.7 mm M6 threaded aluminium breadboard (MB3045/M, Thorlabs) to ensure that they would not move during measurement. The Petri dish chamber was enclosed within a foam box that was ventilated with air passing through a radiator/computer fan assembly. The radiator was continuously flushed with water from an F12-ED constant temperature water bath (Julabo GmbH, Seelbach, Germany) set at 25°C, maintaining the temperature within the respirometry chamber at 25±0.5°C.

Voltage outputs from the pH-1 micro (pH and temperature), LiCOR-7000 (ΔCO_2 and ΔH_2O), SS3 (respirometry chamber incurrent flow rate) and activity detector were sampled at 10 Hz using a Powerlab 16/30 analogue to digital converter (ADInstruments, Bella Vista, NSW, Australia) and recorded on a desktop PC running LabChart 6.1.3 (ADInstruments).

Decapitated cockroach haemolymph pH measurement

The procedure for measuring haemolymph pH was followed as described above, with the exception that after the cockroach was cold-knocked-out and waxed to the bottom of the Petri dish, its head was quickly removed using a razor blade. The neck wound was then sealed with melted wax. Within 1 h of the pH probe being implanted, cockroaches would spontaneously display DGCs. Decapitated cockroaches prepared in this manner all produced DGCs and survived for several days. A minimum 2 h recovery period in 21% O_2 was allowed before manipulation of O_2 or CO_2.

Intratracheal O_2 measurement

Measurements of intratracheal O_2 were made on both intact and decapitated cockroaches. The cockroaches were measured while they were fixed upside down to a square piece of card 35×35 mm using dental modelling wax. They were then placed within a 36×36×25 mm respirometry chamber beneath a dissecting microscope. Intratracheal O_2 was measured using a 140 µm diameter fibre-optic oxygen optode connected to a TX3 O_2 meter (PreSens GmbH) and recorded at 1 Hz. For intratracheal O_2 measurements made on decapitated cockroaches, an unmodified optode with a 140 µm diameter tip was inserted through a small opening in the side of the respirometry chamber directly into one of the two severed major longitudinal tracheal trunks exposed in the neck wound (Fig. 2) using a three-axis micromanipulator (World Precision Instruments). The optode was then sealed into the tracheal trunk with wax. The opening in the side of the respirometry chamber was also sealed with wax, thus making it airtight. For measurements of intact cockroaches, the 140 µm diameter tip of the optode was slightly too large to be inserted through a spiracle directly into the cockroach's tracheal system. To make a tube small enough to cannulate a spiracle, the tapered end of a 10–200 µl polypropylene (PP) pipette tip was held over a soldering iron set at 160°C. Once the tip had melted into a droplet containing an air bubble, the molten plastic was quickly gripped with tweezers and pulled into a thread. This technique produced a continuous lumen within a tube 90–120 µm in diameter. A razor blade was used to trim a ~4 mm length of this PP micro-tube, with one end cut at 45 deg. This 4 mm PP micro-tube was then fixed within a ~2 mm length of 0.8 mm i.d., 1.6 mm o.d. polytetrafluoroethylene (PTFE) tube using cyanoacrylate adhesive (Supa glue, Selleys Pty Ltd, Padstow, NSW, Australia). The optode

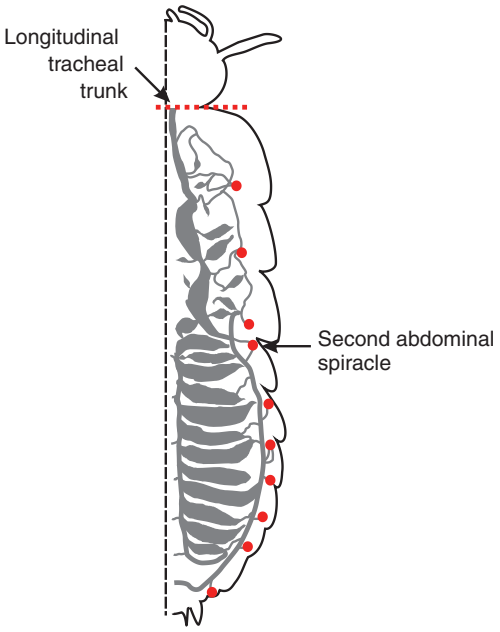

Fig. 2. Diagram of the cockroach tracheal system showing the location of intratracheal O_2 measurement. Red circles indicate spiracles. Optodes were implanted through the second abdominal spiracle in intact cockroaches or placed directly within the severed end of a longitudinal tracheal trunk in decapitated cockroaches. Diagram modified from Miller (Miller, 1981).

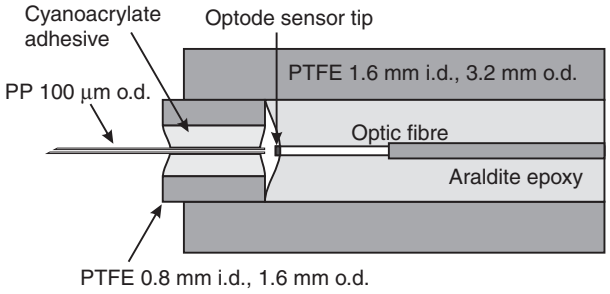

Fig. 3. Schematic of the modified O_2 optode used to measure intratracheal O_2. The 100 μm polypropylene (PP) tube was small enough to insert into the tracheal system through the second abdominal spiracle.

was mounted within a 15 mm length of PTFE tubing (1.6 mm i.d., 3.2 mm o.d., Cole-Parmer, Vernon Hills, IL, USA), such that the O_2-sensitive tip was inside the tubing ~1 mm from the end, while the tube behind the optode tip was filled with Araldite epoxy resin (Selleys Pty Ltd). The smaller PTFE tube containing the PP micro-tube was then fitted inside the larger PTFE tube containing the optode (Fig. 3). Thus, the PP and PTFE tube served as a replaceable tip that could be inserted through a cockroach spiracle, connecting the air space around the optode with the cockroach's tracheal system. Once the PP tube had been inserted in through the second abdominal spiracle (Fig. 2), high vacuum silicone grease (Dow Corning, Midland, MI, USA) was applied around the spiracle, creating an airtight seal around the tube within the spiracle, and between the two PTFE tubes. As the relatively low conductance of the PP micro-tube decreased the speed with which the optode responded to changes in O_2, the system's lag was characterised by placing the PP micro-tube and optode in a stream of pure N_2. The rate of O_2 decline was then recorded, allowing the time constant of the probe to be determined and an instantaneous correction applied to the data (Seymour et al., 1998). The time constant (τ; time taken to reach 63% of the final asymptotic value) of the bare optode was 5 s and the time constant for the PTFE tube/optode assemblies was 42±5 s. The instantaneous corrections applied to the optode data using a z-transform produced a mean τ of 13±2 s.

Data analysis

Rates of CO_2 production (ml h^{-1} g^{-1}) and f_V (cycles min^{-1}) were calculated with LabChart 6.1.3. Statistical analyses were performed using JMP® 8.0.2.2 (SAS Institute Inc., Cary, NC, USA). The effect of CO_2 and O_2 on f_V and pH was analysed using mixed-model ANOVA with individual cockroach identity included as a random effect, followed by Tukey's honestly significant difference (HSD) test. The inclusion of individual cockroach identity accounts for the non-independence of multiple measurements obtained from single individuals. This approach was preferred to repeated-measures ANOVA because in the present study data were not available for all individuals at all treatment levels.

