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RESEARCH ARTICLE

Discontinuous gas exchange in Madagascar hissing cockroaches is not a consequence of hysteresis around a fixed P_{CO_2} threshold

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ABSTRACT

It has been hypothesised that insects display discontinuous gas-exchange cycles (DGCs) as a result of hysteresis in their ventilatory control, where CO_2-sensitive respiratory chemoreceptors respond to changes in haemolymph P_{CO_2} only after some delay. If correct, DGCs would be a manifestation of an unstable feedback loop between chemoreceptors and ventilation, causing P_{CO_2} to oscillate around some fixed threshold value: P_{CO_2} above this ventilatory threshold would stimulate excessive hyperventilation, driving P_{CO_2} below the threshold and causing a subsequent apnoea. This hypothesis was tested by implanting micro-optodes into the haemocoel of Madagascar hissing cockroaches and measuring haemolymph P_{O_2} and P_{CO_2} simultaneously during continuous and discontinuous gas exchange. The mean haemolymph P_{CO_2} of 1.9 kPa measured during continuous gas exchange was assumed to represent the threshold level stimulating ventilation, and this was compared with P_{CO_2} levels recorded during DGCs elicited by decapitation. Cockroaches were also exposed to hypoxic (P_{O_2} 10 kPa) and hypercapnic (P_{CO_2} 2 kPa) gas mixtures to manipulate haemolymph P_{O_2} and P_{CO_2}. Decapitated cockroaches maintained DGCs even when their haemolymph P_{CO_2} was forced above or below the putative ∼2 kPa ventilation threshold, demonstrating that the characteristic oscillation between apnoea and gas exchange is not driven by a lag between changing haemolymph P_{CO_2} and a P_{CO_2} chemoreceptor with a fixed ventilatory threshold. However, it was observed that the gas exchange periods within the DGC were altered to enhance O_2 uptake and CO_2 release during hypoxia and hypercapnia exposure. This indicates that while respiratory chemoreceptors do modulate ventilatory activity in response to haemolymph gas levels, their role in initiating or terminating the gas exchange periods within the DGC remains unclear.

KEY WORDS: Blood gas, *In vivo*, Optode, Episodic gas exchange

INTRODUCTION

Insect ventilation is generally understood to be driven by respiratory chemoreceptors that respond to internal oxygen and carbon dioxide partial pressure (P_{O_2} and P_{CO_2}, respectively) by stimulating appropriate ventilatory responses when either gas deviates from some defended threshold level. Thus, insects exposed to hypoxia and/or hypercapnia respond by opening their spiracles (Case, 1956; Förster and Hetz, 2010; Wigglesworth, 1935) and increasing ventilation frequency (Bustami et al., 2002; Harrison et al., 2006; Henderson et al., 1998; Matthews and White, 2011). While this explains changes in ventilation frequency when gas exchange is continuous, whether these same chemoreceptor responses also drive episodic patterns of gas exchange is not well understood.

Many insects display a pattern of intermittent breathing called a discontinuous gas exchange cycle (DGC) where periods of gas exchange alternate with periods of apnoea, and internal P_{O_2} and P_{CO_2} fluctuate significantly as a consequence (Förster and Hetz, 2010; Harrison et al., 1995). Stereotypical DGCs consist of three repeating phases: the closed (C), flutter (F), and open/ventilation (O or V) phase (Lighton, 1996). During the C-phase, spiracles are closed and both O_2 uptake and CO_2 release are negligible, causing P_{O_2} to fall and P_{CO_2} to rise (Buck and Keister, 1955). The C-phase then transitions into a F-phase, where spiracles begin to open and close rapidly but sporadically. During the F-phase, the rate of O_2 uptake is just sufficient to satisfy the insect's aerobic demands so tracheal P_{O_2} remains low and relatively constant (Lighton, 1996), whereas internal P_{CO_2} continues to increase (Levy and Schneiderman, 1966). Finally, the F-phase transitions to the O-phase when the spiracles open and the accumulated CO_2 is released in a large burst and O_2 is taken up rapidly. This O-phase generally coincides with a period of vigorous abdominal ventilatory movements. During DGCs, these three phases repeat in this order indefinitely.

Fundamental questions remain about the mechanisms responsible for producing DGCs. Specifically, assuming that insects regulate their gas exchange to control internal P_{O_2} and P_{CO_2} at some desired level, what happens during DGCs to cause these gas levels to decouple from these putative threshold levels? Previous research on diapausing cecropia moth pupae (*Hyalophora cecropia*) found that the transitions between closed, flutter and open spiracle states occurred when the pupa's tracheal P_{O_2} and P_{CO_2} crossed hypoxic and hypercapnic thresholds (Burkett and Schneiderman, 1974; Förster and Hetz, 2010). These pupae opened their spiracles once tracheal P_{CO_2} rose above a threshold level of ∼1–1.5 kPa, or when tracheal P_{O_2} dropped below a mean threshold level of ∼2.6 kPa; the actual P_{O_2} threshold was positively dependent on P_{CO_2}, with hypercapnia causing the spiracles to open at a higher P_{O_2}. Spiracle fluttering was observed only occasionally and was assumed to occur when tracheal P_{O_2} fluctuated around the spiracle-opening P_{O_2} threshold. From these experiments, Förster and Hetz (2010) outlined a simple model whereby DGCs arise as tracheal P_{O_2} and P_{CO_2} fluctuate around these fixed hypoxic and hypercapnic thresholds (referred to as the fixed-threshold model: Fig. 1).

These moth pupae data show that internal P_{O_2} and P_{CO_2} thresholds can explain the transitions in spiracle state from the C- to the F-phase, and from the F- to the O-phase, during DGCs: high internal P_{O_2} and low P_{CO_2} result in closed spiracles, and the reverse conditions result in open spiracles. Crucially, though, it cannot explain why so much CO_2 is expelled during the O-phase that internal P_{CO_2} falls below the P_{CO_2} threshold, resulting in a

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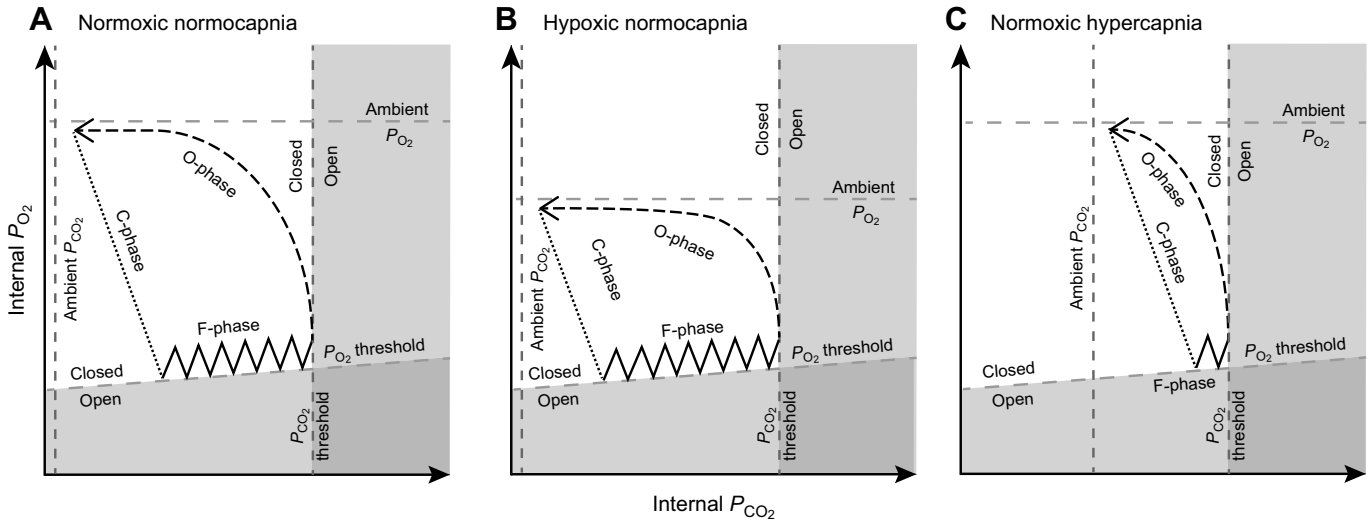

Fig. 1. Fixed-threshold model of the discontinuous gas exchange cycle (DGC) whereby gas exchange occurs when internal P_{O_2} falls below, or P_{CO_2} exceeds, a threshold value that triggers spiracle opening. (A) Behaviour of the insect's spiracles in a normoxic normocapnic atmosphere. (B,C) Predicted effect of moderate hypoxia and hypercapnia, respectively. Horizontal light-grey dashed lines indicate ambient P_{O_2} (top) and the P_{O_2} threshold that triggers the flutter (F)-phase (bottom), while vertical dark-grey dashed lines indicate ambient P_{CO_2} (left) and the P_{CO_2} threshold triggering the open (O)-phase (right). Thus, spiracles open when the insect's internal P_{CO_2} or P_{O_2} fall within the shaded regions and close when they fall in the white region, except during the O-phase, where spiracles remain open and closure is delayed, allowing internal P_{CO_2} to fall below the P_{CO_2} threshold. The black solid/dashed line running counter-clockwise indicates the P_{O_2} and P_{CO_2} within the insect during a complete DGC, with C-, F- and open (O-) phases indicated. Modified from Förster and Hetz (2010).

subsequent C-phase. Indeed, the quantity of CO_2 exhaled during the O-phase can be as much as 90% of the CO_2 accumulated in each cycle (Harrison et al., 1995; Levy and Schneiderman, 1966). A delayed response to changing internal P_{CO_2} has been proposed as one way to explain this behaviour, and thus explain the emergence of DGCs.

