ON THE REGULATION OF INSECT DISCONTINUOUS GAS EXCHANGE CYCLES: THE ROLE OF HEMOLYMPH OXYGEN AND CARBON DIOXIDE ON VENTILATORY RHYTHMS IN MADAGASCAR HISSING COCKROACHES

by

Tormod T.C. Rowe

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Examining Committee:

Philip G.D. Matthews, Associate Professor, Department of Zoology, UBC
Supervisor

William K. Milsom, Emeritus Professor, Department of Zoology, UBC
Supervisory Committee Member

Ben Matthews, Assistant Professor, Department of Zoology, UBC
Additional Examiner

Additional Supervisory Committee Members:

Colin J. Brauner, Full Professor, Zoology, UBC
Supervisory Committee Member

Abstract

Many evolutionarily distant species of insect display an episodic pattern of breathing termed a discontinuous gas exchange cycle (DGC), defined by bursts of ventilation interspersed with long apnoeic periods. Internal O_2 and CO_2 are not tightly regulated during DGCs as PO_2 and PCO_2 fluctuate significantly. It is as of yet unknown what mechanisms drive the emergence of DGCs, although one hypothesis states that DGCs arise from an unstable ventilatory control system that is unable to quickly respond to fluctuations in internal PO_2 and PCO_2 resulting in alternating cycles of ventilation and apnoea. Essentially, this hypothesis suggests that a temporal lag present between chemoreception of CO_2 and a ventilatory response results in CO_2 levels oscillating around a ventilatory CO_2 threshold as ventilation is turned on and off. This hypothesis is tested in this thesis by implanting PO_2 and PCO_2 optodes into the hemocoel of Madagascar hissing cockroaches, to measure hemolymph PO_2 and PCO_2 fluctuations *in vivo* during periods of continuous and discontinuous ventilation. Additionally, rates of CO_2 release were measured using a flow-through respirometry setup, and ventilatory frequency was measured using an infrared phototransistor. The stable hemolymph PCO_2's measured during continuous ventilation were assumed to represent the CO_2 threshold stimulating gas exchange, and these levels were compared with the CO_2 fluctuations during DGCs elicited in decapitated cockroaches. Cockroaches were also exposed to hypoxia (low O_2) and hypercapnia (high CO_2) in order to artificially manipulate hemolymph PO_2 and PCO_2. Decapitated Madagascar hissing cockroaches were observed maintaining DGCs with internal O_2 and CO_2 levels outside of the assumed threshold values. Results suggest that the DGCs displayed by Madagascar hissing cockroaches are not the result of PO_2 and PCO_2 oscillating around fixed ventilatory thresholds. However, it was observed that patterns of DGCs, such as ventilatory burst duration, interburst duration, and ventilation pattern were altered by exposure to hypoxia and hypercapnia.

Lay Summary

Many insects display discontinuous gas exchange cycles (DGCs), characterised by the long periods of breath holding interspersed with ventilatory movements. It is still unknown what drives insects to breathe episodically. However, previous research has hypothesised that DGCs emerge due to an unstable ventilatory control system turning ventilation on or off in response to chemoreception of oxygen and carbon dioxide around relative ventilatory thresholds. Essentially the hypothesis states that by turning ventilation on or off, the unstable system perpetuates further fluctuations in oxygen and carbon dioxide concentrations around threshold levels, resulting in further periods of apnoea or ventilation. To test this hypothesis, oxygen and carbon dioxide probes were implanted into Madagascar hissing cockroaches and DGCs were elicited by decapitation. Cockroaches were then exposed to hypoxia (low oxygen) and hypercapnia (high carbon dioxide) in order to push oxygen and carbon dioxide levels outside of normal ranges. Interestingly, we showed that cockroaches were able to maintain DGCs independently of feedback from specific oxygen and carbon dioxide setpoints. However, we did find that hypoxia and hypercapnia exposure caused changes to certain ventilatory patterns exhibited during DGCs.

Preface

This thesis is the original, unpublished work by the author, Tormod T.C. Rowe, with editorial feedback from my supervisory committee, Drs Philip G.D. Matthews, Colin J. Brauner, and William K. Milsom.

I carried out all experiments and analyses. Dr Matthews was the supervisory author and was involved in the conception of all experiments.

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List of Symbols and Abbreviations

Δ	Change
ºC	Degrees Celsius
CO_2	Carbon dioxide
C- phase	Closed phase of the DGC
DGCs	Discontinuous gas exchange cycles
F- phase	Flutter phase of the DGC
F_ECO_2	Excurrent CO_2 concentration
f_V	Ventilatory frequency
IR	Infrared (e.g. reflection sensor)
IRGA	Infrared gas analyser
kPa	Kilopascal
MR	Metabolic Rate
N_2	Nitrogen
O_2	Oxygen
O- phase	Open phase of the DGC
PCO_2	Partial pressure of carbon dioxide
PO_2	Partial pressure of oxygen
RH	Relative Humidity
RO	Reverse osmosis (i.e. water)
$\dot{V}CO_2$	Volumetric rate of carbon dioxide release
V_I	Incurrent flow rate
$\dot{V}O_2$	Volumetric rate of oxygen uptake

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First and foremost, I wish to thank my supervisor Dr. Philip Matthews. He has given me incredible guidance throughout my degree, imparting a breadth of knowledge that surpasses that which is written in this thesis. Without his help, and enthusiasm for our project, my thesis would not have been possible. I also want to extend gratitude to my supervisory committee members, Drs William Milsom and Colin Brauner, who were both always eager to offer consultation throughout my degree and impart their own expertise in, and enthusiasm for comparative physiology.

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Last but not least I wish to thank my family, I would not have gotten this far without such unwavering support.

Chapter 1: General Introduction

1.1 Fundamentals of Animal Gas Exchange

Animals must provide O_2 to, and remove CO_2 from, their tissues. O_2 is required by mitochondria for the production of ATP by aerobic metabolism (Popel, 1989), and CO_2 is produced as a by-product. As CO_2 reacts with water to form carbonic acid (Hill et al., 1973), dissolved concentrations of CO_2 are inversely related to the pH of body fluids. An exchange of O_2 and CO_2 between the animal and its environment is therefore required to meet energy demands and maintain acid-base balance. The demand an animal has for O_2, and the rate that an animal produces CO_2, is determined by the amount of energy an animal expends over a period of time, referred to as the animal's metabolic rate (MR). If an animal has a low metabolic rate, a body with a high surface area to volume ratio, and possesses a thin, permeable integument, passive diffusion can be sufficient for environmental gas exchange. However, passive diffusion will not exchange sufficient O_2 or CO_2 in relatively large animals, or in animals with elevated metabolic rates. Instead, these animals possess specialised respiratory structures, which enable high gas exchange rates across high surface areas of thin gas permeable membranes. These specialised respiratory structures require active ventilation to meet their gas flux requirements. Animals encounter varying gas exchange demands depending on changing activity states and corresponding changing metabolic rates, as well as changes in ambient O_2 or CO_2 levels. Accordingly, animals have evolved responses to changing internal O_2 and CO_2 levels, notably, modulating responses in ventilatory motor programmes (Ma and Ringstad, 2012). Many animals display continuous ventilation, identified by a constant exchange of O_2 and CO_2 between an animal and its environment. Episodic ventilatory patterns can also be observed across many different taxa, visible as alternating periodic patterns of high and low ventilatory activity. One extreme episodic breathing pattern which has been extensively studied in a variety of insect species is the discontinuous gas exchange cycle (DGC). DGCs are defined by an alternating sequence of apnoeic periods and bursts of ventilation (Lighton, 1996). The remainder of this introduction will review the current understandings of ventilatory control systems, ultimately leading towards a testable hypothesis for the mechanism of DGC control.