RESULTS

Intact cockroaches

Ventilation frequency

Cockroaches were exposed to hypoxic, hyperoxic and hypercapnic atmospheres (N=10). Insects at rest in 21% O_2 and 0% CO_2 ventilated continuously with regularly spaced abdominal movements (Fig. 4) with a mean frequency of 5.8±1 cycles min^{-1} (N=10). Exposure to hypercapnia produced two distinct levels of ventilatory response depending on the level of CO_2. Abdominal ventilation frequency showed a small linear increase as the inspired level of CO_2 increased from 0 to 1%. This was observed in nine of the 10 cockroaches measured but was non-significant (f_V=5.0719×%CO_2+8.7343, R^2=0.83; expressed as percent change relative to f_V in 0% CO_2: Δf_V=0.5985×%CO_2–0.0232, R^2=0.98). Exposure to atmospheres containing more than 1% CO_2 was associated with a significant linear increase in ventilation frequency

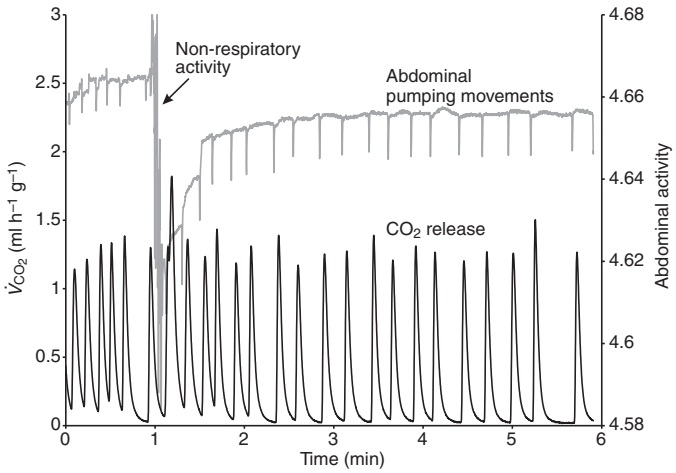

Fig. 4. Relationship between the rate of CO_2 release ($\dot{V}_{CO_2}$; black line) and abdominal pumping movements (arbitrary units; grey line) recorded from a 0.3805 g cockroach in an atmosphere containing 21% O_2 and 0% CO_2.

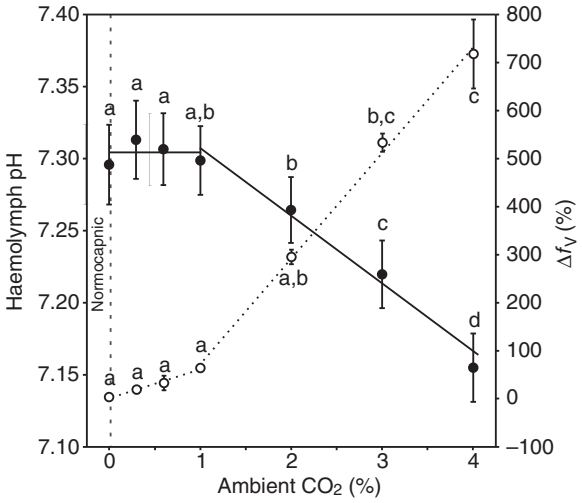

Fig. 5. Haemolymph pH (filled circles, solid lines) and percent change in abdominal ventilation frequency (Δf_V, % change relative to normocapnia; open circles, dotted lines) of cockroaches exposed to hypercapnic atmospheres containing 21% O_2. Points sharing the same letters are not significantly different (Tukey's HSD). Error bars indicate ± s.e.m. Dashed line indicates normocapnic level. Trend lines are least-squares linear regressions.

(f_V=11.849×%CO_2+3.252, R^2=0.99; Δf_V=2.1989×%CO_2–1.5088, R^2=0.997; Fig. 5).

Ventilation frequency showed a slight decrease with increasing O_2 levels in gas mixes containing between 10 and 40% O_2, but this was non-significant (f_V=–0.1188×%O_2+8.5955, R^2=0.91; Δf_V=–0.0209×%O_2+0.5767, R^2=0.74). Ventilation frequency in 5% O_2 was significantly higher than in all other O_2 treatments (Fig. 6) while CO_2 release was effectively continuous (Fig. 7). All cockroaches displayed a protracted apnoeic period upon return to normoxia following hyperventilation in 5% O_2 (Fig. 7).

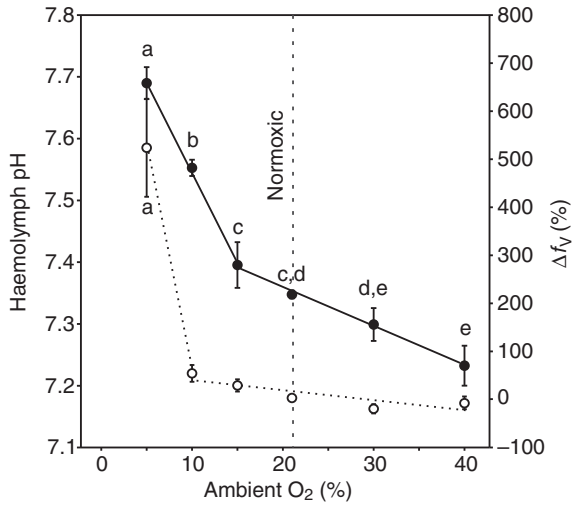

Fig. 6. Haemolymph pH (filled circles, solid lines) and percent change in abdominal ventilation frequency (Δf_V, % change relative to normoxia; open circles, dotted lines) of cockroaches exposed to hypoxic and hyperoxic atmospheres containing 0% CO_2. Points sharing the same letters are not significantly different (Tukey's HSD). Error bars indicate ± s.e.m. Dashed line indicates normoxic level. Trend lines are least-squares linear regressions.

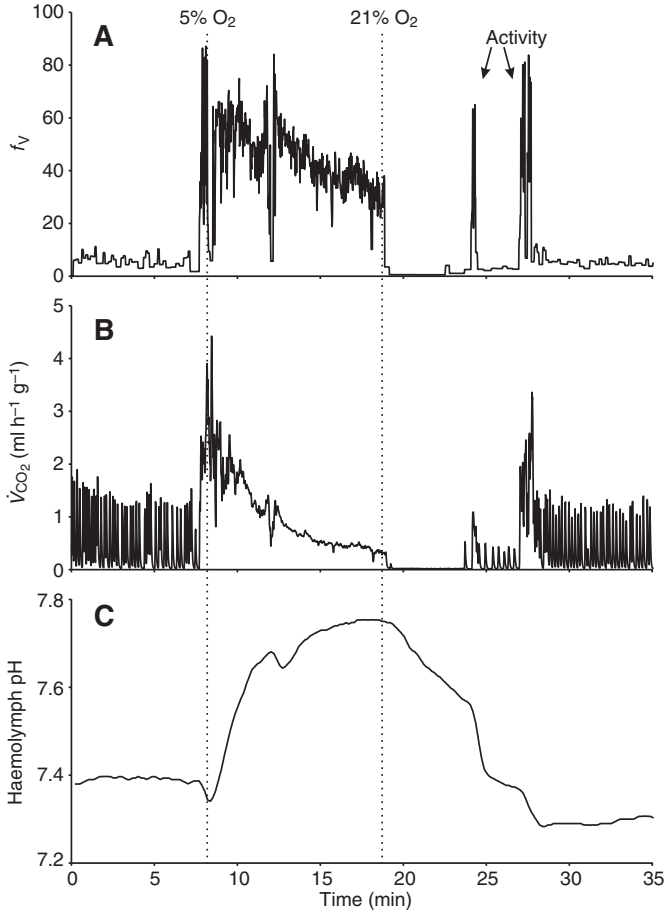

Fig. 7. A typical example of (A) abdominal ventilation frequency (f_V), (B) the rate of CO_2 release ($\dot{V}_{CO_2}$) and (C) haemolymph pH measured from a cockroach exposed to acute hypoxia (5% O_2) for 10 min (dashed lines indicate when the gas mix was switched from 21% O_2 to 5% and back to 21%). Hypoxia causes hyperventilation (A), increasing the rate at which CO_2 is released (B) and increasing haemolymph pH (C). Ventilation and CO_2 emission cease when normoxia is restored, only resuming once haemolymph pH has returned to pre-hypoxic levels.

Haemolymph pH

The cockroaches' haemolymph pH was 7.30±0.03 measured in normoxia (21% O_2 and 0% CO_2; N=10). Exposure to 0–1% CO_2 did not significantly alter haemolymph pH, whereas pH declined significantly in atmospheres containing higher than 2% CO_2 (Tukey's HSD, P=0.05; Fig. 5). Haemolymph pH showed a significant negative correlation with ambient %O_2 (P=0.05; Fig. 6). Hypoxia (5% O_2) caused haemolymph pH to increase by 0.34 units, whereas hyperoxia (40% O_2) resulted in a pH decrease of 0.12 units.

DGCs were displayed by three intact cockroaches in normoxia. In the 25 cycles measured, haemolymph pH declined steadily during the closed/flutter periods of the gas exchange cycle, before rising during the open phase as a burst of CO_2 was released. The mean change in pH was 0.07±<0.01 unit.