Control theory describes how a temporal lag between an animal's changing internal P_{CO_2} level and its detection by a chemoreceptor controlling the ventilatory response can drive the respiratory system into sustained oscillation. This begins with an initial respiratory disturbance that pushes the internal P_{CO_2} above the spiracle-opening threshold level. This causes the spiracles to open (O-phase) and the accumulated CO_2 to be released from the insect's haemolymph and tracheal system into the environment. If a substantial lag existed between the rapidly changing P_{CO_2} within the haemocoel and the P_{CO_2} being detected by the chemoreceptor, then spiracle closure would occur only after haemocoel P_{CO_2} had dropped below the spiracle-opening threshold. This would lead to a protracted corrective apnoea (C-phase), during which internal P_{CO_2} would again rise above the threshold level and the cycle would repeat. For the fixed-threshold model, the expulsion of excess CO_2 during the O-phase is necessary to produce a subsequent C-phase, and currently the origin of this overshoot is assumed to be the result of hysteresis, either due to a temporal lag between the chemoreceptor and haemolymph or somehow built into the chemoreceptor itself, e.g. by incorporating two P_{CO_2} thresholds: a high P_{CO_2} 'open' threshold and a lower P_{CO_2} 'close' threshold (Förster and Hetz, 2010). The critical importance of hysteresis in generating DGC patterns has also been borne out in more complex fixed-threshold models of insect ventilatory control, where including a delay in the response of one of two chemoreceptor feedback loops was found to be essential for the models to produce realistic DGC-like behaviour (Grieshaber and Terblanche, 2015). However, regardless of how the lag is generated, the existence of a hysteresis between CO_2 detection and the spiracle/ventilation response is currently hypothetical. Likewise, the role of active ventilation during the O-phase (as is the normal condition for most non-pupal insects) has never been considered in these models. As such, the accuracy of this fixed-threshold model has not been validated experimentally for either insects or insect pupae, and all relevant physiological parameters have never been measured simultaneously *in vivo*.

This study used Madagascar hissing cockroaches (*Gromphadorhina portentosa*) (Schaum 1853) to determine: (a) how haemolymph P_{O_2} and P_{CO_2} co-vary during continuous gas exchange and DGCs, (b) whether these levels oscillate around a fixed P_{CO_2} ventilatory threshold during DGCs, and (c) how challenging the insect with ambient hypoxia and hypercapnia alters haemolymph P_{O_2}, P_{CO_2} and the gas exchange pattern adopted by the insect. Miniaturised fibre optic P_{O_2} and P_{CO_2} optodes were implanted simultaneously into the haemocoel of these large insects to measure these parameters *in vivo*. Abdominal ventilation frequency and rate of CO_2 production ($\dot{V}_{CO_2}$) were also measured to quantify gas exchange patterns and metabolic rate, respectively. Continuous gas exchange was recorded from intact individuals, but following these measurements, decapitation was used to elicit DGCs. While decapitation is clearly an artificial way to elicit a DGC, the gas exchange pattern produced displays the same C/F/O phases that occur when this pattern is displayed spontaneously by the intact insect. It may, therefore, be safely assumed that DGCs displayed by decapitated individuals are driven by the same underlying ventilatory control system that generates DGCs in intact individuals. This is more plausible than positing the existence of a second redundant DGC control system that replicates the output of the DGC using unrelated mechanisms. As such, these intact and decapitated DGCs must be manifestations of the same control system, regardless of how the pattern was elicited, so examining how DGCs displayed by decapitated cockroaches respond to hypoxic and hypercapnic challenges should reveal how these respiratory gases are involved in the production of this gas exchange pattern.

The ultimate aim of this research was to determine the typical haemolymph gas parameters of these insects during continuous and

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discontinuous gas exchange, then use this information to test the hypothesis that DGCs arise as a natural consequence of a delayed ventilatory response to haemolymph P_{CO_2} resulting in oscillations around a fixed P_{CO_2} threshold.

MATERIALS AND METHODS

Animals

Madagascar hissing cockroaches *G. portentosa* were reared for multiple generations in an insectary at the University of British Columbia. Cockroaches were held in two, 65 l black plastic storage containers at 31°C, on a 12 h:12 h light:dark cycle, and fed fruit, dry cat food (Friskies Chef's Blend, Purina, Mississauga, ON, Canada) and hydrated hydrogel granules *ad libitum*. Only male cockroaches were used for experiments, as the large pronotal horns possessed by the males of this species were a convenient site for the implantation of fibre optic P_{O_2} and P_{CO_2} sensors. Individual male cockroaches (mean±s.d. mass 12.75±1.82 g, n=18) were isolated and fasted for 24 h before experiments. Cockroaches were weighed to 0.01 mg on an electronic balance (XPE205D, Mettler-Toledo Inc., Mississauga, ON, Canada) directly before experimentation.

Respirometry setup

Mixtures of O_2 and CO_2 in N_2 were produced using mass flow controllers (Alicat Scientific, Tucson, AZ, USA) calibrated for air (0–2 l min^{-1}), N_2 (0–500 ml min^{-1}) and CO_2 (0–50 ml min^{-1}) (Fig. 2). A continuous supply of dry, normoxic, CO_2-free air (20.95% O_2, 79.05% N_2) was produced by a purge gas generator (CDA4-CO_2, Puregas, Broomfield, CO, USA) which pressurised, dehumidified, and stripped laboratory air of CO_2. Any remaining trace amounts of CO_2 and water vapour were then removed from the purge gas by passing it through 1 litre columns of soda-lime and Drierite (W. A. Hammond Drierite Co. Ltd, Xenia, OH, USA). A continuous supply of pressurised nitrogen (95–99% purity) was generated using a nitrogen generator (Parker Balston Model N2-O4, Parker Hannifin Corporation, Haverhill, MA, USA). CO_2 (>99.5% pure) was obtained from a pressurised gas cylinder. The total flow rate of all gas mixtures used was 900 ml min^{-1} STPD. The incurrent airstream/gas mixture produced by the mass flow controllers was humidified first by bubbling it through a 500 ml gas-washing bottle half-filled with reverse osmosis (RO) water, then passing it through a dew point generator (DG-4, Sable Systems International, North Las Vegas, NV, USA) which regulated the relative humidity (RH) at 70% at 22°C. This conditioned airstream was then piped into an incubator (I36VL, Percival Scientific Inc., Perry, IA, USA) maintained at 22°C, where it first passed through a chamber (~10 ml) enclosing a RH/temperature probe (HMP60, Vaisala, Helsinki, Finland) before entering an acrylic respirometry chamber housing an individual cockroach (Fig. 2B). On exiting the respirometry chamber, the airstream was split and directed into two parallel drying columns arranged in a shell-and-tube configuration. Each drying column consisted of a 73 cm length of Nafion water-permeable tubing (0.054 inch internal diameter TT-070, CD Nova, Surrey, BC, Canada) which ran through the middle of a 70 cm long, 15.9 mm×9.5 mm outer diameter×inner diameter

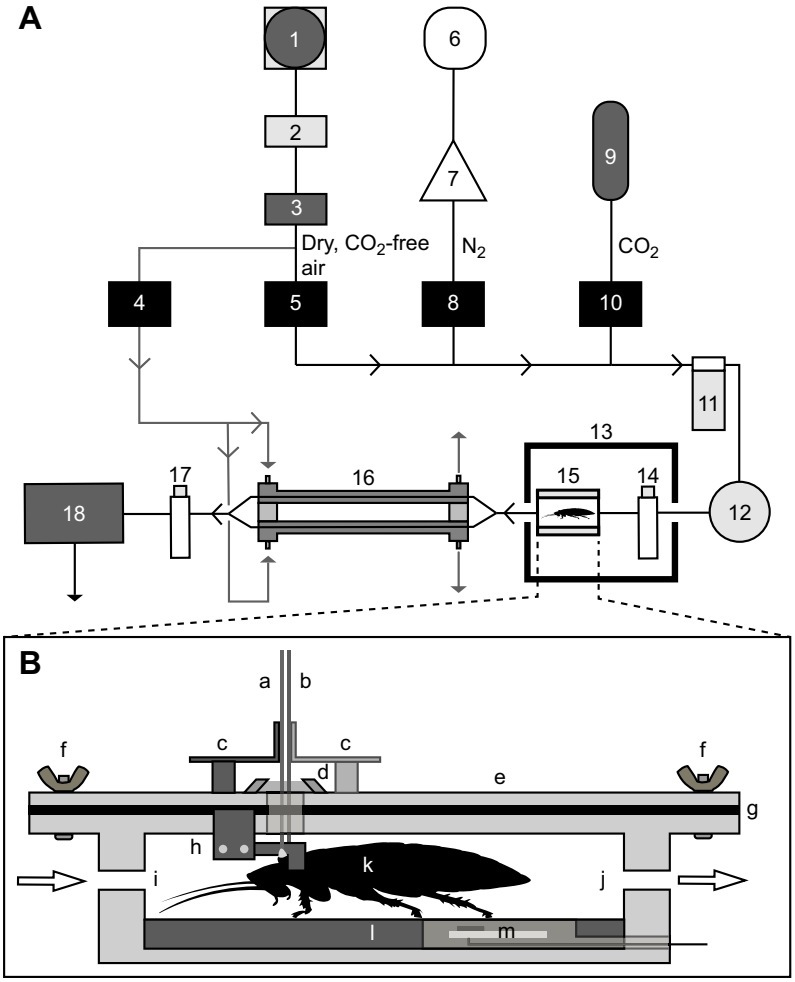

Fig. 2. Respirometry setup and respirometry chamber used for the simultaneous measurement of CO_2 release, haemolymph P_{O_2}, P_{CO_2} and abdominal ventilation. (A) 1, Purge gas generator; 2, column containing soda-lime; 3, column containing Drierite; 4, 2×0–500 ml min^{-1} flow controllers in parallel; 5, 0–2 l min^{-1} flow controller; 6, air compressor; 7, N_2 generator; 8, 0–500 ml min^{-1} flow controller; 9, CO_2 gas cylinder; 10, 0–50 ml min^{-1} flow controller; 11, water-filled gas-washing bottle; 12, dew point generator; 13, incubator; 14, humidity and temperature sensor; 15, respirometry chamber; 16, drying column; 17, humidity and temperature sensor; 18, CO_2 infra-red gas analyser (IRGA). (B) a, P_{CO_2} probe implanted in the left pronotal horn; b, P_{O_2} probe implanted in the right pronotal horn; c, ABS clamp fixing the probe to the chamber lid; d, ABS well filled with polyvinyl siloxane plug; e, respirometry chamber lid; f, wingnuts securing the chamber lid; g, closed-cell neoprene foam gasket; h, ABS thoracic harness attached to chamber lid; i, air inlet; j, air outlet; k, Madagascar hissing cockroach; l, ABS plastic baseplate, covered with acrylic platform; m, infrared activity detector. Arrows indicate the direction of airflow.

clear acrylic tube. The excurrent air was directed through the lumen of the Nafion tube, while the space between the outside of the Nafion tube and the inside of the acrylic tube was flushed with dry purge gas (0% RH), which flowed counter-current to the excurrent airstream at a rate of 500 ml min^{-1} in each column. After exiting the drying columns, the airstreams were recombined, passed through another chamber containing a second identical RH/temperature probe (HMP60, Vaisala), then finally into a LI-820 CO_2 infra-red gas analyser (IRGA; Licor, Lincoln, NE, USA). The concentration of CO_2 in the airstream (in ppm) was sampled at 2 Hz using a Powerlab 8/35 DAQ analog to digital converter (ADInstruments, Bella Vista, NSW, Australia) and recorded using LabChart software (v.8.1.5, ADInstruments) on a desktop PC. This CO_2 concentration was converted into a rate of CO_2 exhaled ($\dot{V}_{CO_2}$) in µl s^{-1} according to the equation:

$$\dot{V}_{CO_2} = \dot{V}_I \times F_{E CO_2}, \quad (1)$$

where $\dot{V}_I$ is incurrent flow rate (µl s^{-1}) and $F_{E CO_2}$ is the excurrent fractional CO_2 concentration (%).