1.2 Regulation of internal O_2/CO_2

For an animal to maintain an internal respiratory environment within physiologically acceptable limits, it requires three things: 1. a mechanism for sensing internal levels of O_2, CO_2 and/or pH, 2. the ability to compare this detected level to a required level, and 3. a ventilatory system regulated in a manner that reduces the difference between the detected and required levels (Matthews and Terblanche, 2015, Khoo, 2000, González et al., 1995)(Fig. 1). Animals possess specialised chemoreceptors which sense internal PO_2, PCO_2 and/or pH. Changes in blood-gas variables detected by chemoreceptors stimulate opposing, corrective changes in ventilation. Animals have a hypoxic ventilatory response, where if chemoreceptors sense declining PO_2, animals will increase the intensity and frequency of their breaths (Ma and Ringstad, 2012). Additionally, animals respond to changing PCO_2/pH either by signalling for increased ventilation if pCO_2 rises/pH falls, or decreased ventilation if PCO_2 falls/pH rises. Chemoreception of O_2 and CO_2/pH will alter ventilation, and any change in ventilation will in turn alter the levels of O_2, CO_2 and/or pH which are stimulating chemoreceptors. Thus, animal ventilation is regulated using feedback loops between chemoreceptors and ventilatory motor programmes (Fig. 1). Ventilatory systems regulate levels of O_2, CO_2 and/or pH through negative feedback around threshold levels, where a suitable ventilatory response is employed if internal O_2 or CO_2 deviates from these levels.

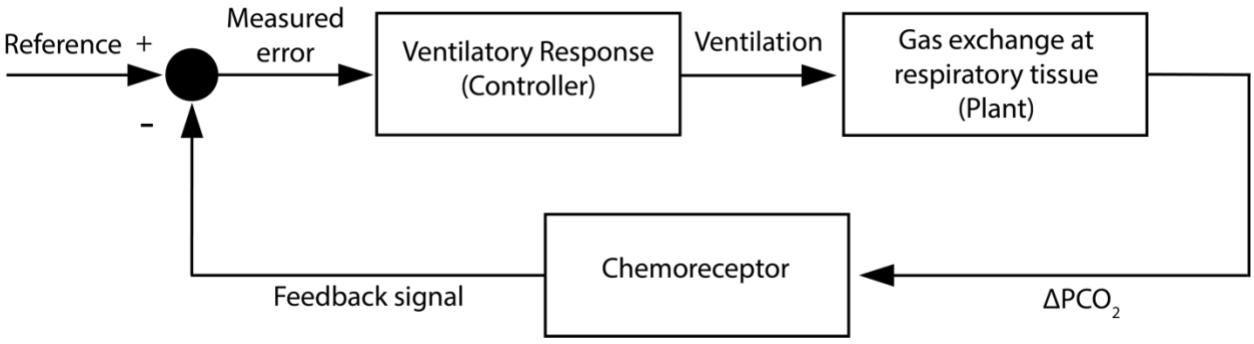

Figure 1. Model illustrating the feedback loop of a generalised ventilatory control system regulating internal PCO_2.

If ventilation responded instantaneously to changing O_2 and CO_2/pH then O_2 and CO_2/pH would never deviate significantly from threshold levels. However, delays exist within a ventilatory control system between the chemoreception of O_2 and CO_2/pH and the instigation of corrective responses. Delayed ventilatory responses result in O_2 and CO_2/pH continually rising above or falling below ventilatory threshold levels. The degree of stability of control within a respiratory system's negative feedback loop will be determined by both controller gain, which is the magnitude of the corrective response that occurs for a given change PO_2 and PCO_2, and plant gain, which is the effect that a corrective response has on changing the level of PO_2 and PCO_2/pH (Khoo, 2000). If the controller gain is too high within a ventilatory system, then deviating O_2 and CO_2/pH will cause an excessive corrective ventilatory response. Equally, if the plant gain is too high, then any corrective ventilatory response will dramatically change PO_2 and PCO_2/pH. The combined effect of controller gain and plant gain is termed loop gain (Khoo, 2000) and is predominantly responsible for determining the stability of a self-regulating system. If loop gain within a ventilatory system is low, then corrective responses to deviating O_2 and CO_2/pH will not result in further significant deviations from the threshold levels, maintaining homeostasis. However, if loop gain is too high then corrective responses to deviating O_2 and CO_2/pH will cause greater deviations around the thresholds, resulting in a sustained instability within the system (Khoo et al., 1982). A repeated pattern of unstable corrective responses from the ventilatory system would result in periodic bouts of elevated and depressed ventilation, exhibited as patterns of episodic breathing.

1.3 Episodic Breathing

A well understood example of how instability in a ventilatory system causes sustained episodic breathing can be seen in humans suffering from cardiac arrest and displaying Cheyne-Stokes respiration. Heart failure during cardiac arrest slows down the circulation of blood within the circulatory system. This causes a significant lag between the time when blood exchanges O_2 and CO_2 with air in the alveoli and the time which blood PO_2 and PCO_2 are sensed by chemoreceptors (Cherniack and Longobardo, 1973). Slower blood flow within a cardiovascular system also causes arterial hypoxemia (low blood pO_2), as the supply of O_2 does not fulfil the respiratory demand. Hypoxemia increases chemosensory sensitivity to PCO_2, the equivalent of increasing controller gain within the system (Cherniack and Longobardo, 1973). The significant

delay between the chemoreceptors and the ventilatory driver, paired with the increased controller gain results in control instability and the elicitation of periodic breathing (Cherniack and Longobardo, 1973).

Episodic breathing is not purely a pathological condition. A multitude of diverse species display episodic breathing naturally, often as part of a repertoire of different ventilatory patterns. The functional significance of episodic breathing is still poorly understood in these animals. However, the appearance of episodic breathing often coincides with particular physiological states. For example, breathing becomes discontinuous in many vertebrate species when metabolic rate and/or respiratory drive are low, such as is common in small mammals entering hibernation (Milsom et al., 1997, Milsom and Jackson, 2011). Another, distantly related example is the periodic ventilation observed in many decapod species, such as the brown crab (*Cancer pagurus*), where this pattern also occurs during inactivity (Burnett and Bridges, 1981, Metzger et al., 2007). The mechanisms responsible for producing episodic breathing are not all universally understood. For example, episodic breathing in the American bullfrog (*Rana catesbeiana*) has been shown to persist even in the absence of internal PO_2 and PCO_2/pH fluctuations (Kinkead and Milsom, 1994), indicating that factors other than feedback loop instability may drive these oscillations.