Intratracheal O_2

Implantation of the optode through an abdominal spiracle was achieved successfully with eight cockroaches. Measurements where silicone grease or haemolymph blocked the PP micro-tube connecting the optode to the tracheal system were easily

distinguished by the sudden onset of a flat O_2 trace, and were discarded. Intratracheal O_2 was measured under conditions of 21% (normoxia), 15% and 30% O_2. In normoxia mean intratracheal O_2 was 18.2±0.1% (N=8). The O_2 level fluctuated between ambient and ~15% O_2 following restricted gas exchange or an increase in activity (Fig. 8). Mean intratracheal O_2 was 25.8±0.2% (N=8) in an atmosphere containing 30% O_2. The level of O_2 in the tracheal system appeared to undergo larger fluctuations in hyperoxia, with restricted gas exchange occurring for longer periods. However, during periods of CO_2 release, intratracheal O_2 still reached ambient levels (Fig. 8). Cockroaches in 15% O_2 had a mean intrtracheal O_2 level of 12.8±0.1% (N=3).

Decapitated cockroaches

Ventilation frequency

All cockroaches (N=3) began breathing discontinuously within 1 h of decapitation. In normoxia and hyperoxia the discontinuous gas exchange cycles lacked a flutter phase, alternating between periods of no gas exchange and periods of active abdominal ventilation. The characteristics of the ventilation periods were remarkably consistent between all decapitated preparations. Gas exchange periods commenced with a high rate of ventilation (15±0.2 cycles min^{-1}), which gradually declined asymptotically to a lower rate (9±0.2 cycles min^{-1}), at which point gas exchange and ventilation would cease (Fig. 9). The ventilation periods tended to increase in duration as the preparation aged, increasing from ~5 min initially to 9 min over 24 h. Exposure to hypoxia or hyperoxia did not change the frequency of abdominal pumping during the periods of gas exchange, except in 5% O_2 where ventilation occurred continuously and more than doubled in frequency. However, hypoxia reduced the duration of the apnoeic periods between bouts of ventilation, as well as causing the appearance of a flutter phase. The flutter phase was characterised by several brief bursts of CO_2 preceding the ventilatory period. During the flutter period, the cockroach's abdomen would pump at a lower frequency than during the ventilatory phase (Fig. 10), but the activity trace shows a smaller voltage change during these pulsations, possibly indicating that the stroke volume of the abdomen was lower. When the cockroaches were returned to normoxia

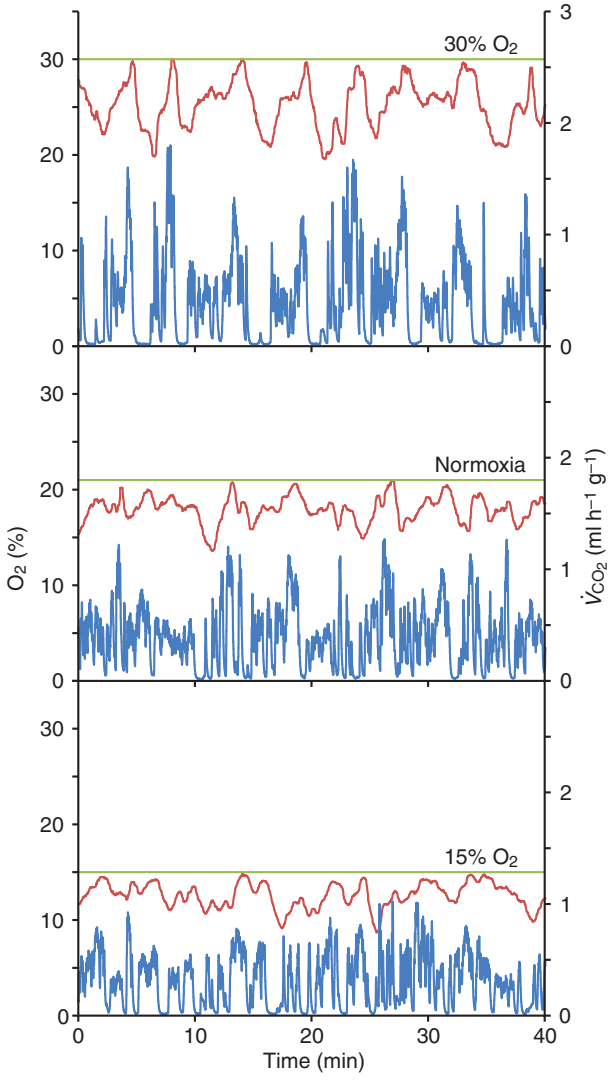

Fig. 8. Measurement of intratracheal % O_2 (red lines) and the rate of CO_2 release ($\dot{V}_{CO_2}$; blue lines) of a cockroach exposed to 30, 21 (normoxia) and 15% O_2 (green lines). The O_2 optode was inserted into the tracheal system through the second abdominal spiracle. Lag in the response time of the O_2 optode was corrected with a z-transformation (Seymour et al., 1998).

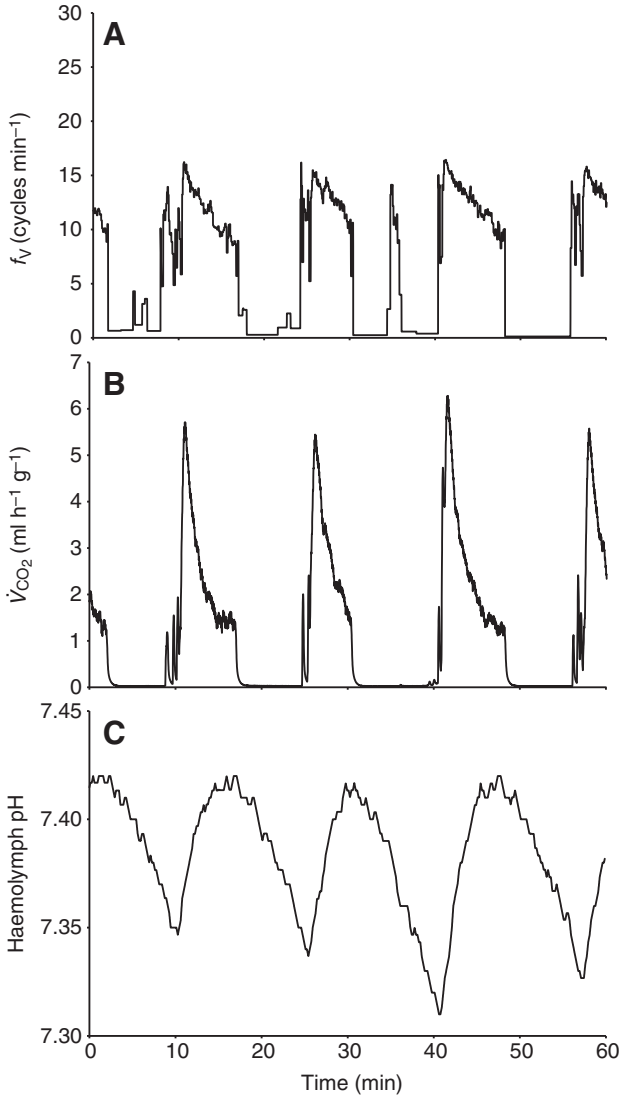

Fig. 9. Measurement of (A) abdominal ventilation frequency (f_V, cycles min^{-1}), (B) the rate of CO_2 release ($\dot{V}_{CO_2}$) and (C) haemolymph pH in a cockroach exposed to normoxia, 18 h after decapitation.