Respirometry chamber and cockroach harness

For each experiment, a cockroach was secured within a 120×35×40 mm (L×W×H; 168 cm^3) clear acrylic respirometry chamber using a custom-built harness. The harness was 3D printed from ABS plastic and fitted over the cockroach's pronotum, posterior to the pronotal horns. Individual cockroaches were anaesthetised by a 30 s exposure to pure CO_2, before being fixed into the harness using a commercially available mixture of paraffin, beeswax and colophony (Brazilian and Bikini Wax, Nads, NSW, Australia) as an adhesive. A beam (15×5×5 mm) containing two horizontal holes projected from the harness over the cockroach's head, allowing the harness to be secured into a 3D printed bracket that was attached to the inside of the respirometry chamber lid using two M3 screws (Fig. 2B). When assembled, this fixed the cockroach so its pronotum was held directly beneath two 10 mm diameter holes in the lid, one above each pronotal horn, while its abdomen was positioned above an IR reflection sensor (SFH 9202, Osram Opto Semiconductors, Regensburg, Germany) mounted in the floor of the chamber. The IR sensor was connected to a circuit which produced a variable voltage in response to the distance between the sensor and the abdomen, allowing abdominal pumping movements associated with gas exchange to be measured. This voltage was measured at 2 Hz by the Powerlab 8/35 DAQ and recorded using LabChart (v.8.1.5, ADInstruments) on a desktop computer, providing a quantitative measure of abdominal ventilation frequency.

Haemolymph P_{O_2} and P_{CO_2} measurements

The P_{O_2} optodes (flat-tip, 230 µm diameter fibre, IMP-PSt7, PreSens GmbH, Regensburg, Bavaria, Germany) were connected to a Microx 4 trace meter (PreSens GmbH) and calibrated using a two-point calibration: 0 kPa P_{O_2} water was produced by mixing sodium sulphite into reverse osmosis (RO) water and submerging the P_{O_2} optodes in this solution for 10 min before calibration; room air was bubbled through RO water using an air stone for 30 min to thoroughly saturate the water with ambient oxygen, before calibrating the probe at 21 kPa P_{O_2}. The calibrations were performed at 22°C inside the same incubator as used for the experiment.

The P_{CO_2} optodes (flat-tip, 250 µm diameter fibre, IMP-CDM1, PreSens GmbH) were calibrated in 100 ml of RO water containing 0.154 mol l^{-1} NaCl which was equilibrated with 0, 0.5, 1, 2, 3, 4 and 5 kPa P_{CO_2} in N_2. The calibration saline was maintained at 22°C in a 200 ml glass bottle suspended in a temperature-controlled water bath (F33-ME, Julabo, Seelbach, Baden-Württemberg, Germany). Two 500 ml min^{-1} flow controllers (MC-500SCCM-D/5M, Alicat Scientific), controlled by gas-mixing software (Flow Vision, Alicat Scientific) running on a desktop PC, were used to generate P_{CO_2} in a stepwise fashion by combining 99.998% N_2 with a certified mix of 5% CO_2 in a balance of N_2 (Praxair, Mississauga, ON, Canada). Gas mixtures were bubbled through an air stone submerged in the calibration saline at 500 ml min^{-1}. Probes were held for 1 h at 0 and 0.5 kPa P_{CO_2}, and 30 min at 1, 2, 3, 4 and 5 kPa P_{CO_2} to ensure complete equilibration of the calibration solution. During calibration, P_{CO_2} was measured at 5 min intervals with a CO_2 meter (pCO_2 micro, PreSens) and recorded onto a desktop PC using pCO_2 micro View software (v.1.0.0, PreSens). Monitoring P_{CO_2} over time guaranteed that the probes had reached equilibrium with the CO_2 level that was bubbled through the calibration saline. The final CO_2 measurement recorded at each CO_2 level was used to produce a multipoint calibration curve for the optode, composed of 7 points ranging from 0 to 5 kPa CO_2.

While the cockroach was still CO_2 narcotised from being mounted in the harness, two small holes were drilled into the insect's haemocoel, one hole in each pronotal horn, using a 0.84 mm diameter carbide drill bit attached by a flexible shaft to a rotary tool (Dremel 3000 series 1.2 Amp Rotary Tool, Dremel, Racine, WI, USA). As each hole was cut it was sealed temporarily by applying a dab of 2-part polyvinyl siloxane casting material (President light body dental impression material, Coltène Whaledent, Altstätten, Switzerland). Following this operation, the cockroach was secured to the respirometry chamber lid using the previously attached ABS harness (Fig. 2B). To implant a calibrated P_{O_2} optode, first the polyvinyl siloxane plug sealing the hole in the right pronotal horn was removed, then the optode was lowered through a 10 mm hole in the chamber lid using a micromanipulator (M3301, World Precision Instruments, Sarasota, FL, USA) and carefully inserted ∼2 mm into the haemocoel. The optode was sealed into the horn by application of more polyvinyl siloxane casting material around the optic fibre. The fibre of the optode was then secured to the respirometry chamber lid using a custom-built clamp, 3D printed from ABS plastic, that was bolted to the top of the lid. A calibrated P_{CO_2} probe was implanted into the haemocoel within the cockroach's left pronotal horn using the same method as described above, but with the optode passing through a second 10 mm hole in the lid and being secured using a second clamp. A 3D-printed ABS plastic ring with inwardly sloping walls had previously been epoxied to the chamber lid, forming a well around both holes. Strips of aluminium foil were placed inside this ring around each optode to cover the two holes in the lid before being covered liberally with polyvinyl siloxane, completely filling the well and forming a gas-tight seal. Low sampling frequencies were used to ensure that any photobleaching of the optode tips was minimised during the 2 day experimental run. *In vivo* haemolymph P_{CO_2} was recorded every 2 min and *in vivo* haemolymph P_{O_2} measurements were recorded every 30 s. No signal drift or change in sensor amplitude was observed for either the P_{O_2} or P_{CO_2} optode during the experiments. All experiments were conducted inside an incubator (22°C, 12 h:12 h light:dark) (Percival Scientific Inc.).

Experimental protocol

For the first 5 h of experimentation, the cockroaches were exposed to normoxic normocapnia (21 kPa P_{O_2}, 0 kPa P_{CO_2}) to stabilise after

surgery. The next 18 h were split into two 9 h treatments. Treatments began with either a further 9 h exposure to normoxic normocapnia, or a 9 h exposure to either hypoxic normocapnia (10 kPa P_{O_2}, 0 kPa P_{CO_2}) or normoxic hypercapnia (21 kPa P_{O_2}, 2 kPa P_{CO_2}). The order of the treatments alternated between experiments such that if one experiment started with a 9 h normoxic normocapnia treatment, the next experiment began with either the 9 h hypoxia or hypercapnia exposure. After the first 23 h, the respirometry chamber was removed from the incubator, and the cockroach was anaesthetised by a 30 s exposure to pure CO_2. The lid of the respirometry chamber was removed with cockroach and optodes attached, and the cockroach was swiftly decapitated using fine scissors. The neck wound was sealed using melted beeswax. Decapitation reliably elicited sustained DGCs that began shortly after surgery and continued for at least a week thereafter. Once decapitated, the cockroach was returned to the chamber, and the chamber lid was again fixed in place. The chamber was then returned to the incubator for another 23 h of experimentation. The final 18 h of DGC experiments were again divided into two 9 h blocks, repeating the same modified atmosphere manipulations as were carried out before decapitation.

Measurements of O_2 and CO_2 levels were recorded as percentage of total gas dissolved in haemolymph. These raw values were converted into partial pressures (P_{O_2} or P_{CO_2} in kPa) by dividing each value by 100 then multiplying by 101.3 kPa. Mean continuous P_{CO_2} and P_{O_2} values were taken from the longest sustained period of stable haemolymph P_{CO_2} in each treatment. When DGCs were displayed, the maximum and minimum P_{CO_2} and P_{O_2} values were determined for each C–F–O cycle in the final 6 h of each treatment using the peak analysis function in LabChart software (ADInstruments) and averaged. The durations of different phases of the DGCs were measured in both treatment gases and compared. The O-phase and interburst duration were determined using the haemolymph P_{O_2} trace. The interburst duration was defined as the period of continuous haemolymph P_{O_2} decline, while the O-phase duration was defined as the period when P_{O_2} began to rise before a subsequent fall. Total cycle duration was determined by adding the O-phase and interburst phase durations. The C-phase duration was determined by the absence of ventilatory movements, whereas the F-phase was determined by the presence of relatively low-frequency ventilatory movements which corresponded with no observable change in haemolymph P_{O_2}. The experiment also measured the mean rate of CO_2 release during each DGC in hypoxic and normoxic conditions. The cockroach's rate of CO_2 release was recorded using the IRGA and then averaged over each DGC to calculate mean $\dot{V}_{CO_2}$ (µl s^{-1}). The method used to identify specific parts of the DGC for analysis is illustrated in Fig. 3 using typical data traces recorded from cockroaches displaying continuous ventilation and DGCs.