Discontinuous gas exchange cycles (DGCs) are an episodic breathing pattern described in a diverse range of insect species, generally consisting of long periods of apnoea interspersed with clusters of ventilatory bursts. The functional significance of DGCs is still widely disputed (Buck et al., 1953, Lighton, 1998, Hetz and Bradley, 2005), and the underlying mechanisms driving the appearance of DGCs remain unknown. It has been predicted that DGCs arise from unstable ventilatory control systems responding to PO_2 and PCO_2 fluctuations (Chown and Holter, 2000). However, this prediction has not yet been tested experimentally. The general insect respiratory control system is outlined in the following section, leading to a hypothesis for DGC regulation by an unstable ventilatory control system.

1.4 Insect Respiratory Physiology

Insects, unlike most animals, do not rely on a circulatory system for gas exchange. Instead, most insects transport respiratory gasses directly between the atmosphere and respiring tissues via an extensive air-filled tracheal system (Fig. 2). The tracheal system is comprised of tracheae, which are a network of cuticular tubes responsible for the transport of respiratory gasses throughout the insect body (Chapman and Chapman, 1998, Westneat et al., 2003). Also distributed within the tracheal system may be relatively larger air sacs (Chapman and Chapman, 1998), acting as bellows to increase the volume of air pumped during active ventilation (Wigglesworth, 1963) and assisting the movement of gas through the trachea. Tracheae subdivide into finer tracheoles, which are blind ending tubes with a ~1μm diameter (Wigglesworth, 1983), and contribute the majority of the tracheal system's surface area. Tracheoles penetrate directly into respiring tissues and are responsible for approximately 90% of the tracheal system's lateral diffusing capacity (Snelling et al., 2011). The entire tracheal system is gated from the atmosphere by pores called spiracles. Usually, eight to ten pairs of spiracles are located along the lateral sides of the thorax and abdomen. Spiracles possess muscular valves which open or close in response to stimulation by nerves originating from their corresponding ganglia (Case, 1957). Open spiracles facilitate the exchange of respiratory gas between the atmosphere and the tracheal system.

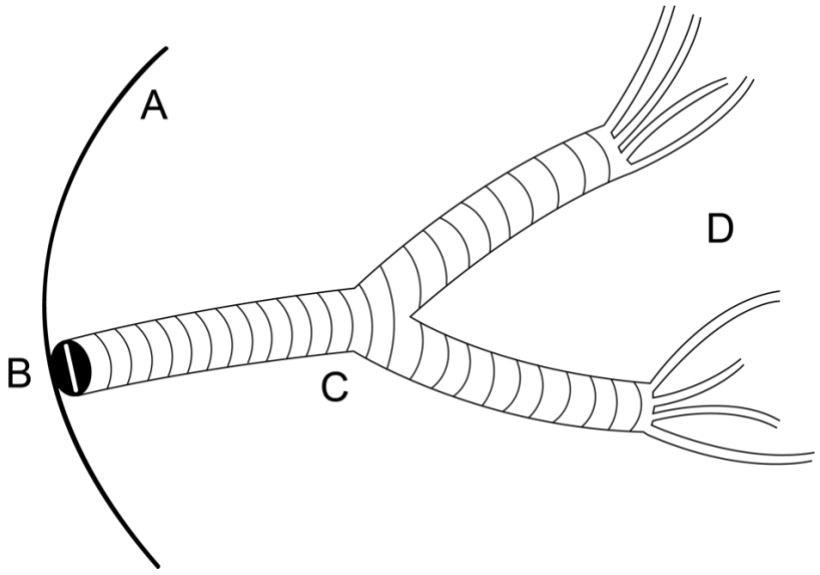

Figure 2. Simplified drawing of the insect tracheal system. A) represents the insect cuticle; B) illustrates the insect spiracle; C) illustrates trachea branching into two divisions; D) illustrates multiple branching tracheoles.

Gas exchange in small or inactive insects is characterised by passive diffusion through partially open spiracles (Lighton, 1996). However, larger insects, or insects with high MRs require a combination of convection and diffusion to meet their gas-exchange needs (Harrison et al., 2013, Matthews and Terblanche, 2015). Ventilatory patterns, likely controlled by pattern generators responding to feedback from chemoreceptors (Burrows and Horridge, 1974, Miller, 1960), drive the convective flow of respiratory gases through the tracheal systems of these larger insects. Dorsoventral muscles located in each abdominal segment compress the hemolymph, in turn compressing air sacs and trachea, forcing a convective flow of air through the tracheal system (Socha et al., 2008). Actively ventilating insects often display unidirectional airflow from anterior to posterior (Weis-Fogh, 1967, Harrison et al., 2013). Unidirectional air flow through the tracheal system can be produced by inhaling air through open thoracic spiracles while abdominal spiracles are closed, and exhaling air through open abdominal spiracles while thoracic spiracles are closed (Heinrich et al., 2013). It is important to note that the activity state of the insect will alter its spiracle activity and ventilatory movements, altering the pattern of airflow within the tracheal system. For example, the differential grasshopper (*Melanoplus differentialis*) transitions from unidirectional air flow to tidal breathing in distinct body compartments when exposed to different temperatures or changing ambient PCO_2 (Henderson et al., 1998). Different patterns of spiracle activity and abdominal contractions give rise to distinct ventilatory patterns.

1.5 Insect Ventilatory patterns

Ventilatory patterns occur across a spectrum ranging from continuous to episodic. The most commonly described and clearly defined patterns of insect gas exchange are:

1. Continuous gas exchange, identified by the lack of clear and sustained spiracle closure, typically resulting in a non-zero value of gas exchange (Matthews and White, 2011b)
2. Cyclic gas exchange, identified by alternating periods of high and low ventilation (Marais et al., 2005)
3. Discontinuous Gas exchange Cycles (DGCs), identified primarily by a sequence of prolonged apnoeic periods alternating with bursts of ventilation (Lighton, 1996)

The following sections discuss the regulation of continuous gas exchange and consider whether these same regulatory mechanisms are responsible for the production of discontinuous gas exchange cycles.