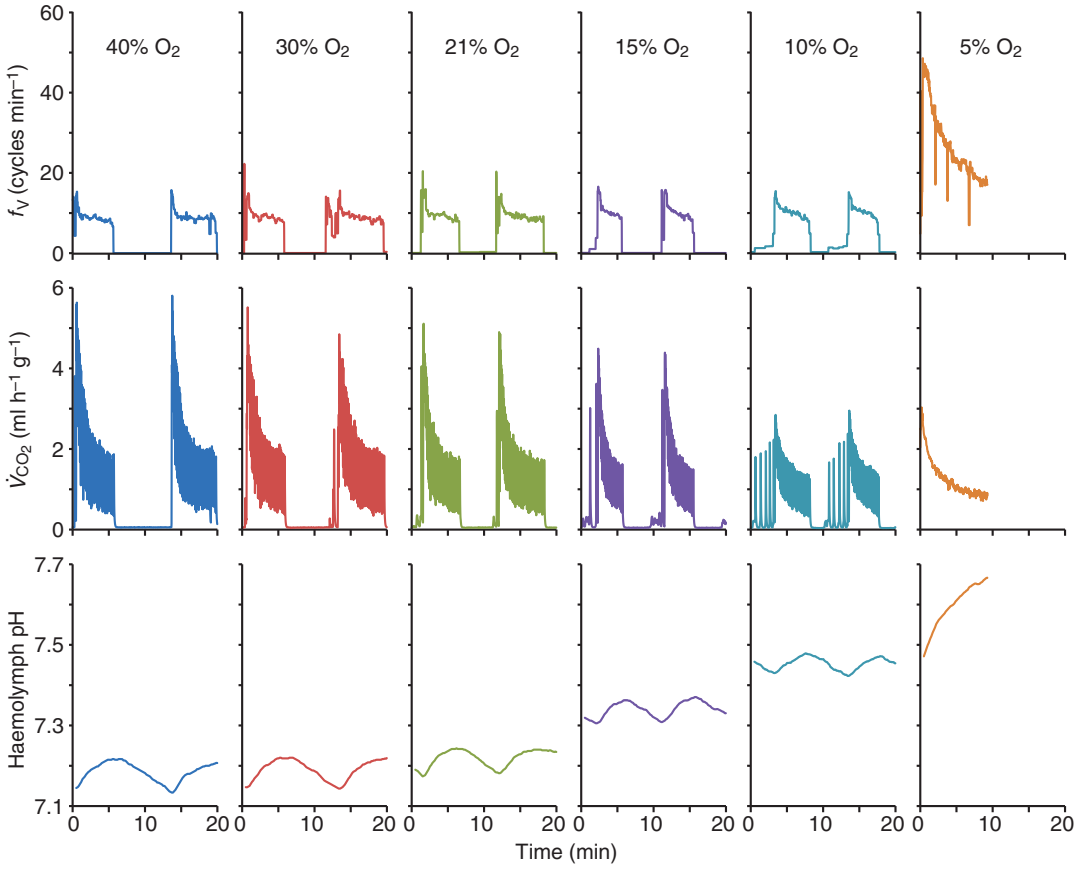

Fig. 10. Measurement of abdominal ventilation frequency (f_V, cycles min^{-1}), the rate of CO_2 release ($\dot{V}_{CO_2}$) and haemolymph pH in a decapitated cockroach exposed to 40, 30, 21, 15, 10 and 5% O_2. Data presented show the last 20 min of a 30 min exposure to the treatment gas. Decreasing O_2 levels below normoxia (21%) are coincident with the appearance of a flutter phase (short, low-frequency bursts of gas exchange preceding the main open phase) and a rise in haemolymph pH. The 5% O_2 shows the first 10 min of exposure and is not in equilibrium.

following continuous ventilation in 5% O_2, there was no extended apnoea. Instead, the periods of gas exchange continued as regularly as before, but with the volume of CO_2 released during each burst greatly reduced for the first two gas exchange periods.

Exposure to hypercapnia changed the characteristics of the ventilation periods, so that instead of a gradual decline in f_V over the course of a burst, ventilation would change in a U shape, beginning with 15 cycles min^{-1}, dropping to ~13 cycles min^{-1} and then rising back to 15 cycles min^{-1} at the end of the burst. Hypercapnia in excess of 4% CO_2 caused abdominal pumping movements to become continuous, but because of the high level of CO_2 it was impossible to determine from the respirometry trace whether gas exchange also became continuous. Upon return to normocapnia following hypercapnic exposure, the cockroach performed a protracted ventilatory period while pH returned to its normoxic, normocapnic level.

Haemolymph pH

A total of 82 DGCs were measured in normoxia. Alternating periods of no gas exchange and active ventilation caused haemolymph pH to fluctuate by 0.11±0.004 units (Figs 9 and 10). Exposure to 30% O_2 had no effect on either cycle duration or haemolymph pH; the duration of the closed phase increased in 40% O_2 (Fig. 10). Exposure to hypoxia caused pH to increase, coincident with the appearance of a flutter phase comprising low-frequency abdominal pumping movements and discrete bursts of CO_2 before the open phase (Fig. 10). Exposure to hypercapnia was associated with a drop in pH.

Intratracheal O_2

Decapitated cockroaches all displayed DGCs. During the periods of no gas exchange, intratracheal O_2 levels fell continuously, but rose rapidly to a plateau level during periods of ventilation (Fig. 11). Mean intratracheal O_2 in normoxia was 16.8%.

DISCUSSION

Ventilation frequency, hypercapnia, hypoxia and hyperoxia

Exposure to hypercapnic, hyperoxic and hypoxic atmospheres demonstrate that respiratory gas exchange in the cockroach is stimulated by O_2 and CO_2 in a manner comparable to most other air-breathing animals: both hypoxia and hypercapnia stimulate ventilation, but small increases in ambient CO_2 levels effect far greater increases in ventilation frequency than equivalent decreases in O_2 (Fig. 12). Thus, while O_2 stimulates ventilation only under conditions of moderate to extreme hypoxia (i.e. <10% O_2), low levels of hypercapnia (i.e. >1% CO_2) dramatically increase ventilation frequency (Figs 5 and 6). It can be seen that ventilation is regulated according to the accumulation of intratracheal CO_2, as the mean O_2 level within the tracheal system of an active cockroach remains close to 18% in normoxic air and intratracheal O_2 levels do not reach the hypoxic trigger point. These observations lead to the general conclusion that, under normal atmospheric conditions of 21% O_2

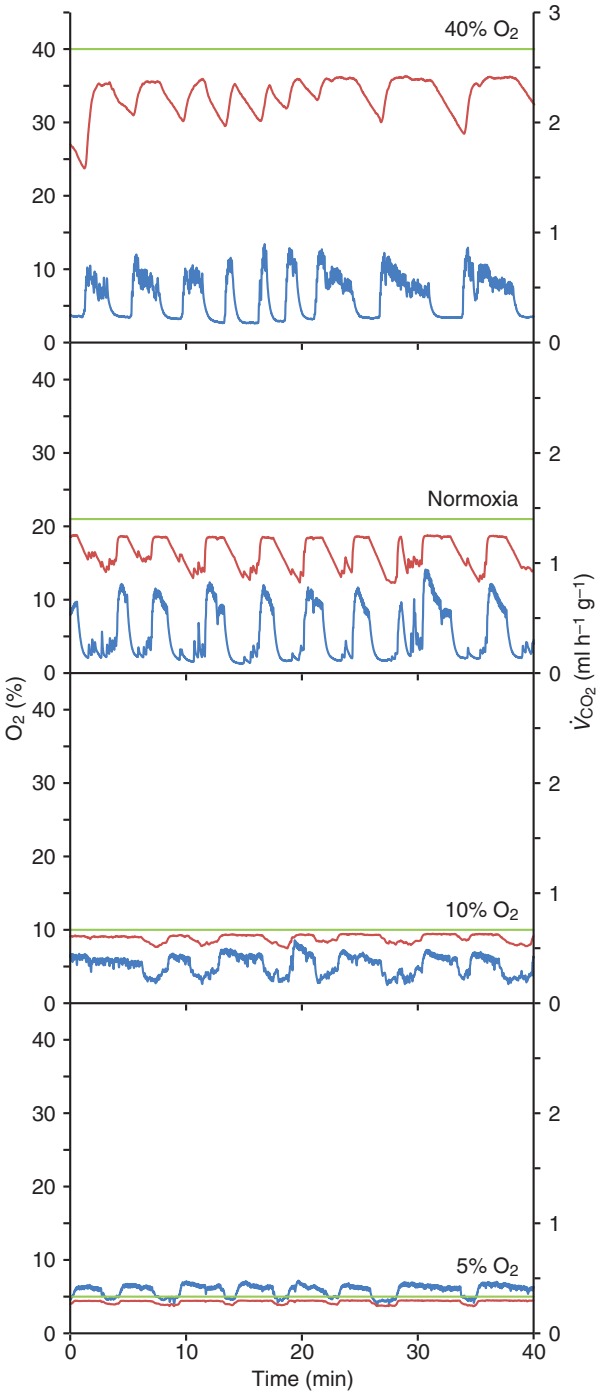

Fig. 11. Measurement of intratracheal % O_2 (red lines) and the rate of CO_2 release ($\dot{V}_{CO_2}$; blue lines) of a decapitated cockroach exposed to 40, 21 (normoxia), 10 and 5% O_2 (green lines). The O_2 optode was inserted into the tracheal system through the severed longitudinal tracheal trunks following removal of the head.