Statistical analysis

All DGC data from individual cockroaches represent the average obtained from the final 6 h of DGCs recorded in each treatment gas. All data are available from Dryad (https://doi.org/10.5061/dryad.b8gtht7ct). Paired measurements obtained from individual cockroaches represent measurements made during exposure to normoxic normocapnia (21 kPa P_{O_2}, 0 kPa P_{CO_2}), and a treatment of repeated normoxic normocapnia (control), hypoxic normocapnia (10 kPa P_{O_2}, 0 P_{CO_2}) or normoxic hypercapnia (21 kPa P_{O_2}, 2 kPa P_{CO_2}). Differences between values obtained in normoxic normocapnia exposure and the treatment gas were tested using a 2-tailed paired *t*-test in Prism 8 (GraphPad Software Inc., San Diego, CA, USA) with statistical significance being set at α=0.05. The Benjamini–Hochberg (BH) procedure with an α=0.05 was applied to control the false discovery rate associated with performing multiple tests on each treatment group: 11 tests on data from the normoxia–hypercapnia manipulation and 12 tests on the normoxia–hypoxia and normoxia–normoxia manipulations. *t*-Tests were used despite often low ($n \leq 5$) sample sizes, as any significant changes in values resulting from different gas exposures would still be detected so long as the data showed strong correlation coefficients within treatments and a large effect size (De Winter, 2013). However, overall the statistical analysis employed here is conservative. Given that the small sample sizes increase the likelihood of Type II errors, while the BH procedure controlled for Type I errors, this study is more likely to commit errors of omission, rather than generate false positives.

RESULTS

Median changes in P_{O_2} and P_{CO_2} during continuous ventilation and DGC, as well as median changes in phase duration are summarised in Table 1.

Haemolymph P_{O_2} and P_{CO_2} during continuous breathing

Changes in mean haemolymph P_{O_2} and P_{CO_2} are shown in Fig. 4. Intact *G. portentosa* displaying continuous ventilation in normoxic normocapnia maintained stable haemolymph P_{O_2} and P_{CO_2} levels with a mean (±s.d.) of 17.1±2.9 kPa (n=14) and 1.9±0.4 kPa (n=15), respectively. Neither mean haemolymph P_{O_2} nor P_{CO_2} changed significantly between the first and second 9 h period of exposure to the normoxic normocapnic gas mixture (t_4=1.7417, P=0.1565 and t_4=1.1952, P=0.2980, respectively). Three of six intact cockroaches exposed to hypoxia (10 kPa P_{O_2}) exhibited sustained DGCs in lieu of continuous breathing. Unfortunately, equipment failure meant that reliable P_{CO_2} measurements could be obtained from only two of the three continuously breathing cockroaches. Mean haemolymph P_{O_2} was significantly lower in continuously ventilating cockroaches exposed to hypoxia (t_2=9.8835, P=0.0101), falling by between 7.9 and 11 kPa. There was no significant change in haemolymph P_{CO_2} when cockroaches were exposed to hypoxia (t_1=3.333, P=0.1855). During exposure to hypercapnia (2 kPa P_{CO_2}), neither haemolymph P_{O_2} nor P_{CO_2} changed significantly relative to levels in normoxic normocapnia (t_2=1.152, P=0.3809, and t_3=2.8947, P=0.0628, respectively).

DGCs in decapitated cockroaches

All decapitated cockroaches displayed robust DGCs. Minimum P_{O_2} level fell close to 0 kPa during the C-phase in most experiments. Exposure to 10 kPa P_{O_2} or 2 kPa P_{CO_2} did not cause any of the decapitated cockroaches to stop displaying DGCs. Changes in minimum and maximum haemolymph P_{O_2} during DGCs in decapitated cockroaches exposed to control, hypoxia and hypercapnia treatments are shown in Fig. 5A,B. Cockroaches exposed to two periods of normoxic normocapnic air showed no significant change in either maximum P_{O_2} (t_4=1.4698, P=0.2156) or minimum P_{O_2} (t_4=1.000, P=0.3739). When cockroaches were exposed to hypercapnia, there was no change in mean minimum P_{O_2} (t_4=2.2361, P=0.089) or mean maximum P_{O_2} (t_4=1.0461, P=0.3546). Unsurprisingly, maximum haemolymph P_{O_2} was reduced significantly during exposure to hypoxia (t_4=17.3003, P=0.0001), but minimum P_{O_2} did not change significantly (t_4=1.7833, P=0.1491). However, minimum P_{O_2} was still reduced in all cockroaches that did not reach 0 kPa during their normoxic normocapnia control treatment.

Changes in minimum and maximum haemolymph P_{CO_2} during DGCs in decapitated cockroaches exposed to control, hypoxia and

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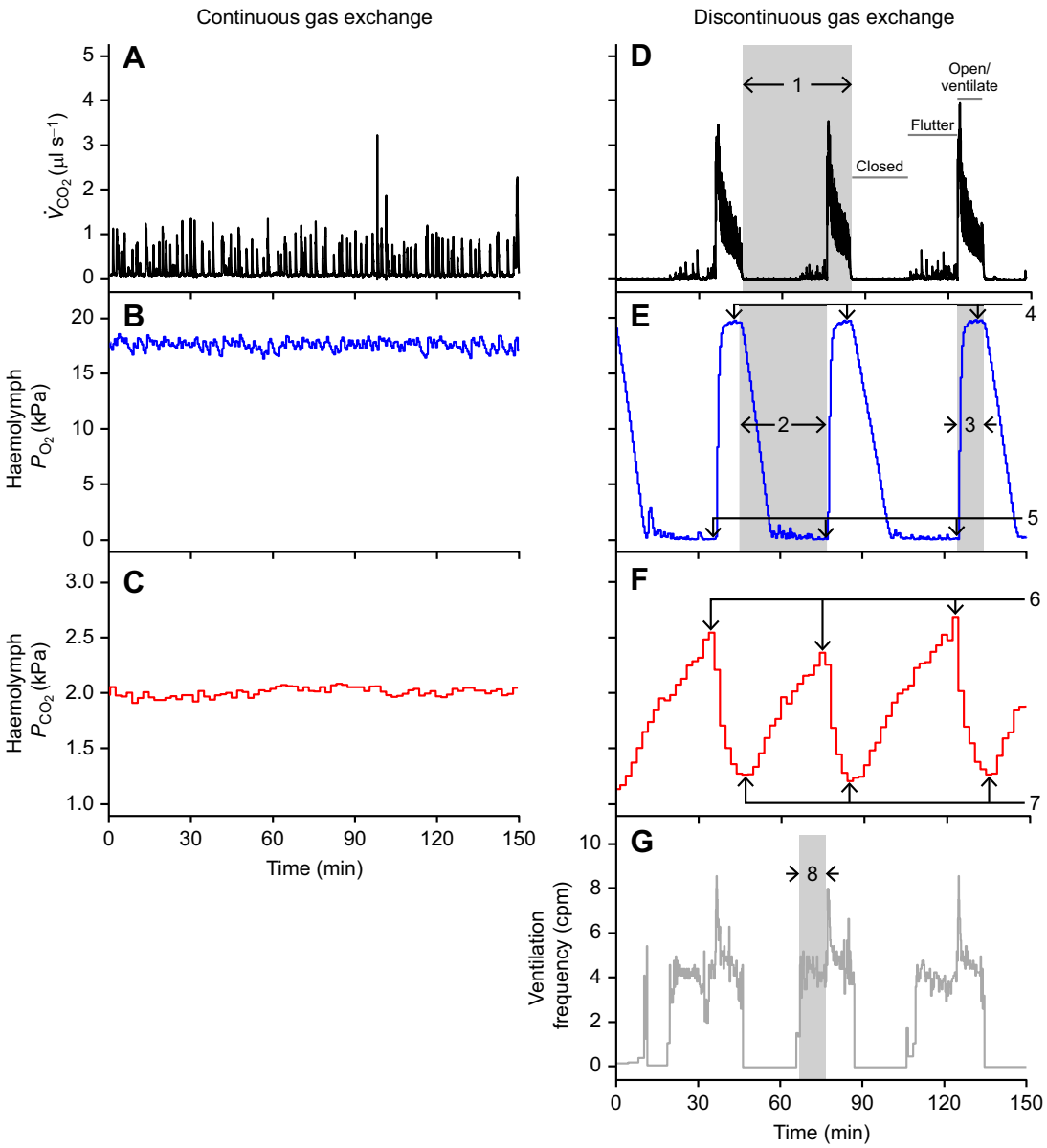

Fig. 3. Typical measurements taken from continuously (left) and discontinuously (right) ventilating *Gromphadorhina portentosa* in normoxia. Numbers, arrows and bars indicate how DGC parameters were defined. (A,D) $\dot{V}_{CO_2}$, (B,E) haemolymph P_{O_2}, (C,F) haemolymph P_{CO_2} (kPa), (G) ventilation frequency (cycles per minute, cpm). (1) Total CO_2 exhaled per cycle (µl); (2) inter-burst duration (s); (3) open phase duration (s). Indicated partial pressures were used to calculate: (4) average maximum P_{O_2} (kPa); (5) average minimum P_{O_2} (kPa); (6) average maximum P_{CO_2} (kPa); (7) average minimum P_{CO_2} (kPa); and (8) flutter phase duration (s).

hypercapnia treatments are shown in Fig. 5C,D. Sustained exposure to normoxic normocapnia did not significantly change either mean maximum P_{CO_2} (t_5=0.1152, P=0.9128) or mean minimum P_{CO_2} (t_5=0.0000, P=1). However, of the six cockroaches measured, one showed increasing and one showed decreasing haemolymph P_{CO_2} over time, despite constant ambient P_{O_2} and P_{CO_2} levels. Cockroaches exposed to hypercapnia had significantly increased mean maximum and mean minimum haemolymph P_{CO_2} (t_4=4.2055, P=0.0136 and t_4=5.8987, P=0.0041, respectively). Conversely, cockroaches exposed to hypoxia had significantly lower mean maximum and minimum haemolymph P_{CO_2} (t_4=4.1386, P=0.0144 and t_4=3.5, P=0.0249).

Plotting haemolymph P_{CO_2} against P_{O_2} on an *x*/*y* scatter plot revealed that, relative to DGCs in normoxia, hypoxia reduces overall haemolymph P_{CO_2} and P_{O_2} (Fig. 6A), while exposure to normoxic hypercapnia only elevates P_{CO_2} (Fig. 6B). The range of gas tensions seen in the haemolymph of cockroaches exposed only to normoxia was usually consistent (Fig. 6C), but could drift over time (Fig. 6D). Of the five control cockroaches, one experiment showed haemolymph P_{CO_2} increasing, and another showed haemolymph P_{CO_2} decreasing over time.