1.5.1 Continuous Gas Exchange

Continuous gas exchange is displayed by all insect species, most commonly when they are active (Contreras and Bradley, 2009). As in other animals, it is generally assumed that insects displaying continuous gas exchange must regulate their internal PO_2 and PCO_2/pH within acceptable limits by sensing deviations outside of these levels and then enacting a suitable ventilatory response via a feedback loop. Insects exposed to hypoxia (low ambient PO_2) and hypercapnia (high ambient PCO_2) display appropriate corrective responses by opening their spiracles and increasing ventilation. Thus, the frequency of spiracle opening has been shown to be inversely related to ambient PO_2 (Case, 1956, Wigglesworth, 1935), as is ventilatory frequency (Harrison et al., 2006, Bustami et al., 2002). Exposure to hypercapnia, too, stimulates both spiracle opening (Förster and Hetz, 2010) and hyperventilation (Matthews and White, 2011b, Henderson et al., 1998). The PCO_2 or PO_2 levels required to stimulate insect breathing vary between species (Matthews and Terblanche, 2015), although in terrestrial insects an increase in inhaled PCO_2 will have a greater effect on breathing than an equal decrease in inhaled PO_2 (Matthews and White, 2011b). Ventilation can be driven independently by either hypoxia or hypercapnia, or synergistically, where exposure to both hypoxia and hypercapnia results in a greater ventilatory response than the sum of the independent responses (Matthews and White, 2011b). It has also been shown in the desert locust, *Schistocerca americana,* that either elevating tracheal PO_2 above, or reducing tracheal PCO_2 below normal resting levels, result in a marked decrease in ventilatory frequency (Gulinson and Harrison, 1996). These experiments show that continuously breathing insects sense internal PO_2 and PCO_2 levels and modulate their gas exchange by generating an appropriate corrective response when either PO_2 or PCO_2 deviate from some defended threshold level.

1.5.2 Discontinuous Gas Exchange Cycles

DGCs are distinguished from cyclic gas exchange patterns by the presence of complete apnoea between bursts of gas exchange. Stereotypical DGCs consist of three repeating phases of spiracle activity: The closed phase, the flutter phase, and the open phase (Lighton, 1996) (Fig. 3). During the closed phase, spiracles are closed and both O_2 uptake and CO_2 release is negligible (Buck and Keister, 1955). As a result, tracheal O_2 is consumed and internal PCO_2 increases. Due

to the high solubility of CO_2 in hemolymph (Buck and Keister, 1955), and a respiratory quotient ($\dot{V}CO_2/\dot{V}O_2$) less than one, there is less CO_2 released into the tracheal system than there is O_2 consumed. This causes the pressure inside the tracheal lumen to decline during the closed phase. The closed phase then transitions into a flutter phase, where spiracles begin to open and close rapidly but sporadically. At the beginning of the flutter phase, the endotracheal pressure in the tracheal system can be lower than the external atmosphere, and thus when the spiracles open initially, there is a convective flow of air into the tracheal system. As a result, some O_2 is taken up but minimal CO_2 is released. During the flutter phase, the rate of O_2 uptake is just sufficient to satisfy the insect's aerobic demands so tracheal PO_2 remains low and relatively constant (Lighton, 1996), whereas internal PCO_2 continues to increase (Levy and Schneiderman, 1966). Finally, the flutter phase gives way to the open phase as all spiracles open. This often coincides with a burst of abdominal ventilation. The quantity of CO_2 exhaled during the open phase can be as much as 90% of the CO_2 accumulated in each cycle (Levy and Schneiderman, 1966, Harrison et al., 1995). These three phases reoccur in this order indefinitely during DGCs.

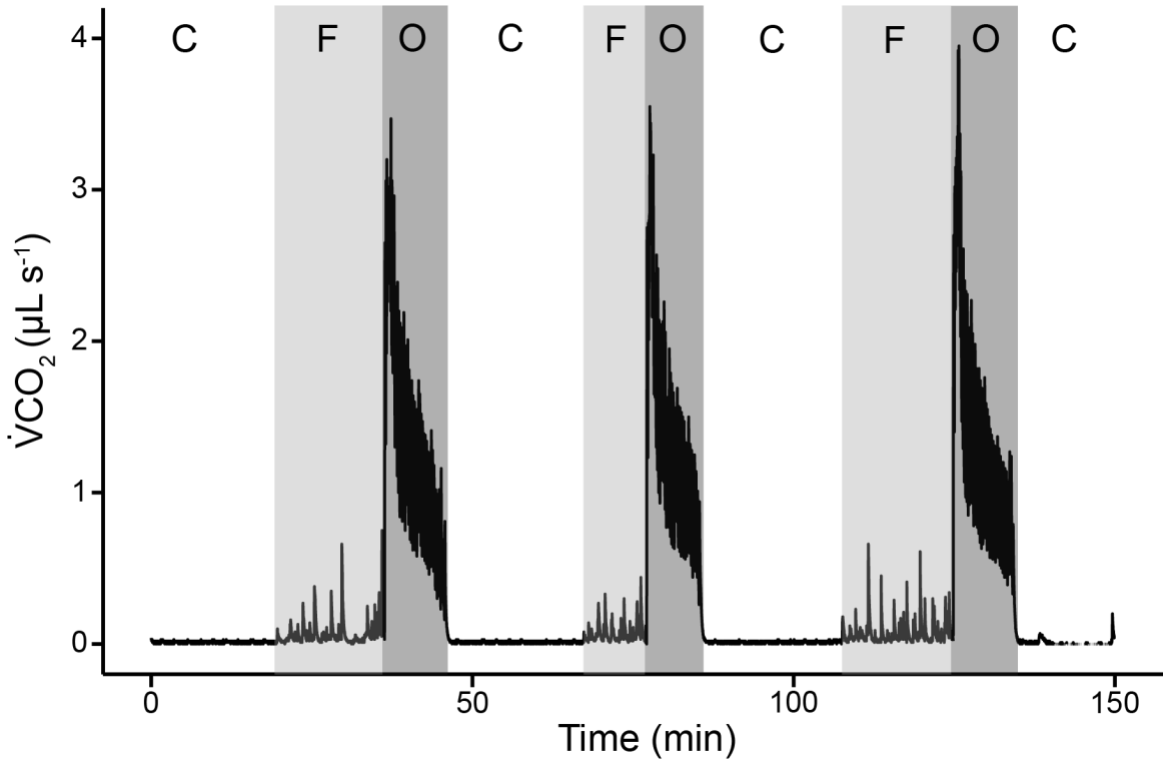

Figure 3. Respirometry trace showing $\dot{V}CO_2$ release during a textbook example of the repeating three-phase DGC from an individual *G. portentosa*. The white regions indicate the closed phases (C), the light grey regions indicate the flutter phases (F), and the dark grey regions indicate the open phases (O).

1.5.3 Proposed Mechanism of DGC Regulation

Basic questions still remain about the nature of the mechanisms that drive DGCs. Specifically, what drives the transitions between the three phases? The Emergent Property Hypothesis (Chown and Holter, 2000) proposes that DGCs are a nonadaptive outcome of interactions between the O_2 and CO_2 set points that regulate spiracle opening and closure (Chown et al., 2006). Essentially, the same hypoxic and hypercapnic thresholds which appear responsible for stimulating continuous ventilation are conserved between breathing patterns. In support of this, previous research on cecropia moth pupae (*Hyalophora cecropia*) found that the transitions between the closed, flutter, and open phases occurred when tracheal PO_2 and PCO_2 levels crossed fixed hypoxic and hypercapnic thresholds (Burkett and Schneiderman, 1974). Förster and Hetz (2010) further demonstrated that *H. cecropia* moth pupae would open their spiracles at a specific hypoxic or hypercapnic threshold level. All spiracles were observed to open once tracheal PCO_2 rose above a threshold level of ~1-1.5 kPa, or when tracheal PO_2 dropped below a threshold level of ~2.6 kPa. These thresholds operate independently, but the PO_2 threshold was identified as showing some dependence on PCO_2. Spiracular fluttering was observed as the internal PO_2 approached the hypoxic threshold, a behaviour that was hypothesized to result from rapid changes in O_2 influx due to the rapid opening and closing of the spiracles, resulting in slight fluctuations in PO_2 around these thresholds. From these experiments, Förster and Hetz (2010) proposed a two-phase model whereby DGCs arise as tracheal O_2 and CO_2 levels fluctuate around these fixed hypoxic and hypercapnic thresholds (Fig. 4).