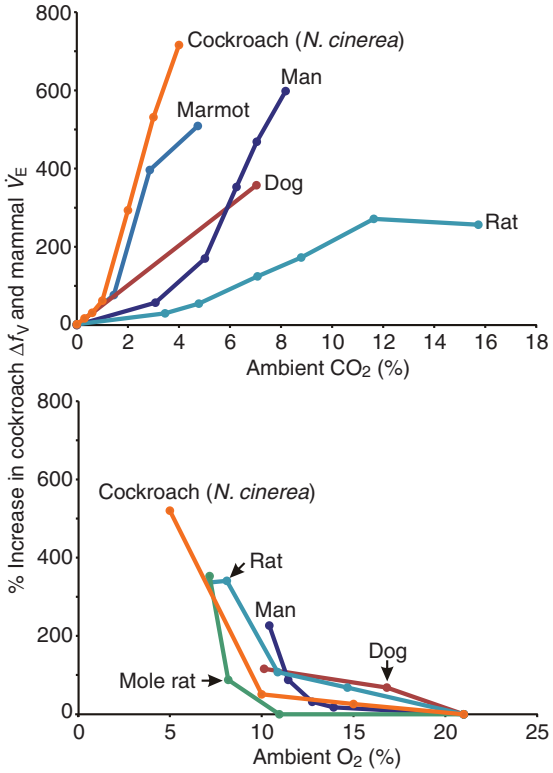

Fig. 12. Comparison between the percent increase in ventilation frequency (f_V) of the cockroach and the ventilation rate ($\dot{V}_E$) of a range of mammals exposed to varying levels of CO_2 and O_2 relative to normoxia and normocapnia. Mammal data modified from Arieli and Ar (1979).

and 0.03% CO_2, the regulation of gas exchange depends primarily on the level of CO_2 within the insect.

The role of CO_2 as the primary stimulus driving ventilation is also clearly apparent during exposure to hypoxia and hyperoxia. The cockroaches' ventilation proved to be insensitive to hyperoxia, with ambient O_2 levels of 30–40% causing no significant changes in abdominal pumping frequency compared with normoxia (Fig. 6). However, the significant reduction in haemolymph pH in atmospheres containing 40% O_2 requires comment. A previous study on moth pupae provides evidence that the CO_2 sensitivity of their spiracles is depressed by high intratracheal levels of O_2, elevating the CO_2 threshold that causes them to open (Schneiderman, 1960). This behaviour would explain the observed pH decrease, as a transient depression of ventilation frequency at the onset of hyperoxic exposure causes intratracheal CO_2 to increase until it once again stimulates spiracular opening. From then on, the cockroach resumes ventilation at the same frequency as before, but with higher intratracheal levels of O_2 and CO_2, as well as a lower haemolymph pH. The resulting surfeit of intratracheal O_2 is apparently of little consequence to these animals; a finding that is at odds with recent assertions that insects regulate their gas exchange specifically to maintain low intratracheal O_2 levels (Bradley, 2007). However, the results presented here show, perhaps not surprisingly, that O_2 influences gas exchange more through its absence than by its presence. As such, cockroaches respond to hypoxia by hyperventilating, increasing their ventilation frequency fivefold in atmospheres containing only 5% O_2 (Figs 6 and 7). While this behaviour is necessary to maintain adequate O_2 uptake in hypoxia, it has the unintended side effect of increasing the rate of CO_2 clearance above its rate of production, resulting in internal hypocapnia. The end result is that, upon their return to normoxia following hypoxic hyperventilation, cockroaches display an extended apnoeic period to correct their internal CO_2 deficit (Fig. 7). Although hypoxia alone increases ventilatory drive, compared with the effect of hypercapnia, it fails to stimulate ventilation to the same degree. For example, in 5% O_2, ventilation frequency increased fivefold above resting, whereas 4% CO_2 caused ventilation frequency to increase

sevenfold. It is possible that the stimulatory effect of hypoxia is counteracted in part by the decrease in internal CO_2 due to hyperventilation. To test this, cockroaches (N=4) were exposed simultaneously to hypoxia and hypercapnia (5% O_2 and 3% CO_2). Under these conditions, mean ventilation frequency was 54±2 cycles min^{-1}, compared with 32±15 cycles min^{-1} in 5% O_2 or 40±12 cycles min^{-1} in 3% CO_2. This experiment indicates that the effects of hypoxia and hypercapnia are synergistic, as together they drive greater increases in ventilation frequency than either could alone.

Exposing cockroaches to selected levels of O_2 and CO_2 demonstrates four critical points necessary for understanding insect gas exchange: (1) small increases in ambient CO_2 levels elicit greater increases in ventilation than equivalent decreases in O_2; (2) hypoxia stimulates ventilation whereas hyperoxia has no significant effect; (3) hypoxia stimulates ventilation independently, regardless of CO_2 levels; and (4) apnoeic periods occur when internal CO_2 levels fall below a threshold value and when internal O_2 levels are not so low as to stimulate ventilation. It is interesting to note that the conclusions presented here on cockroach ventilation are essentially the same as those of Haldane and Priestly in their classic paper examining lung ventilation in humans (Haldane and Priestley, 1905). Although the respiratory systems of mammals and cockroaches are separated by hundreds of millions of years of independent evolution, natural selection under the same physiochemical constraints associated with breathing air has caused them to converge on essentially identical mechanisms to regulate their gas exchange: ventilation is stimulated by internal CO_2 levels except when hypoxia demands hyperventilation.

Intratracheal CO_2 and haemolymph pH

The implantation of micro pH optodes within the cockroaches' haemolymph reveals that changes in ventilation frequency caused by hypoxia and hypercapnia have significant effects on haemolymph pH. In normoxia, cockroaches maintained a haemolymph pH of 7.30±0.03 (Figs 5 and 6). Breathing atmospheres containing up to 1% CO_2 did not cause this pH to change. Although there was a non-significant trend towards higher ventilation frequencies with increasing CO_2 (Fig. 5), an increase in ventilation frequency, amplitude or some combination of the two is the only plausible mechanism to explain a stable pH in the face of increasing hypercapnia. By increasing the convective ventilation of their tracheal system, cockroaches would maintain a constant $\dot{V}_{CO_2}$ in the face of a decreased P_{CO_2} gradient between their tissues and tracheal system, and thus maintain a stable haemolymph pH. However, in hypercapnic atmospheres containing more than 1% CO_2, ventilation rate increased significantly, while haemolymph pH dropped (Fig. 5). These changes in haemolymph pH and ventilation frequency in hypercapnia reveal that cockroaches maintain an intratracheal CO_2 level between ~1 and 2%. The significant decrease in haemolymph pH associated with a rapid increase in ventilation when breathing gas mixes containing 2% or more CO_2 demonstrates that this level of CO_2 exceeds that normally maintained within the cockroaches' tracheal system, such that no increase in ventilation can reduce their internal CO_2 back to their desired level. Further confirmation that intratracheal CO_2 levels are close to 2% in normoxia can be found in the experiment where cockroaches were forced to hyperventilate in hypoxic atmospheres (5% O_2) containing up to 4% CO_2 (Fig. 13). The loss of internal CO_2 due to excessive ventilation in hypoxia caused the cockroach's haemolymph pH to become alkaline relative to its usual normoxic level. However, this pH disturbance was fully reversed in an atmosphere containing 2% CO_2, suggesting that this is the usual intratracheal CO_2 level. An intratracheal CO_2 level regulated at ~2% is in very close agreement with ~1.5% measured for the grasshopper *Schistocirca americana* (Gulinson and Harrison, 1996). Similarly, the spiracles of the atlas moth *Attacus atlas* remain open when their tracheal system is perfused with 1.5% CO_2 (Förster and Hetz, 2009), and the spiracles of the flea *Xenopsylla cheopis* remain open in 2% CO_2 (Wigglesworth, 1935).