DGC phase duration

Changes in cycle duration, interburst duration, and C-, F- and O-phase duration during DGCs in decapitated cockroaches exposed to normoxia, hypoxia and hypercapnia treatments are shown in Fig. 7. Cockroaches exposed to repeated normoxic normocapnia exposure showed no significant change in O-phase duration (t_5=2.3284, P=0.0673), F-phase duration (t_3=2.9765, P=0.0588) or interburst duration (t_5=2.2158, P=0.0775). After applying the BH procedure,

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Table 1. Direction and magnitude of change in median P_{CO_2} and P_{O_2} levels during continuous and discontinuous gas exchange, and changes in DGC phase duration in response to normoxic normocapnia, hypoxic normocapnia and normoxic hypercapnia treatment, relative to measurements made in normoxic normocapnia (control)

	Normoxia/normoxia			Normoxia/hypoxia			Normoxia/hypercapnia		
	Change	ΔMedian (kPa)	*n*	Change	ΔMedian (kPa)	*n*	Change	ΔMedian (kPa)	*n*
DCG phase duration (min)									
C-phase	=	10.7	4	=	−7	4	=	−0.6	5
F-phase	=	−6.4	4	=	−5.3	4	=	−9.9	5
O-phase	=	1.1	6	=	−0.3	6	↑	4.8*	6
Interburst	=	5.9	6	↓	−12.4*	6	=	−13.9	6
Total cycle	=	7.5	6	↓	−12.4*	6	=	−10.8	6
Continuous (kPa)									
P_{O_2}	=	−0.9	5	↓	−9.35*	3	=	0.2	3
P_{CO_2}	=	−0.1	5	=	−1	2	=	0.35	4
DGC (kPa)									
P_{O_2} min.	=	0	5	=	−0.4	5	=	0.1	5
P_{O_2} max.	=	3.5	5	↓	−10.9*	5	=	0.55	5
P_{CO_2} max.	=	0.15	6	↓	−1*	4	↑	0.8*	5
P_{CO_2} min.	=	0.05	6	↓	−0.3*	4	↑	0.7*	5
DGC $\dot{V}_{CO_2}$ (ml min^{-1})									
MR	=	−1.1	6	=	0.2	6		N/A	

DGC, discontinuous gas exchange cycle; *n*, number of pairs. *Significant changes as determined by paired 2-tailed *t*-test and Benjamini and Hochberg procedure.

changes in mean total cycle duration and C-phase duration were also non-significant (t_5=4.6515, P=0.0056, and t_3=3.7444, P=0.0332, respectively). In cockroaches exposed to hypercapnia, there was no significant change in mean total cycle duration (t_5=2.5012, P=0.0544), F-phase duration (t_4=2.4057, P=0.0739), C-phase duration (t_4=0.5380, P=0.6191) or mean interburst duration (t_4=3.1219, P=0.0262). However, mean O-phase duration did increase significantly (t_5=4.3797, P=0.0072). Cockroaches exposed to hypoxia showed no significant change in C-phase duration (t_3=1.6903, P=0.1896), F-phase duration (t_3=0.4430, P=0.6878) or O-phase duration (t_5=1.2295, P=0.2736). However, cockroaches exposed to hypoxia showed significantly decreased mean total cycle duration (t_5=4.4931, P=0.0064) and mean interburst duration (t_5=5.9467, P=0.0019).

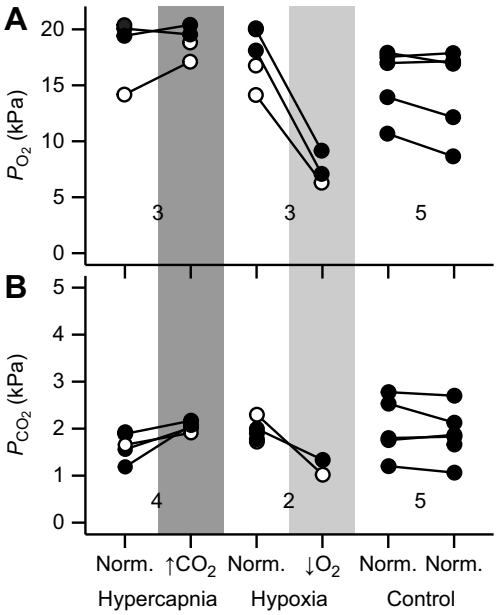

Fig. 4. Mean haemolymph P_{O_2} and P_{CO_2} in individual *G. portentosa* displaying continuous gas exchange during sequential exposure to normoxia and normoxia, hypoxia or hypercapnia. (A) Mean haemolymph P_{O_2} and change relative to normoxia. (B) Mean haemolymph P_{CO_2} and change relative to normoxia. Lines indicate paired means from the same individual. Black circles represent cockroaches exposed to normoxia in the first 9 h of measurement and to treatment gas in the second 9 h; white circles represent the reverse order. Unpaired circles indicate data from single observations. Numbers below circles indicate the number of paired observations for that treatment. Statistical analyses are presented in Table 1.

Mean $\dot{V}_{CO_2}$ during DGCs

Mean $\dot{V}_{CO_2}$ was not significantly different in the control group when exposed to repeated normoxic normocapnia (t_5=1.1773, P=0.2920), or in the hypoxia group between control and treatment (t_5=0.928, P=0.3959). As $\dot{V}_{CO_2}$ could not be measured in a hypercapnic atmosphere, any changes in $\dot{V}_{CO_2}$ associated with this treatment could not be determined.

DISCUSSION

Continuous ventilation

The mean haemolymph P_{O_2} and P_{CO_2} values measured during continuous gas exchange (17.1 and 1.9 kPa, respectively) are similar to values observed in other insect species displaying continuous ventilation (Förster and Hetz, 2010; Harrison et al., 1991; Matthews and White, 2011). Assuming that continuous ventilation displayed by *G. portentosa* in normoxic normocapnia is due to respiratory chemoreceptors stimulating a continuous ventilatory drive, this suggests that a haemolymph P_{CO_2} of ∼1.9 kPa and P_{O_2} of ∼17.1 kPa represent steady-state threshold levels required to maintain this continuous ventilatory drive. As such, exposure to either the 2 kPa hypercapnia or 10 kPa hypoxia treatment should elicit a corrective ventilatory response. Exposing cockroaches to hypercapnia did indeed cause them to hyperventilate, as haemolymph P_{CO_2} did not increase by 2 kPa, instead showing a non-significant median increase of only 0.35 kPa (Table 1), while exposure to hypoxia (10 kPa P_{O_2}) has previously been shown to induce hyperventilation in continuously breathing *G. portentosa* (Harrison et al., 2016). These same O_2 and CO_2 partial pressures were also sufficient to increase ventilation in the speckled feeder roach *Nauphoeta cinerea* (Matthews and White, 2011). Thus, during continuous gas exchange, *G. portentosa* respond to hypoxic and hypercapnic challenges in the same way as other insects, and most air-breathing animals, by increasing ventilation. But once

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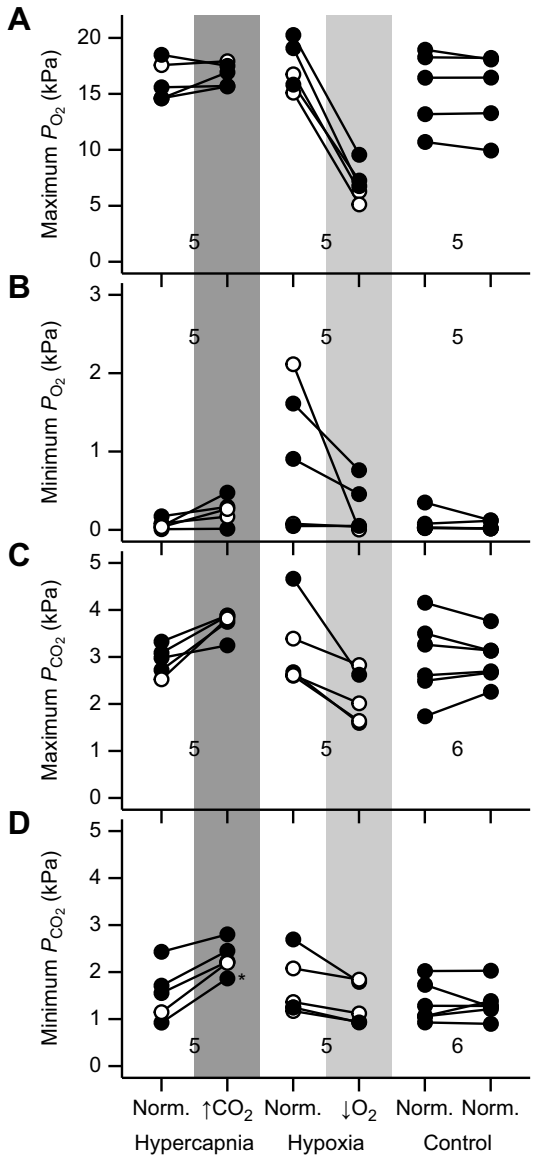

Fig. 5. Mean maximum and minimum haemolymph P_{O_2} and P_{CO_2} in individual *G. portentosa* displaying DGCs during sequential exposure to normoxia and normoxia, hypoxia or hypercapnia. (A,B) Mean maximum (A) and minimum (B) haemolymph P_{O_2} and change relative to normoxia. (C,D) Mean maximum (C) and minimum (D) haemolymph P_{CO_2} and change relative to normoxia. *P_{CO_2} value lower than ambient P_{CO_2}, but within equipment error limits. Lines indicate paired means from the same individual. Black circles represent cockroaches exposed to normoxia in the first 9 h of measurement and treatment gas in the second 9 h; white circles represent the reverse order. Numbers above/below circles indicate the number of paired observations for that treatment. Statistical analyses are presented in Table 1.

DGCs were elicited by decapitation, the ventilatory responses of these cockroaches changed substantially.