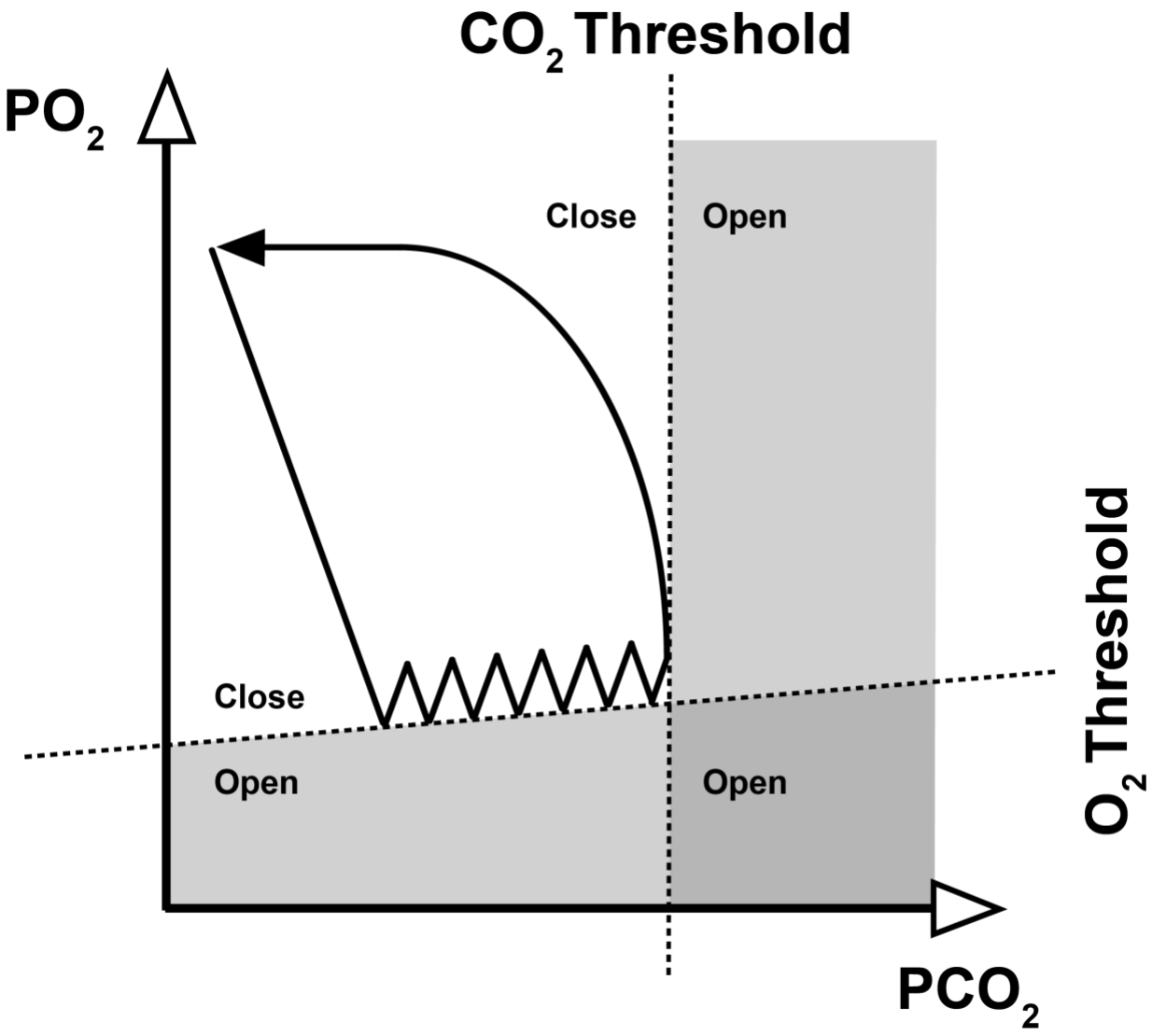

Figure 4. Phase space map of spiracle behaviour producing DGCs. Open/Close labels along the threshold lines denote the spiracle activity of the respective control system. The line running counterclockwise denotes PO_2 and PCO_2 fluctuations during the DGC. Spiracles remain open in any of the shaded region. Redrawn from Förster & Hetz (2010).

In the proposed two-phase model, high internal PO_2 and low PCO_2 would result in closed spiracles, and the reverse would result in open spiracles. When spiracles are closed, no gas exchange occurs which results in decreasing internal PO_2 and increasing internal PCO_2. If internal PO_2 declines below the O_2 threshold, spiracles would then open, resulting in an uptake of O_2. If spiracles responded to this hypoxic threshold fast enough, this elevated PO_2 would result in spiracles closing again as internal PO_2 rose above the threshold. The rapidly opening and closing spiracles resulting from this non-steady state would be observed as the flutter phase. During the flutter phase, tracheal PO_2 remains stable whereas tracheal PCO_2 increases. The increasing internal PCO_2 would eventually reach the hypercapnic threshold level, and all spiracles would open. If a delayed response to falling PCO_2 is included in the model, internal PCO_2 would fall far below threshold levels, and tracheal O_2 would rise back to the original levels. These exceedingly low PCO_2 levels and high PO_2 levels would result in an extended period with closed spiracles, and the cycle would repeat itself.

For the phase space model to be plausible, chemoreceptors with both a fast response to PO_2 and a delayed response to PCO_2 chemoreception would be required. It is still not understood why spiracles remain open when both PO_2 and PCO_2 are on the 'closed' side of the spiracle thresholds during the protracted open phase. The delayed response to falling CO_2 is hypothetical and does not currently have experimental support. However, supporting the plausibility of this hypothesis, computer modelling by Grieshaber and Terblanche (2015) has shown that with physiologically relevant parameters, but including a hypothetical delayed response in one of two chemoreceptor feedback loops, realistic DGCs can be produced. However, the validity of this threshold model has not been confirmed experimentally and measuring all relevant parameters has never been attempted in a pupal insect, let alone a non-quiescent insect.