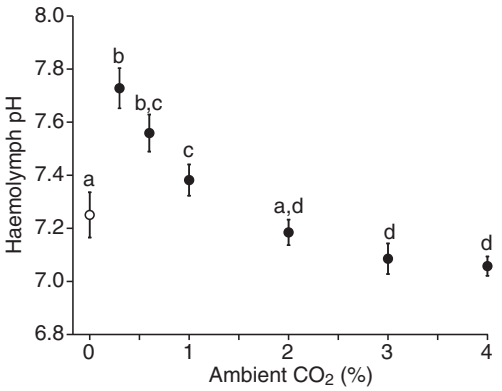

Fig. 13. Cockroach haemolymph measured *in situ* in normoxia (21% O_2, 0% CO_2; open circle) and hyperventilating in hypoxic, hypercapnic atmospheres containing 5% O_2 and 0.3, 0.6, 1, 2, 3 and 4% CO_2 (filled circles). Points sharing the same letters are not significantly different (Tukey's HSD). Error bars indicate ±s.e.m.

The internal regulation of a relatively constant internal CO_2 level is also apparent from experiments where cockroaches hyperventilated when exposed to severe hypoxia (5% O_2). Hyperventilation caused a rapid initial increase in $\dot{V}_{CO_2}$, which then declined asymptotically to a constant level, while haemolymph pH increased by 0.39±0.04 units (Fig. 6). Assuming that the constant $\dot{V}_{CO_2}$ approximates the cockroach's metabolic rate, then the increase in $\dot{V}_{CO_2}$ over and above this level must be due to the liberation of CO_2 stored in the cockroach's haemolymph. This assumption is supported by the rapid rise in haemolymph pH. Therefore, by integrating the area below the $\dot{V}_{CO_2}$ burst and using the stable $\dot{V}_{CO_2}$ as the baseline, it was estimated that the cockroaches' lost 0.071±0.01 ml of CO_2 in total from their haemolymph, or 0.146±0.02 ml g^{-1} body mass (Fig. 7). When normoxia was restored, cockroaches stopped ventilating almost immediately and closed their spiracles, preventing gas exchange. During this apnoeic period, haemolymph pH dropped steadily as internal levels of CO_2 increased. Once haemolymph pH approached or exceeded its pre-hypoxia level, ventilation and gas exchange would resume as before (Fig. 7). The respiratory alkalosis induced by hypoxic hyperventilation, and the subsequent apnoea, show that cockroaches maintain their haemolymph in a state of compensated respiratory acidosis. It also demonstrates that a constant pH and/or P_{CO_2} is defended by the regulation of gas exchange.

Patterns of gas exchange

Insects display a range of respiratory patterns by varying when they open their spiracles and when they generate convective airflow through their tracheal system. In this study, *N. cinerea* displayed two distinct patterns of gas exchange: a continuous pattern consisting of a train of small CO_2 'breaths' spaced at regular intervals (Fig. 4) and a DGC consisting of extended breath-hold periods punctuated by periods of active ventilation (Fig. 9). Cockroaches ordinarily display DGCs when they are quiescent, but in this study decapitation

was necessary to elicit this respiratory pattern consistently. The DGC has long been considered so unusual a gas exchange pattern that its occurrence demands special explanation. It was initially proposed that the long periods of spiracular closure typical of the DGC may have evolved to reduce respiratory water loss [the hygric hypothesis (Buck and Keister, 1955)]. More recently, other hypotheses have been put forward suggesting that DGCs may facilitate gas exchange in hypoxic or hypercapnic burrows [the chthonic hypothesis (Lighton and Berrigan, 1995)], or reduce intratracheal O_2 levels to protect against oxidative damage [the oxidative damage hypothesis (Bradley, 2000)]. While the hygric hypothesis is based on the assumption that respiratory water loss is lower during DGCs compared with other patterns of gas exchange, both the chthonic and oxidative damage hypotheses are based on assumptions regarding how insects regulate their gas exchange patterns in response to varying levels of atmospheric and intratracheal O_2 and CO_2. Therefore, the results of the present investigation can be used to test the validity of these assumptions.

The chthonic hypothesis is based on the assumption that an insect can facilitate gas exchange when exposed to ambient hypoxia or hypercapnia by employing the closed phase of the DGC to increase intratracheal hypoxia and hypercapnia above ambient levels, thereby maximising the partial pressure gradients driving O_2 and CO_2 between the insect and its environment when the spiracles eventually open (Lighton and Berrigan, 1995). However, it is apparent from experimental manipulation of ambient O_2 and CO_2 that a subterranean or chthonic origin of DGCs is unlikely. Rather than favouring the emergence of DGCs, both hypoxic and/or hypercapnic conditions stimulate hyperventilation, directly opposing the emergence of cyclic or periodic gas exchange patterns. Similar findings have been reported for beetles that switch from a discontinuous to continuous pattern of gas exchange when exposed to hypoxia (Chappell and Rogowitz, 2000; Chown and Holter, 2000), while the grasshopper *Taeniopoda eques* abandons DGCs in atmospheres containing more than 2% CO_2 (Harrison et al., 1995). Ambient hypoxia and hypercapnia have also been shown to prevent the occurrence of DGCs in *Samia cynthia* moth pupae (Terblanche et al., 2008). All of these examples demonstrate that insects possess essentially the same respiratory sensitivities to low O_2 (below approximately 10% O_2) and high CO_2 (above 2% CO_2) during DGCs as during continuous gas exchange. This illustrates the fundamental problem with the chthonic hypothesis, specifically that using periodic apnoea to enhance gas exchange when exposed to deleterious atmospheres requires the insect to suffer even greater intratracheal hypoxia and hypercapnia than if it simply abandoned the DGC altogether. A corollary of this issue is that for DGCs to have evolved in a subterranean atmosphere, insects must also possess a respiratory system capable of distinguishing whether hypoxia or hypercapnia is of ambient or intratracheal origin and then respond in one of two contradictory ways: ambient hypercapnia or hypoxia would need to inhibit gas exchange to produce the DGC, whereas high intratracheal CO_2 and low intratracheal O_2 produced by routine aerobic activities must promote gas exchange. This scenario is paradoxical. Following the above lines of reasoning, and the consistent responses of other insects exposed to hypoxia and hypercapnia, we conclude that the chthonic hypothesis cannot explain the origin or maintenance of DGCs in insects.

The oxidative damage hypothesis was proposed following the observation that moth pupae performing DGCs maintained a low, stable intratracheal O_2 level of ~4% for the duration of the flutter phase, irrespective of whether they were exposed to atmospheres containing 6 or 50% oxygen (Hetz and Bradley, 2005; Levy and Schneiderman, 1966). Because the DGC restricts O_2 entry to the tracheal system during both the closed and flutter phases, it is possible that this gas exchange pattern is a mechanism to limit the exposure of the insect's tissues to high levels of O_2, thereby protecting them from excessive oxidative damage. However, diapausing pupae are a highly modified insect life stage, and the mean intratracheal levels of O_2 and CO_2 regulated by a pupa may not be typical of most other active insect life stages. Until now, lack of data has prevented any comparison. Here we show that the mean intratracheal O_2 level is 18.2±0.1% measured in intact cockroaches, and 16.8% in the longitudinal tracheal trunks in decapitated preparations displaying DGCs. Even during the closed phase of a DGC, intratracheal O_2 never fell lower than 10% and rarely dropped below 13%. Cockroaches do not match the pattern observed in moth pupae: low and stable levels of intratracheal O_2, either during DGCs or when active, do not occur (Figs 8 and 9). Likewise, the duration of DGCs displayed by western lubber grasshoppers (*Taenepodia eques*) and American cockroaches (*Periplaneta americana*) were unaffected by hyperoxic atmospheres containing up to 40–60% O_2, indicating that, under these conditions, intratracheal O_2 levels never decreased to the hypoxic trigger point during the closed phase of the cycle (Harrison et al., 1995; Woodman et al., 2008). In these insects, too, stable and low intratracheal O_2 levels apparently do not occur. Measured levels of O_2 in the locusts' tracheal system [18.6±0.6% in a normoxic atmosphere (Gulinson and Harrison, 1996)] are also the same as those recorded from cockroaches in the present study. Given the exceptionally close agreement between these two intratracheal O_2 measurements made on insects belonging to different orders, it would appear that insects do not seek to avoid near-ambient levels of O_2 within their tracheal systems, contrary to the assumption that gas exchange patterns function to limit O_2 uptake (Bradley, 2007). However, it should be noted that O_2 levels within the insects' tissues are likely to be lower and more stable than those recorded within the tracheal system.