Normoxic normocapnia

During DGCs in normoxic normocapnia, ventilation during the O-phase caused haemolymph P_{O_2} to rise to the same near-ambient levels as observed in continuously breathing individuals, but during the C-phase, haemolymph P_{O_2} fell to ∼0 kPa (Fig. 6). Haemolymph P_{O_2} often remained at or near 0 kPa for a substantial period of time during the C- and F-phases, before rising rapidly during the O-phase once ventilation began. Given this protracted internal hypoxia, the O-phase is clearly not triggered when haemolymph P_{O_2} reaches some hypoxia threshold. This is in agreement with previous studies on moth pupae, locusts and other species of cockroach, which all indicate that while internal hypoxia does not trigger the open phase, it does appear to stimulate spiracular fluttering as a corrective response (Matthews and White, 2011; Matthews et al., 2012; Schneiderman, 1960). Assuming that respiratory chemoreceptor thresholds drive the transition from the C- to the O-phase, this leaves only elevated P_{CO_2} as the trigger.

While continuously breathing cockroaches maintained a stable internal P_{CO_2}, once decapitated and breathing discontinuously, all cockroaches displayed a mean maximum haemolymph P_{CO_2} (i.e. the P_{CO_2} reached immediately preceding O-phase ventilation) that was higher than the P_{CO_2} observed during continuous ventilation in the same individual (Fig. 8), indicating internal CO_2 was accumulating above the level regulated during continuous gas exchange. Furthermore, during exposure to normoxic normocapnia, spiracles were generally observed to open at the same maximum P_{CO_2} during each DGC. However, in two of the six control experiments, it was observed that minimum and maximum haemolymph P_{CO_2} drifted over time. In the first of these experiments, minimum and maximum haemolymph P_{CO_2} decreased and in the second experiment, they increased (Fig. 6D). These two experiments indicate that strict P_{CO_2} thresholds may not be responsible for maintaining DGCs, as the F→O and O→C transitions did not occur at a fixed P_{CO_2}. Exposing cockroaches displaying DGCs to hypoxia and hypercapnia further tests whether fixed thresholds are responsible for driving the transitions between phases required to produce DGCs.

Hypercapnia exposure

Hypercapnia-exposed *G. portentosa* experienced an ambient P_{CO_2} of 2 kPa, a level that approximates the mean haemolymph P_{CO_2} observed during continuous ventilation in normoxic normocapnic air (1.9±0.4 kPa, n=15). Assuming insects actively regulate internal P_{CO_2} around this level, a P_{CO_2} of 2 kPa hypercapnia should force cockroaches displaying DGCs to instead breathe continuously as they could never become hypocapnic – a condition required to generate the C- and F-phases of the DGC, assuming hysteresis around a fixed threshold. However, this did not occur. During hypercapnia exposure, both minimum and maximum haemolymph P_{CO_2} increased significantly relative to levels in normoxic normocapnia (Fig. 8B), while internal P_{O_2} continued to vary between ∼0 kPa and near ambient. If a fixed P_{CO_2} threshold was present then the maximum P_{CO_2} should not vary between normoxic and hypercapnic exposures as breath holding would be terminated once haemolymph P_{CO_2} reached this threshold value. However, the data presented here show that the transitions from F→O and from O→C continued to occur in hypercapnia, but at a significantly higher haemolymph P_{CO_2} compared with normocapnic air (Fig. 8B). Given that these cockroaches were all capable of transitioning from the O- to C-phase while their minimum internal P_{CO_2} was elevated significantly demonstrates that oscillations in haemolymph P_{CO_2} around a fixed CO_2 chemosensory threshold cannot account for the appearance of DGCs in *G. portentosa*.

To examine whether a higher haemolymph P_{CO_2} would abolish DGCs in *G. portentosa*, a single decapitated cockroach was exposed to an ambient P_{CO_2} of 3 kPa. This cockroach continued to display DGCs despite its haemolymph P_{CO_2} never once falling below levels previously recorded in normoxic normocapnia (Fig. 9). Although this is only one individual, it demonstrates that in this instance the DGC could not be explained by hysteresis around a fixed

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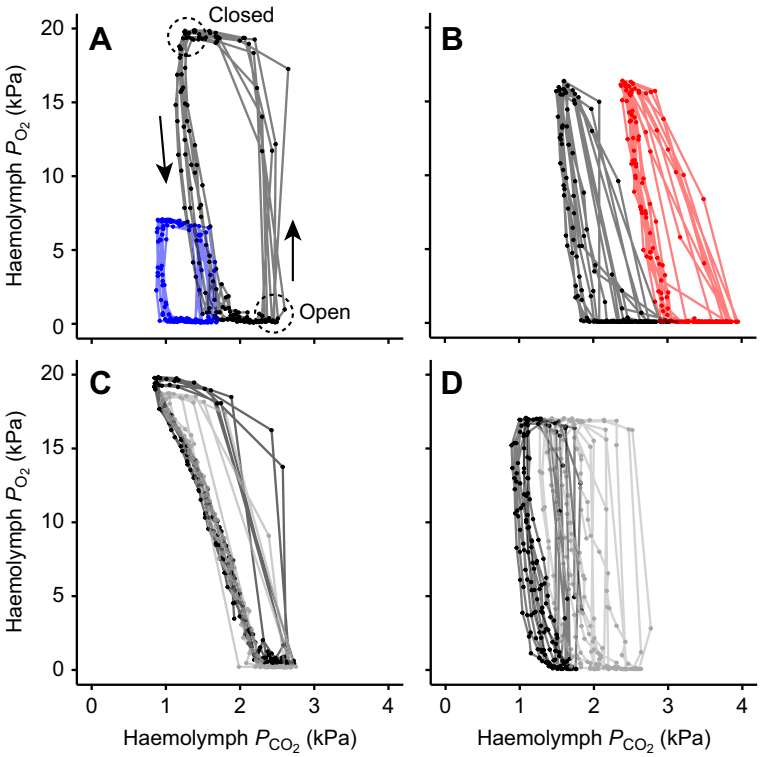

Fig. 6. Scatterplot illustrating simultaneous changes in haemolymph P_{CO_2} and P_{O_2} during DGCs exhibited by four different *G. portentosa* cockroaches exposed to normoxia, hypoxia and hypercapnia. The loops cycle counter-clockwise over time between open and closed spiracle states (shown in A). Data were recorded from DGCs observed in the final 6 h of exposure to normoxic normocapnia (black, all plots) and the final 6 h of exposure to hypoxic normocapnia (blue; A), normoxic hypercapnia (red; B), or a control treatment of normoxic normocapnia (grey; C,D). C illustrates P_{CO_2} and P_{O_2} fluctuations which remained similar throughout the experiment. D shows P_{CO_2} and P_{O_2} in another experiment shifting over time despite constant ambient P_{O_2} and P_{CO_2}.

P_{CO_2} threshold, as cycles continued even though the minimum haemolymph P_{CO_2} never once fell below the maximum P_{CO_2} value recorded in normoxia. However, this extreme hypercapnia exposure also caused both maximum and minimum haemolymph P_{O_2} to increase (Fig. 9), again showing that although persistent hypercapnia does not abolish the DGC, it does stimulate increased ventilation during the O-phase. Likewise, cockroaches exposed to hypercapnia significantly increased the duration of their O-phase (Fig. 7). Thus, exposure to hypercapnia results in a longer O-phase and enhanced ventilation to compensate for elevated haemolymph P_{CO_2}.

These results point to substantial differences between the DGCs displayed by different insect orders. For example, previous research has shown that exposure to hypercapnia >2.9 kPa eliminates DGCs in intact grasshoppers and moth pupae (Harrison et al., 1995; Terblanche et al., 2008), whereas our results show that decapitated Madagascar hissing cockroaches would probably require their haemolymph P_{CO_2} to be elevated well beyond these levels to abolish DGCs – an effect that may be physiologically irrelevant. This apparent insensitivity of the DGC to hypercapnia has been shown for at least one other species of cockroach, with Miller (1981) reporting that quiescent burrowing cockroaches (*Blaberus craniifer*) switch from DGC to continuous breathing only when ambient P_{CO_2} exceeded 5–10 kPa.

Hypoxia exposure

Decapitated *G. portentosa* maintained DGCs in hypoxia despite this treatment resulting in significantly depressed minimum and maximum haemolymph P_{CO_2} (Fig. 8C). This trend was previously observed in decapitated *N. cinerea* displaying DGCs (Matthews and White, 2011), indicating either that hypoxia increases sensitivity to CO_2, reducing the P_{CO_2} threshold that initiates the O-phase, or that these cycles are being generated by a ventilatory rhythm that is largely insensitive to CO_2 chemosensory feedback (Matthews, 2018). The latter explanation appears to be the case here, as haemolymph P_{O_2} before the start of the O-phase was around 0 kPa in both the normoxia and hypoxia treatments while the average maximum P_{CO_2} was 2.9 kPa in normoxia but only 2.1 kPa in hypoxia. Thus, equally hypoxic insects initiated their O-phase at different P_{CO_2}, indicating that neither a hypoxia-induced change in P_{CO_2} sensitivity nor crossing a fixed P_{CO_2} ventilatory threshold appears to initiate the O-phase. However, while alternating episodes of gas exchange and apnoea are not eliminated by hypoxia, some aspects of the DGC are modulated by hypoxia. For example, the decreased minimum haemolymph P_{CO_2} during exposure to 10 kPa O_2 indicates that hypoxia stimulates increased ventilation during the O-phase, resulting in increased CO_2 clearance. Hypoxia was also associated with a significantly decreased interburst duration which contributed to a significantly decreased DGC duration (Fig. 7).

The changes in DGC phases observed in *G. portentosa* here are similar to the findings of Chown and Holter (2000), who found that both C- and F-phase duration in the scarabid beetle *Aphodius fossor* decreased in response to hypoxia, resulting in more frequent ventilatory periods. However, research on the locust *L. migratoria* showed that although hypoxia reduced the C-phase duration, it increased the F-phase duration, resulting in no net change in interburst duration (Snelling et al., 2011). In addition, whereas our research found no significant effect of hypoxia on O-phase duration, hypoxia has variously been observed to decrease (Chown and Holter, 2000), increase or have no substantial effect (Lighton and Garrigan, 1995) on O-phase duration in other insect species. Clearly, there is substantial variation in the effects of hypoxia on DGC phase duration in different species. This considerable interspecific variation is further evidence that DGCs do not arise from predictable ventilatory responses to oscillating gas tension. Overall, our results suggest that although the gas exchange portions of the DGC are modulated by hypoxia, a specific P_{O_2} threshold is not required to trigger phase transitions.