This thesis aims to investigate whether significant fluctuations in PO_2 and PCO_2 levels around ventilatory thresholds are responsible for the production of insect DGCs. This study uses Madagascar hissing cockroaches (*Gromphadorhina portentosa*) (Schaum 1853) as a model organism for the study of DGCs. This species of cockroach was ideal for this study due to the reliable display of DGCs following decapitations. The DGCs observed showed apnoeic periods which were considerably longer (~30min) than those observed in other species of cockroach, leading to substantial fluctuations in hemolymph PCO_2 and PO_2. Additionally, males of this species possess two enlarged pronotal horns which were beneficial for instrumentation.

Miniaturized fiber optic PO_2 and PCO_2 probes were implanted into the hemocoel of multiple *G. portentosa*, enabling the first successfully reported *in vivo* measurements of hemolymph PO_2 and PCO_2 fluctuations during different breathing patterns in varying ambient gas mixtures. Additionally, both abdominal ventilation frequency and exhaled CO_2 were measured in order to further measure how exposure to different gas mixtures affected patterns of gas exchange during DGCs.

The ultimate goal of this thesis is to test the hypothesis that DGCs arise as a natural consequence of a delayed ventilatory response to PCO_2 resulting in oscillations around a fixed PCO_2 threshold.

Chapter 2: Chemoreception of O_2 and CO_2 During DGCs

2.1 Introduction

Insects transition between a variety of respiratory patterns depending on their physiological state and life stage (Terblanche and Woods, 2018), ranging from continuous to episodic breathing patterns. Continuous gas exchange is generally observed in insects during states of elevated activity or MR and is defined by the continuous exchange of respiratory gases between insect and environment, observed as a non-zero value of CO_2 release. Conversely, insects are capable of displaying episodic breathing patterns, identified by bouts of ventilation alternating with bouts of apnoea. One such extreme episodic breathing pattern is the discontinuous gas exchange cycle (DGC) which consists of three repeating phases of ventilatory activity (Fig. 4). First, the closed phase (C-phase) is identified by closed spiracles and an absence of gas exchange. Next, a flutter phase (F-phase) can sometimes be observed, whereby spiracles open and close rapidly and asynchronously. During the F-phase it has been shown that some O_2 can be taken up by the insect, resulting in a relatively constant tracheal PO_2, whereas internal PCO_2 continues to increase (Levy and Schneiderman, 1966). Finally, the open phase (O-phase) occurs, identified by open spiracles, significant ventilation, and burst of O_2 uptake and CO_2 release. These three phases repeat themselves during a sustained DGC.

It has been proposed that DGCs lay on a continuum of ventilatory patterns driven by metabolic demand, with episodic breathing patterns displayed during states of low MR (Bradley, 2007). In support of this metabolic rate hypothesis, DGCs are commonly observed in insects displaying reduced physical activity and therefore decreased MR. Artificially reducing the MR of the ailanthus silkmoth (*Samia synthia)* by exposure to cold temperature correlates with increased DGC occurrence (Moerbitz and Hetz, 2010). However, other studies have reported DGCs observed independently of changing MR (Heinrich and Bradley, 2014, Williams et al., 2010). The presence of DGCs may therefore be attributed a state of quiescence, rather than the reduced MR which coincides with this state. In agreement, Kestler (1985) showed DGCs were more readily expressed in *Periplaneta americana* exposed to cold temperatures but only when the animal was undisturbed. Additionally while testing if DGCs are indicative of a sleep like state independent of MR, Matthews and White (2013) reliably elicited DGCs in the cockroach *Nauphoeta cinerea* by applying a cold block to the heads of the insects. In the absence of

ventilatory control from higher neural centres during states of quiescence, abdominal and thoracic ganglia were hypothesised to control ventilation, resulting in the emergence of DGCs (Matthews and White, 2011a). This hypothesis, called the neural hypothesis, may also explain why many species of insects, particularly *G. portentosa*, display DGCs following decerebration, as DGCs emerge as a result of a lack control from cerebral ganglia.

There is currently little consensus on the adaptive function of DGCs, with the purported benefits to displaying DGCs not found universally between all species. Instead, DGCs have been hypothesised to emerge as a nonadaptive outcome of interactions between the O_2 and CO_2 set points that regulate spiracle opening and closure during periods of low respiratory demand (Chown et al., 2006). Research by Förster and Hetz (2010) on the atlas moth pupae (*Attacus atlas*) observed clear hypoxic and hypercapnic ventilatory thresholds. If intratracheal PCO_2 was fixed above the perceived hypercapnic threshold, all spiracles opened, and if PCO_2 was fixed below this threshold then all spiracles closed. Alternately, if intratracheal PO_2 was fixed above the hypoxic threshold spiracles would close, whereas if PO_2 was fixed below this threshold then all spiracles were fixed in an open state. The presence of these O_2 and CO_2 thresholds clearly allow these pupae to enact a ventilatory response to correct deviating O_2 or CO_2 levels, controlling internal PO_2 and PCO_2 around preferred setpoints. Förster and Hetz (2010) illustrate a mechanism for how DGCs emerge through unstable fluctuations in PO_2 and PCO_2 around these hypoxic and hypercapnic thresholds (Fig. 4). They hypothesised that DGCs may emerge due to a delayed ventilatory response to changing CO_2 levels. Thus, a delayed response to rising PCO_2 during the closed phase causes CO_2 to over accumulate within an insect, and when ventilation is finally triggered (i.e., the O- phase begins), a delayed response to falling CO_2 causes internal PCO_2 to fall past the CO_2 threshold, resulting in a compensatory ventilatory apnoea (C- phase), further perpetuating the unstable cycle. A separate hypoxic ventilatory threshold was proposed to be responsible for the F-phase, where a faster responding system opened and closed spiracles as O_2 was inhaled and respired. Grieshaber and Terblanche (2015) attempted to simulate the behaviour of a DGC using a numerical model. Using physiologically relevant parameters obtained from measurements on orthoptera, they showed that incorporating hysteresis around a CO_2 ventilatory threshold could produce an episodic pattern of gas exchange that matched that of a DGC. However, how such a protracted temporal delay between CO_2 chemoreception and a

ventilatory response could arise in an insect has not been determined, and the proposed hysteresis mechanism for the production of DGCs remains hypothetical.

The primary aim of this thesis was to determine if hysteresis around a CO_2 threshold was responsible for the production of DGCs. To test this, micro-fiber PO_2 and PCO_2 optodes were implanted into the hemocoel of *G. portentosa*. Hemolymph PO_2 and PCO_2 were measured simultaneously in intact cockroaches displaying continuous ventilation, as well as in decapitated cockroaches displaying DGCs. Cockroaches were also exposed to different ambient O_2 and CO_2 gas tensions in order to manipulate hemolymph PO_2 and PCO_2 beyond expected ventilatory thresholds. In addition, rate of CO_2 release ($\dot{V}CO_2$) was measured using an infrared gas analyser, while a reflection sensor monitored the insect's abdominal ventilatory movements. We predicted that if hysteresis around a PCO_2 threshold was necessary for the production of DGCs, then altering the level of these gases in the insect's hemolymph above the threshold would eliminate episodic gas exchange.