The current adaptive hypotheses cannot explain why DGCs have evolved among such a diverse range of insects. Therefore, explanations for the origin and maintenance of this pattern of gas exchange must be sought elsewhere. A non-teleological approach can be found in the mechanistic hypotheses that explain DGCs as an emergent property of the insect's respiratory system. These theories are based on the assumption that the DGC may not be adapted for any particular purpose; rather, it is a pattern that arises spontaneously due to interactions between the regulatory mechanisms controlling gas exchange, but only under particular circumstances (Chown et al., 2006; Chown and Holter, 2000). The one condition common to all insects displaying DGCs is that they are inactive, either as quiescent adult insects or diapausing pupae. This then begs the question: what happens when an insect becomes inactive that causes them to breathe periodically rather than continuously? One obvious change is that the insect's metabolic rate decreases at rest. However, if the same respiratory control mechanism responsible for producing individual breaths during continuous ventilation continued to operate when an insect became quiescent and its respiratory demand decreased, then individual breaths would still occur, but less frequently, as gas exchange would continue to be triggered by the ~2% CO_2 threshold. Instead, quiescent insects display DGCs that are characterised by large fluctuations in intratracheal CO_2, intratracheal O_2 and haemolymph pH due to rhythmically occurring apnoeas punctuated by bouts of vigorous gas exchange. This difference in stability is indicative of changes in the underlying respiratory control mechanism driving gas exchange. In the case of the decapitated cockroaches, this change

in regulatory control can be linked directly to the absence of a head and, consequently, the absence of the cephalic ganglion.

The spontaneous occurrence of DGCs in decapitated cockroaches, and their response to hypoxia and hypercapnia, reveals fundamental properties of this pattern. Firstly, it is apparent that decerebration unmasks a periodic gas exchange pattern that is hardwired into the cockroaches' ventilatory neural networks. Secondly, the occurrence of this pattern in a decapitated preparation indicates that pattern generators within the cockroaches' thoracic or abdominal ganglia generate this ventilatory periodicity. A central pacemaker located in the metathoracic ganglion has previously been identified from electrophysiological studies on the cockroach *P. americana* (Farley et al., 1967). This suggests that a tonic input from the cockroach's brain to the thoracic ganglia is necessary to produce a continuous pattern of breathing, whereas the absence or reduction of this input leads to discontinuous gas exchange. This situation is analogous to that found in vertebrates, where *in vitro* brainstem–spinal cord preparations spontaneously display periodic ventilatory activity, but only in the absence of a functional pons or midbrain (Fong et al., 2009). The nature of the input from the cephalic ganglion to the thoracic ganglia can be hypothesised from a third observation: the decapitated preparation does not respond to hypocapnia or hypercapnia, but does respond to hypoxia. This suggests that the CO_2 and/or pH chemoreceptors that normally modulate the ventilatory pattern are present in the cockroaches' head (see Miller, 1960a), whereas O_2 chemoreceptors are located in the thorax or abdomen. Thus, in hypoxia, the open and closed phases of the DGC continue to occur at regular intervals in the absence of an intratracheal hypercapnic trigger (as indicated by increasingly alkaline haemolymph; Fig. 10). The flutter phase is then superimposed on this basic pattern, emerging only if intratracheal O_2 falls below the hypoxic threshold before the spontaneous initiation of the next open phase (Fig. 10). This shows that the flutter phase is not an intrinsic component of the DGC, but an independent phenomenon that serves to correct intratracheal hypoxia.

Although there are clear differences between the DGCs displayed by intact and decapitated insects (i.e. sensitivity to CO_2), the decerebrated preparation is informative insofar as it shows that a periodic breathing pattern can occur spontaneously without requiring the presence of other mechanisms, such as chemosensory hysteresis. This finding is important for explaining the origin of the periodicity that characterises the DGC. It has long been known that the open and flutter phases of the DGC are triggered in response to changing intratracheal levels of O_2 and CO_2: the flutter phase is a graded response to intratracheal hypoxia whereas the open phase is a threshold all-or-nothing response induced by hypercapnia (Levy and Schneiderman, 1958). But precisely what determines the duration of the open phase, and what causes it to terminate, is a longstanding question that is fundamental for understanding the DGC, but which has thus far gone unanswered. Previously, models describing the behaviour of DGCs have been forced to assume a large temporal lag between changing intratracheal P_{CO_2} and the chemoreceptors stimulating gas exchange, thus driving the system to intratracheal hypocapnia during the open phase which is then followed by a corrective apnoeic period, i.e. the closed phase. But as of yet, no evidence has been put forward to demonstrate that this delay exists (Förster and Hetz, 2009). It has also been hypothesised that because the tracheal system can deliver O_2 at far higher rates than are required for oxidative metabolism at rest, the closed phase is initiated and maintained by a combination of high intratracheal O_2 and low intratracheal CO_2 so the spiracles remain closed to reduce internal O_2 to 'safe' levels (Bradley, 2007). However, this does not explain why DGCs occur, because low intratracheal CO_2 is sufficient to induce spiracle closure; the presence of high intratracheal O_2 during the open phase has no independent effect on the spiracles, except in the presence of low CO_2 (i.e. the spiracles will only close in the presence of both low CO_2 and high O_2). This being the case, gas exchange should continue with discrete breaths, not periodic bursts, each breath causing intratracheal CO_2 to drop below the hypercapnic threshold level while intratracheal O_2 remains high.

There is reason to believe that the spontaneous ventilatory periods observed in the decapitated cockroaches are the fundamental pattern underlying the emergence of the DGC. These gas exchange cycles appear to be an intrinsic property of thoracic central pattern generators that rhythmically produce ventilatory bursts of regular duration and frequency (e.g. Figs 10 and 11). Assuming an intrinsic ventilation pattern of fixed duration explains the tendency for insects to display burst phases of consistent duration during DGCs while their interburst duration may vary in response to respiratory conditions (i.e. Lighton and Garrigan, 1995; Woodman et al., 2008). However, while the ventilatory periods appear to begin and end spontaneously in decerebrated cockroaches, in intact insects it is generally accepted that the open phase is initiated when intratracheal CO_2 levels cross a hypercapnic threshold (Harrison et al., 1995; Levy and Schneiderman, 1966). It is plausible that crossing this threshold initiates the all-or-nothing response of the thoracic central pattern generators and a protracted ventilatory period of fixed duration then ensues. The long duration of this burst would be sufficient to produce intratracheal hypocapnia, increasing the time required for intratracheal CO_2 to accumulate to the hypercapnic threshold, resulting in a corrective apnoea and delaying the occurrence of the next ventilatory period. This would produce the basic periodicity of the DGC. In insects with high spiracular conductance and low respiratory demand, this automatic ventilatory burst is more than sufficient to remove accumulated CO_2 while increasing intratracheal O_2 to near-ambient levels (e.g. Figs 9 and 10). However, experimental evidence shows that the situation described above is overly simplistic. In intact insects, a multitude of afferent inputs must further modify the basic bursting pattern depending on the insect's respiratory demands and ambient conditions. For example, progressively blocking a moth pupa's spiracles gradually lengthens the duration of its open phase (Hetz, 2007; Moerbitz and Hetz, 2010), indicating that the ventilatory burst may be extended. Clearly, a more complete understanding of how and why DGCs occur could be gained from investigating the intrinsic and extrinsic factors that affect the thoracic central pattern generators.

CONCLUSIONS

This study reveals that cockroaches control their gas exchange to regulate intratracheal levels of CO_2 and O_2 in manner comparable to most other air-breathing animals. Under normal conditions, ventilation frequency is varied to maintain a constant internal CO_2 level and haemolymph pH. Intratracheal P_{O_2} is not regulated so long as it remains above a hypoxic threshold. This control is abandoned and DGCs emerge when regulation of gas exchange switches from the insect's brain to central pattern generators in the thoracic and abdominal ganglia. The experiments presented in this paper all point toward DGCs being symptomatic of reduced or absent brain activity (Matthews and White, 2011), unmasking a central pattern generator that is further modified by afferent inputs.

ACKNOWLEDGEMENTS

This research was supported by the Australian Research Council (projects DP0879605 and DP0987626). Our thanks go to the anonymous reviewers whose comments improved this paper; R. S. Seymour, who read an earlier version of this paper; and S. K. Hetz, for providing advice on constructing the movement detector circuit.