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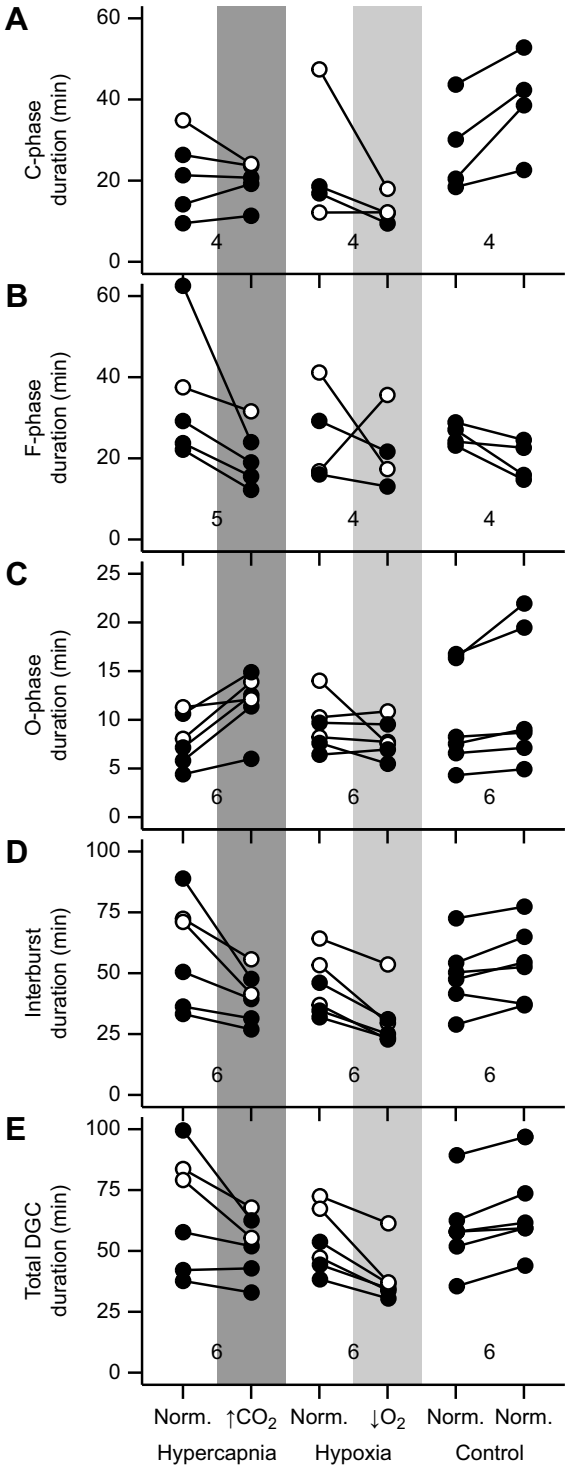

Fig. 7. Mean closed, flutter, open, interburst and total DGC duration in individual *G. portentosa* displaying DGCs during sequential exposure to normoxia and normoxia, hypoxia or hypercapnia. (A) Mean C-phase duration and change relative to normoxia. (B) Mean F-phase duration and change relative to normoxia. (C) Mean O-phase duration and change relative to normoxia. (D) Mean interburst duration and change relative to normoxia. (E) Mean total DGC duration and change relative to normoxia. Lines indicate paired means from the same individual. Black circles represent cockroaches exposed to normoxia in the first 9 h of measurement and treatment gas in the second 9 h; white circles represent the reverse order. Unpaired circles indicate data from single observations. Numbers below circles indicate the number of paired observations for that treatment. Statistical analyses are presented in Table 1.

DGC model predictions and experimental results

Several models of the DGC have been developed that explicitly incorporate fixed P_{O_2} and P_{CO_2} ventilatory thresholds as the key mechanism for generating an episodic gas exchange pattern (Förster and Hetz, 2010; Grieshaber and Terblanche, 2015). The data presented here provide the first complete record of multiple respiratory parameters from a single insect species displaying DGCs in both hypoxia and hypercapnia, thus allowing the predictions of these fixed-threshold models to be tested against empirical observations.

From the fixed-threshold model of Förster and Hetz (2010), it may be predicted that exposure to hypoxia would decrease the duration of the C-phase by reducing the time taken for internal P_{O_2} to fall to the F-phase threshold, and that this in turn would result in a longer F-phase (Fig. 1B). The effect of hypoxia on the duration of the O-phase is not clear as the mechanism that determines O-phase duration has not been defined, other than it is likely to be governed by some unspecified hysteresis. Finally, the maximum haemolymph P_{CO_2} level during the DGC would continue to reach (or exceed) the P_{CO_2} ventilatory threshold to trigger the start of the O-phase. The data presented here do not agree with any of these predictions as exposure to hypoxia caused both the interburst period and total DGC duration to decrease significantly, while O-phase duration did not change. Maximum P_{CO_2} coincident with the initiation of the O-phase also fell significantly in hypoxia. Likewise, the predicted effects of hypercapnia on a fixed-threshold DGC are also not met. As ambient P_{CO_2} approaches the insect's P_{CO_2} threshold, DGC cycle duration should decrease (Fig. 1C), ultimately reaching zero as ambient P_{CO_2} converges on the threshold level and the insect transitions to continuous gas exchange. Assuming this P_{CO_2} threshold is approximately 2 kPa in *G. portentosa*, breathing air with a P_{CO_2} of 2 kPa should have caused their ventilation to become continuous, or at the very least severely curtailed the duration of the DGC. But the data show that not only did DGCs persist in all cockroaches exposed to hypercapnia but also it was associated with only a statistically insignificant reduction in DGC cycle duration (an average decrease of 18±14%, n=6).

A set of mathematical models describing multiple ways in which DGCs could be generated has been developed by Grieshaber and Terblanche (2015), again assuming hysteresis around fixed P_{O_2} and P_{CO_2} thresholds, but using haemolymph pH as a proxy for P_{CO_2}. Three models were presented that could replicate the main features of the DGC, and explicit predictions were made describing how hypoxia or hypercapnia would change the C-, F- and O-phases of these model DGCs. Moderate hypoxia was predicted to reduce the duration of the F-phase and increase the O-phase. Again, the data presented here do not support these predictions: F- and O-phase duration were both unchanged by hypoxia. All three DGC models predicted the same responses to hypercapnia: an increase in both F-phase duration and total DGC duration, while in extreme hypercapnia the breathing pattern would shift to continuous ventilation. These predictions are also not supported, as both F-phase and total DGC duration did not change significantly (and trended towards shorter durations), while O-phase duration, which was not predicted to change, increased significantly in hypercapnia.

Models of the DGC that assume the pattern is governed by fixed-level thresholds fail to accurately predict the behaviour of DGCs displayed by Madagascar hissing cockroaches when perturbed by exposure to hypoxic and hypercapnic atmospheres. This mismatch between predictions and observations leads to an obvious conclusion: a fixed P_{CO_2} threshold is not required to generate DGCs.

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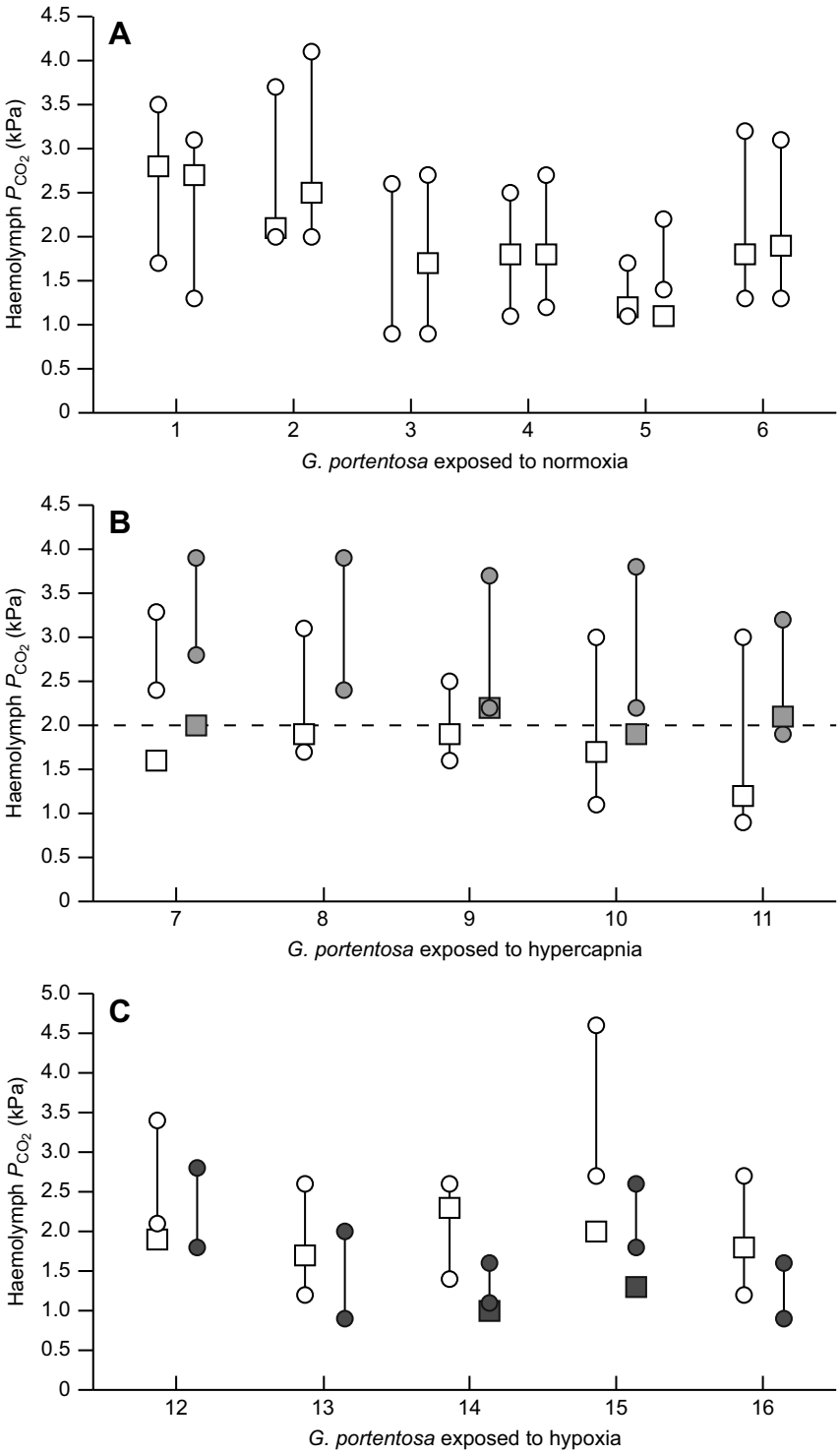

Fig. 8. Mean haemolymph P_{CO_2} measured in 16 individual *G. portentosa* during continuous (squares) and discontinuous (circles) gas exchange, and during exposure to normoxic normocapnia and normoxia, hypercapnia or hypoxia. Paired data show the haemolymph P_{CO_2} of individual cockroaches breathing continuously and discontinuously during exposure to: (A) only normoxic normocapnia (white), (B) normoxic normocapnia and normoxic hypercapnia (light grey) or (C) normoxic normocapnia and hypoxic normocapnia (dark grey). P_{CO_2} during continuous gas exchange was recorded from intact cockroaches during the first 24 h of measurement and DGCs were recorded from the second 24 h following decapitation to induce DGCs. Vertical lines connect the mean minimum and maximum P_{CO_2} during DGCs. The horizontal dashed line in B indicates ambient P_{CO_2} during hypercapnia. P_{CO_2} measured during continuous gas exchange could not be recorded for 5 individuals.