2.2 Materials and Methods

2.2.1 Experimental Cockroaches

Colonies of *G. portentosa* were maintained in an insectary at the University of British Columbia in two, 65 L black plastic storage containers. The lids of the containers were modified to include a 40 cm × 10 cm ventilation hole covered with aluminium mesh, while the containers were half filled with vertically stacked 12" × 12" cardboard egg flats. The containers were placed on top of resistance heater mats maintained at 31 °C, while a lamp in the room provided light on a 12:12 daylight cycle (Herpstat 4 vivarium controller, Spyder Robotics, Rochelle, IL, USA). Colonies were provided with dry cat food (Friskies Chef's Blend Dry Cat Food, Purina, Mississauga, ON, Canada), apples, oranges, and hydrated polyacrylamide water granules (Soil Moist, JRM Chemical Inc., OH, USA) *ad libitum*. Only adult male cockroaches were used for experimentation to take advantage of their enlarged pronotal horns for instrumentation. Individual cockroaches were isolated and starved for 24 h before experiments. The ages of cockroaches used could not be precisely determined, so similarly sized cockroaches were selected. Cockroaches were weighed to 0.01 mg on an electronic balance (XPE205D, Mettler-Toledo Inc., Mississauga, ON, Canada) directly before experimentation. Mean cockroach mass in g was 12.69 ± 1.86 s.d (n = 18).

2.2.2 Respirometry

2.2.2.1 Generating Different Gas Tensions

Mixtures of O_2 and CO_2 in N_2 were produced using three mass flow controllers (Alicat Scientific, Tucson, Arizona, USA) calibrated for: O_2 /N_2 (0-2 L min^{-1}), N_2 (0-500 mL min^{-1}), and CO_2 (0-50 mL min^{-1}) (Fig. 5a). The accuracy of all flow controllers was checked using an NIST-calibrated primary flow standard (Definer 220, Mesa Laboratories, Inc., Lakewood, CO, USA). Flow rates of each flow controller were regulated using a multi-drop breakout controller (BB3-232, Alicat Scientific) connected to a desktop PC running Flow Vision software (v1.3.13.0, Alicat Scientific). Regardless of the gas composition, the total flow rate of the desired mixture was maintained at 900 mL min^{-1}. A continuous supply of dry normoxic, acapnic 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 in the purge gas were then removed by passing it through 1 L columns of soda-lime and Drierite (W.A. Hammond Drierite Co. Ltd, Xenia, OH, USA). A continuous supply of pressurized 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.

2.2.2.2 Respirometry Setup

Flow-through respirometry was used to quantify the gas exchange pattern of the insect and as a proxy for metabolic rate (Fig. 5a). The total flow rate through the setup was maintained at 900 mL min^{-1}. 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, USA), which regulated the relative humidity (RH) at 70 %. 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 an ABS chamber enclosing a RH/temperature probe (HMP60, Vaisala, Helsinki, Finland), and then into an acrylic respirometry chamber (120 × 35 × 40 mm L×W×H; 168 cm^3) housing an individual cockroach (Fig. 5b). On exiting the respirometry chamber, the airstream was piped out of the incubator,

split in two, and was then directed into two parallel, custom-built, 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” internal diameter TT-070, CD Nova, Surrey, BC, Canada), which ran through the middle of a 70 cm long 0.625" O.D. × 0.375" I.D. 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 by the purge gas (0 % RH), which flowed counter-current to the excurrent airstream at a rate of 500 mL min^{-1} in each column. The two airstreams were recombined after exiting the drying columns, passed through another ABS chamber containing a second identical RH/temperature probe (Vaisala), then finally directed 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 analogue 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:

$$\dot{V}CO_2 = \dot{V}_I \times (F_ECO_2 / 1{,}000{,}000)$$

Where V_I is incurrent flow rate (μL s^{-1}) and F_ECO_2 is the excurrent CO_2 concentration (ppm).

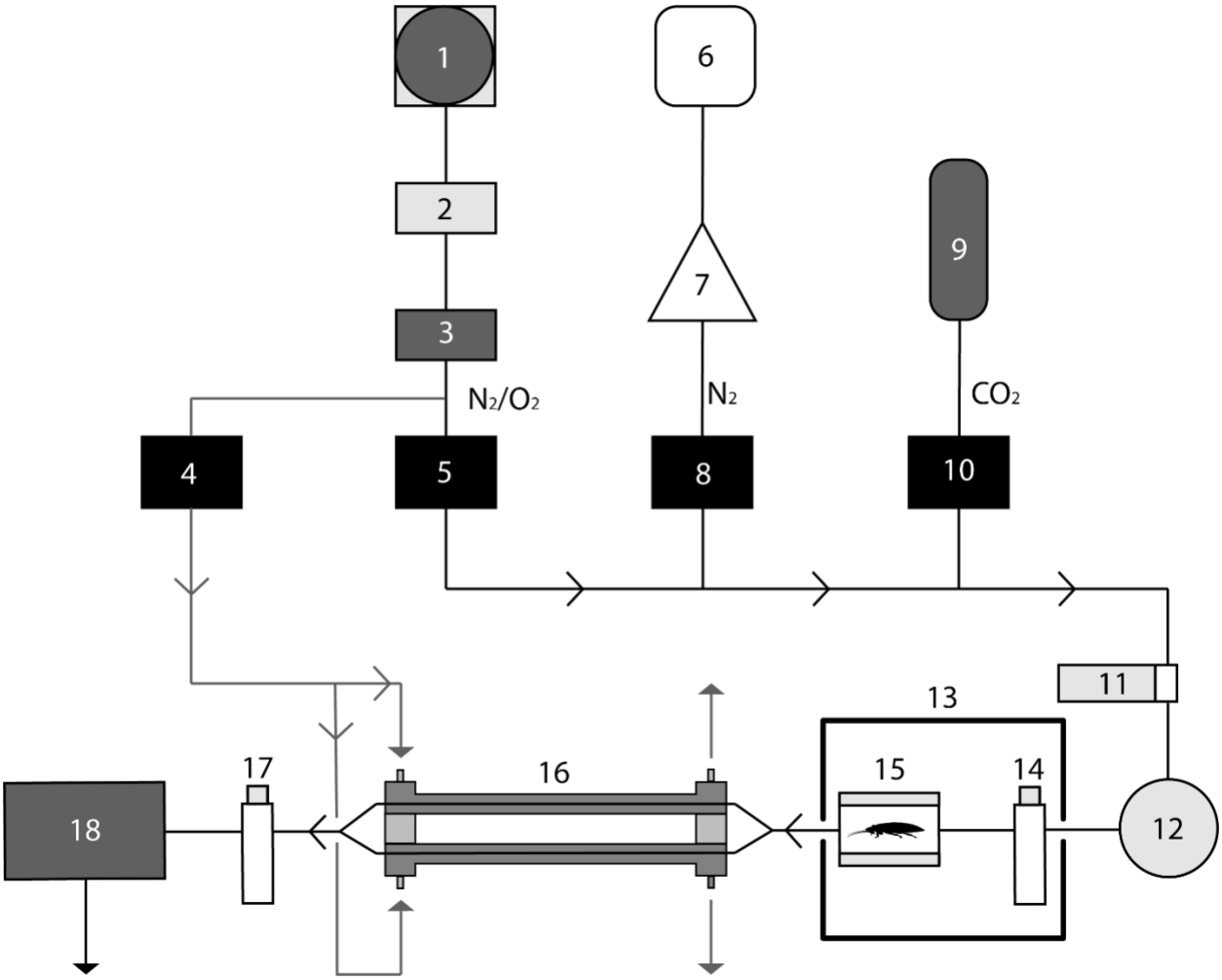

Figure 5a. Respirometry setup.1. Purge gas generator; 2. Sealed column containing Soda-lime; 3. Sealed column containing DRIERITE; 4. 0-500mL min^{-1} flow controller; 5. 0-2L min^{-1} flow controller; 6. Air compressor; 7. N_2 generator; 8. 0-500mL min^{-1} flow controller; 9. CO_2 gas cylinder; 10. 0-50mL 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 IRGA.