REFERENCES

Arieli, R. and Ar, A. (1979). Ventilation of a fossorial mammal (*Spalax ehrenbergi*) in hypoxic and hypercapnic conditions *J. Appl. Physiol.* **47**, 1011-1017.
Barnhart, M. C. (1992). Acid-base regulation in pulmonate molluscs. *J. Exp. Zool.* **263**, 120-126.
Bradley, T. J. (2000). The discontinuous gas exchange cycle in insects may serve to reduce oxygen supply to the tissues. *Am. Zool.* **40**, 952.
Bradley, T. J. (2007). Control of the respiratory pattern in insects. In *Hypoxia And The Circulation*, Vol. 618 (ed. R. C. Roach, P. D. Wagner and P. H. Hackett), pp. 211-220. New York: Springer.
Buck, J. and Keister, M. (1955). Cyclic CO_2 release in diapausing *Agapema* pupae. *Biol. Bull.* **109**, 144-163.
Buck, J. and Keister, M. (1958). Cyclic CO_2 release in diapausing pupae – II: tracheal anatomy, volume and pCO_2; blood volume; interburst CO_2 release rate. *J. Insect Physiol.* **1**, 327-340.
Burggren, W. W. (1992). Respiration and circulation in land crabs: novel variations on the marine design. *Am. Zool.* **32**, 417-427.
Case, J. F. (1957). Differentiation of the effects of pH and CO_2 on spiracular function of insects. *J. Cell. Comp. Physiol.* **49**, 103-113.
Case, J. F. (1961). Effects of acids on an isolated insect respiratory center. *Biol. Bull* **121**, 385.
Chappell, M. A. and Rogowitz, G. L. (2000). Mass, temperature and metabolic effects on discontinuous gas exchange cycles in eucalyptus-boring beetles (Coleoptera: Cerambycidae). *J. Exp. Biol.* **203**, 3809-3820.
Chown, S. L. and Holter, P. (2000). Discontinuous gas exchange cycles in *Aphodius fossor* (Scarabaeidae): a test of hypotheses concerning origins and mechanisms. *J. Exp. Biol.* **203**, 397-403.
Chown, S. L., Gibbs, A. G., Hetz, S. K., Klok, C. J., Lighton, J. R. B. and Marais, E. (2006). Discontinuous gas exchange in insects: a clarification of hypotheses and approaches. *Physiol. Biochem. Zool.* **79**, 333-343.
Dejours, P. (1988). From comparative physiology of respiration to several problems of environmental adaptations and to evolution. *J. Physiol.* **410**, 1-19.
Farley, R. D., Case, J. F. and Roeder, K. D. (1967). Pacemaker for tracheal ventilation in cockroach *Periplaneta americana* (L). *J. Insect Physiol.* **13**, 1713-1728.
Fong, A. Y., Zimmer, M. B. and Milsom, W. K. (2009). The conditional nature of the "Central Rhythm Generator" and the production of episodic breathing. *Respir. Physiol. Neurobiol.* **168**, 179-187.
Förster, T. and Hetz, S. K. (2009). Spiracle activity in moth pupae – the role of oxygen and carbon dioxide revisited. *J. Insect Physiol.* **56**, 492-501.
Gulinson, S. L. and Harrison, J. F. (1996). Control of resting ventilation rate in grasshoppers. *J. Exp. Biol.* **199**, 379-389.
Haldane, J. S. and Priestley, J. G. (1905). The regulation of the lung-ventilation. *J. Physiol.* **32**, 225-266.
Harrison, J. F. (2001). Insect acid-base physiology. *Annu. Rev. Entomol.* **46**, 221-250.
Harrison, J. F., Hadley, N. F. and Quinlan, M. C. (1995). Acid-base status and spiracular control during discontinuous ventilation in grasshoppers. *J. Exp. Biol.* **198**, 1755-1763.
Harrison, J. M. (1989). Temperature effects on intra- and extracellular acid-base status in the American locust, *Schistocerca nitens*. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **158**, 763-770.
Hetz, S. K. (2007). The role of the spiracles in gas exchange during development of *Samia cynthia* (Lepidoptera, Saturniidae). *Comp. Biochem. Physiol.* **148A**, 743-754.
Hetz, S. K. and Bradley, T. J. (2005). Insects breathe discontinuously to avoid oxygen toxicity. *Nature* **433**, 516-519.
Hetz, S. K. and Wasserthal, L. T. (1993). Miniaturized pH-sensitive glass-electrodes for the continuous recording of hemolymph pH in resting butterfly pupae. *Verh. Dtsch. Zool. Ges.* **86**, 92.
Hetz, S. K., Wasserthal, L. T., Hermann, S., Kaden, H. and Oelßner, W. (1993). Direct oxygen measurements in the tracheal system of lepidopterous pupae using miniaturized amperometric sensors. *Bioelectrochem. Bioenerg.* **33**, 165-170.
Kitchel, R. L. and Hoskins, W. M. (1935). Respiratory ventilation in the cockroach in air, in carbon dioxide and in nicotine atmospheres. *J. Econ. Entomol.* **28**, 924-933.
Krolikowski, K. and Harrison, J. (1996). Haemolymph acid-base status, tracheal gas levels and the control of post-exercise ventilation rate in grasshoppers. *J. Exp. Biol.* **199**, 391-399.
Levy, R. I. and Schneiderman, H. A. (1958). Experimental solution to the paradox of discontinuous respiration in insects. *Nature* **182**, 491-493.
Levy, R. I. and Schneiderman, H. A. (1966). Discontinuous respiration in insects-II. Direct measurement and significance of changes in tracheal gas composition during respiratory cycle of silkworm pupae. *J. Insect Physiol.* **12**, 83-104.
Lighton, J. R. B. and Berrigan, D. (1995). Questioning paradigms: caste-specific ventilation in harvester ants, *Messor pergandei* and *M. julianus* (Hymenoptera: Formicidae). *J. Exp. Biol.* **198**, 521-530.
Lighton, J. R. B. and Garrigan, D. (1995). Ant breathing: testing regulation and mechanism hypotheses with hypoxia. *J. Exp. Biol.* **198**, 1613-1620.
Marais, E., Klok, C. J., Terblanche, J. S. and Chown, S. L. (2005). Insect gas exchange patterns: a phylogenetic perspective. *J. Exp. Biol.* **208**, 4495-4507.
Matthews, P. G. D. and White, C. R. (2011). Discontinuous gas exchange in insects: is it all in their heads? *Am. Nat.* **177**, 130-134.
Miller, P. L. (1960a). Respiration in the desert locust: I. The control of ventilation. *J. Exp. Biol.* **37**, 224-236.
Miller, P. L. (1960b). Respiration in the desert locust: II. The control of the spiracles. *J. Exp. Biol.* **37**, 237-263.
Miller, P. L. (1981). Respiration. In *The American Cockroach* (ed. W. J. Bell and K. G. Adiyodi), pp. 87-116. London: Chapman and Hall.
Moerbitz, C. and Hetz, S. K. (2010). Tradeoffs between metabolic rate and spiracular conductance in discontinuous gas exchange of *Samia cynthia* (Lepidoptera, Saturniidae). *J. Insect Physiol.* **56**, 536-542.
Reeves, R. B. (1977). The interaction of body temperature and acid-base balance in ectothermic vertebrates. *Annu. Rev. Physiol.* **39**, 559-586.
Schneiderman, H. A. (1960). Discontinuous respiration in insects: role of the spiracles. *Biol. Bull.* **119**, 494-528.
Seymour, R. S., Withers, P. C. and Weathers, W. W. (1998). Energetics of burrowing, running, and free-living in the Namib Desert golden mole (*Eremitalpa namibensis*). *J. Zool.* **244**, 107-117.
Snyder, G. K., Ungerman, G. and Breed, M. (1980). Effects of hypoxia, hypercapnia, and pH on ventilation rate in *Nauphoeta cinerea*. *J. Insect Physiol.* **26**, 699-702.
Terblanche, J. S., Marais, E., Hetz, S. K. and Chown, S. L. (2008). Control of discontinuous gas exchange in *Samia cynthia*: effects of atmospheric oxygen, carbon dioxide and moisture. *J. Exp. Biol.* **211**, 3272-3280.
Wigglesworth, V. B. (1935). The regulation of respiration in the flea, *Xenopsylla cheopis*, Roths. (Pulicidae). *Proc. R. Soc. Lond. B* **118**, 397-419.
Woodman, J. D., Cooper, P. D. and Haritos, V. S. (2008). Neural regulation of discontinuous gas exchange in *Periplaneta americana*. *J. Insect Physiol.* **54**, 472-480.