Location of haemolymph P_{O_2} and P_{CO_2} measurement

A key assumption in this study is that the P_{O_2} and P_{CO_2} levels recorded by optodes implanted into the cockroach's pronotal horns are the same as, or at least a proxy for, the levels sensed by respiratory chemoreceptors located elsewhere within the insect. For this to be true, either haemolymph P_{O_2} and P_{CO_2} must be homogeneous within the insect's haemocoel or, if gas tensions vary regionally within the haemocoel, any change in P_{O_2} or P_{CO_2} at the chemoreceptor must be associated with equivalent, coincident changes in these gas tensions throughout the rest of the insect. While regional variation in P_{O_2} and P_{CO_2} within *G. portentosa*'s haemocoel cannot be ruled out, there are three lines of evidence that suggest that haemolymph within the insect's haemocoel is well mixed. First, the rapid changes in haemolymph P_{O_2} and P_{CO_2} measured by the implanted optodes during gas exchange (e.g. Figs 3 and 9) indicate that both these gases equilibrate quickly with the tracheal system at the location of measurement. Second, because the recorded haemolymph P_{O_2} and P_{CO_2} values remained close to ambient levels during periods of gas exchange, this indicates that the pronotal horns are not regionally hypoxic or

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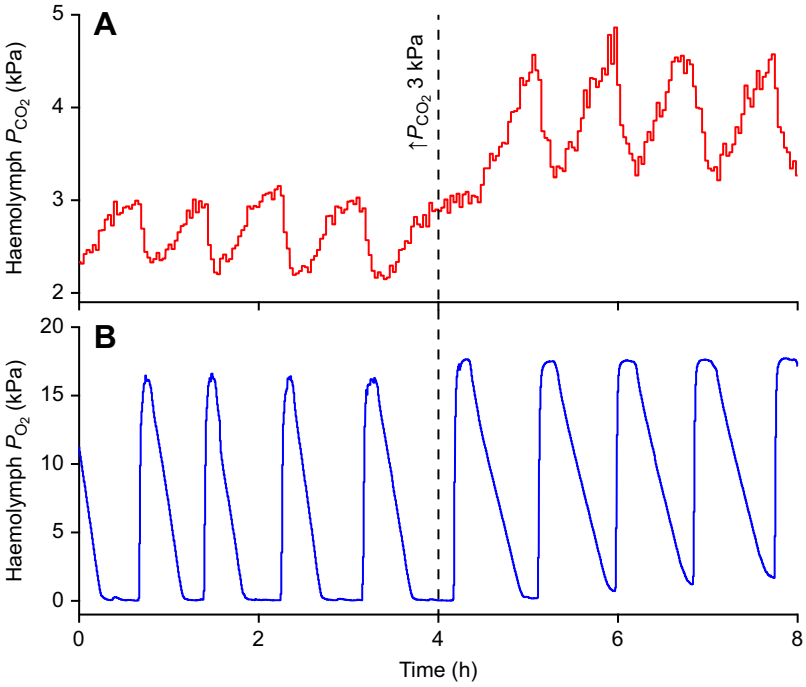

Fig. 9. Traces showing simultaneously recorded *in vivo* haemolymph P_{CO_2} and P_{O_2} from a single decapitated *G. portentosa* during exposure to CO_2-free normoxic air and normoxic hypercapnia (P_{CO_2}=3 kPa). (A) Haemolymph P_{CO_2} (kPa) and (B) haemolymph P_{O_2} (kPa) when exposed to normoxic normocapnia (first 4 h) and normoxic hypercapnia (second 4 h). The dashed vertical line indicates the point at which hypercapnia exposure began.

hypercapnic and that gas exchange at the measurement location occurs without restriction. Finally, as the average duration of the DGC is very long in this cockroach species (~1 h), there is ample time for any localized difference in haemolymph gas tension generated during the C-phase to equilibrate with the air spaces in the tracheal system and the rest of the circulating haemolymph pool. Taken together, these points strongly suggest that haemolymph gas tension within the pronotal horns does not depart significantly from values in the rest of the cockroach's haemocoel.

Conclusion

Although the low sample sizes presented in this study increase the likelihood of type II errors, the results presented here nonetheless provide compelling evidence that insect ventilatory control may be more nuanced than previously described. Continuously breathing *G. portentosa* appear to regulate their ventilation in response to internal P_{CO_2}, keeping haemolymph P_{CO_2} between 1 and 2 kPa in normoxic normocapnic air and hyperventilating to defend this value during exposure to hypercapnia, in agreement with observations made on other insect taxa. DGCs have been hypothesised to emerge as a result of a hysteresis between haemolymph P_{CO_2} and a ventilatory response that is triggered at some fixed P_{CO_2} threshold, with the P_{CO_2} observed during continuous gas exchange being a plausible threshold level. However, our results indicate that this is not the case. Preventing haemolymph P_{CO_2} from falling below the putative 2 kPa ventilatory threshold did not prevent decapitated cockroaches from displaying DGCs despite their significantly elevated haemolymph P_{CO_2}. DGCs also persisted during exposure to hypoxia, despite both maximum and minimum haemolymph P_{CO_2} being significantly reduced compared with levels in normoxic normocapnia. It is possible that the shift from continuous to discontinuous gas exchange is associated with an increase in the ventilatory P_{CO_2} threshold above that seen during continuous gas exchange, as this could explain why DGCs continue when haemolymph P_{CO_2} is significantly elevated above levels that occur while breathing continuously. However, even if this were so, a higher threshold cannot explain why DGCs then persist in hypoxia when haemolymph P_{CO_2} is maintained at a significantly lower level. The data plotted in Fig. 6 clearly illustrate that there are no fixed P_{CO_2}, P_{O_2} or combination of these partial pressures that coincide with the F- to O-phase transition point, in contrast with the assumptions of the fixed-threshold model of the DGC (Fig. 1). Therefore, we conclude that hysteresis around a fixed P_{CO_2} ventilatory threshold, including the P_{CO_2} regulated during continuous gas exchange or some DGC-specific elevated threshold, cannot be responsible for the production of DGCs in these cockroaches. That this long-standing assumption does not apply to at least one species of insect highlights the need to critically test this assumption in other insects. Furthermore, if this exception turns out to be widespread then this could be one explanation why models of insect gas exchange patterns generally fail (Terblanche and Woods, 2018).

If hysteresis around fixed thresholds cannot explain the origin of the DGC, then what other mechanisms could be responsible? Plasticity in respiratory chemoreceptor thresholds could be one possibility. For example, continuous exposure to hypercapnia or hypoxia could cause blunting or acclimation, whereby the respiratory chemoreceptor's threshold sensitivity shifts to a new level relative to ambient conditions. However, this explanation would still require the existence of hysteresis in the insect's ventilatory control loop so that oscillations in P_{CO_2} could emerge around this shifted threshold, thereby generating the alternating phases of the DGC. Alternatively, a protracted refractory period following an O-phase could temporarily suppress any subsequent ventilation from occurring, giving rise to a periodic apnoea. This explanation benefits from the fact that a refractory period could occur independently of chemosensory feedback, allowing DGCs to continue irrespective of ambient hypoxia or hypercapnia.

If DGCs arise as a result of hysteresis, then this pattern must be caused by the insect oscillating between internal hypercapnia and hypocapnia. From this point of view, internal P_{CO_2} should periodically fall well below the ventilation threshold, with the C- and F-phases occurring to correct this deficit. However, given that the maximum P_{CO_2} during a DGC was found to substantially

exceed the P_{CO_2} during continuous gas exchange for every individual and every condition examined here (Fig. 8), it is not unreasonable to suggest that the physiological function of the DGC is to suppress ventilation in order to elevate haemolymph P_{CO_2}, rather than to prevent it from falling too low. This function has been attributed to episodic breathing patterns displayed by dormant land snails, whereby periodic apnoea causes the snail to accumulate CO_2, thereby depressing its blood pH and, potentially, metabolic rate (Barnhart, 1986). Unfortunately, the effect of hypercapnia on the cockroaches' metabolic rate could not be determined in this study, as the proxy for metabolic rate used here ($\dot{V}_{CO_2}$) could only be measured in acapnic treatment gases. However, this explanation for the function of the DGC is worth further investigation, as the P_{CO_2} data presented here reveal that DGCs are associated with P_{CO_2} levels that are substantially elevated relative to those in intact, continuously ventilating cockroaches. But ultimately, what causes the transitions from continuous to discontinuous gas exchange, or transitions between the F-, O- and C-phases within the DGC? These questions still remain unanswered.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: T.T.R., P.G.M.; Methodology: T.T.R.; Validation: M.S.G.; Formal analysis: T.T.R.; Investigation: T.T.R.; Resources: M.S.G., P.G.M.; Data curation: T.T.R.; Writing - original draft: T.T.R.; Writing - review & editing: T.T.R., M.S.G., P.G.M.; Visualization: T.T.R.; Supervision: P.G.M.; Project administration: P.G.M.; Funding acquisition: P.G.M.

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Data availability
All data are available from Dryad (Matthews et al., 2022): https://doi.org/10.5061/dryad.b8gtht7ct).

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