2.2.3 Abdominal Ventilation Frequency

To measure abdominal pumping movements associated with gas exchange, the respirometry chamber was outfitted with an IR reflection sensor beneath the cockroach's abdomen (Fig. 5b). The IR sensor (SFH 9202, Osram Opto Semiconductors, Regensburg, Germany) was mounted beneath a window (30 × 20 mm) cut into a removable acrylic plate (120 × 35 × 3 mm) that served as the floor of the chamber. The sensor was connected to a circuit which produced a variable voltage in response to the distance between the sensor and the abdomen. This voltage was sampled at 2 Hz by the Powerlab 8/35 DAQ analogue to digital

converter and recorded using LabChart (v.8.1.5, ADInstruments) on a desktop computer. Abdominal movements provided a quantitative measure of abdominal ventilation frequency.

The cockroach was secured within the respirometry chamber using a custom-built harness, 3D printed from ABS plastic, that fitted over the posterior portion of the cockroach's pronotum. Individual cockroaches were anaesthetised by a 30-second 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, Norwest, 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's lid using two M3 screws (Fig. 5b). When assembled, this held the cockroach with its pronotum fixed beneath two 10 mm diameter holes in the lid and positioned its abdomen above the reflection sensor.

2.2.4 Hemolymph O_2 and CO_2 Measurements

Hemolymph PO_2 and PCO_2 were measured *in vivo* simultaneously using implantable fiber-optic microsensors. The PO_2 optodes (IMP-Pst7, PreSens GmbH, Regensburg, Bavaria, Germany) were calibrated using a two-point calibration: 0 kPa PO_2 water was produced by mixing anhydrous sodium sulphite into RO water and submerging the PO_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 PO_2. The calibrations were performed at 22 °C inside the same incubator as mentioned previously.

The PCO_2 optodes (IMP-CDM1, PreSens GmbH) were calibrated in 100 mL of RO water containing 0.154 M NaCl which was equilibrated with 0, 0.5, 1, 2, 3, 4, and 5 kPa PCO_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 Flow Vision gas mixing software running on a desktop PC, were used to generate the PCO_2s 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 PCO_2, and 30

min at 1, 2, 3, 4, and 5 kPa PCO_2 to ensure complete equilibration of the calibration solution. During calibration, measurements of CO_2 (%) were taken 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 PCO2 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, comprised of 7 points ranging from 0-5 % CO_2.

After the harness had been affixed to the cockroach (procedure described above) the cockroach was prepared for optode implantation. While the cockroach was still CO_2 narcotised, two small holes were drilled into the insect's hemocoel, 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, Wisconsin, USA). As each hole was cut it was sealed temporarily by applying a dab of 2-part polyvinylsiloxane 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. 5b). To implant a calibrated PO_2 optode, first the polyvinylsiloxane 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 hemocoel. The optode was sealed into the horn by application of more polyvinylsiloxane casting material around the optic fibre. 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 PCO_2 probe was implanted into the hemocoel within the cockroach's left thoracic 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 single raised 3D-printed ABS plastic ring had previously been epoxied to the chamber lid, forming a well around the holes. Strips of aluminium foil were placed inside of this ring around each probe and were covered liberally with polyvinyl siloxane to form a plug in the well. This ensured a gas tight seal around the holes in the chamber lid.

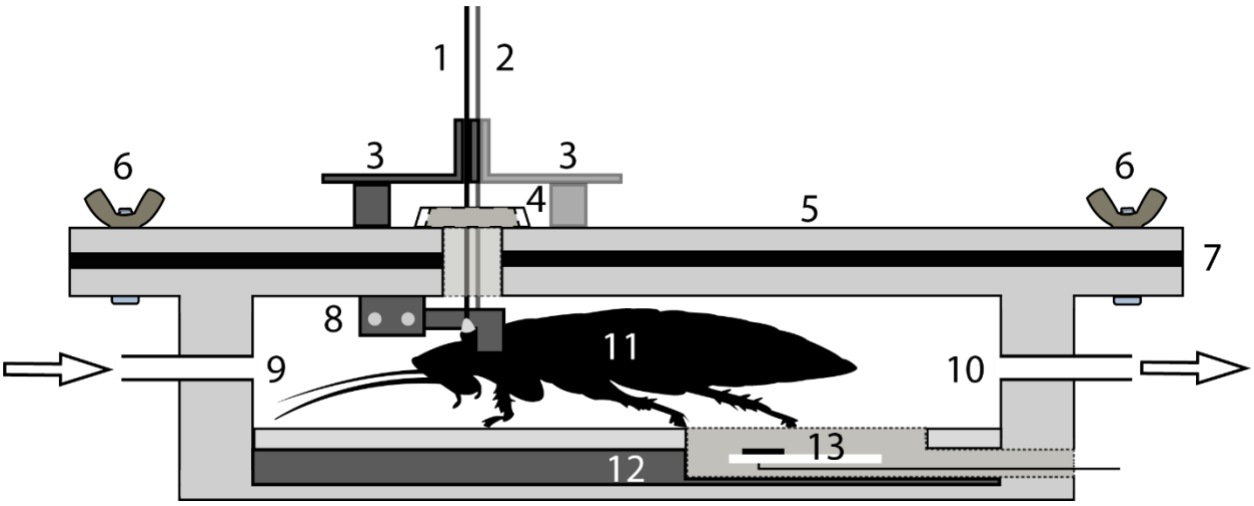

Figure 5b. Respirometry chamber for simultaneous measurement of CO_2 release, hemolymph PO_2, hemolymph PCO_2, and abdominal ventilation. PCO_2 probe implanted in the left thoracic horn; 2. PO_2 probe implanted in the right thoracic horn; 3. ABS clamp fixing probe to chamber lid; 4. ABS well filled with polyvinyl siloxane plug, sealing the chamber; 5. Respirometry chamber lid; 6. Wingnuts securing the chamber lid; 7. Closed-cell foam neoprene gasket; 8. ABS thoracic harness attached to chamber lid; 9. Air inlet; 10. Air outlet; 11. Madagascar hissing cockroach; 12. ABS plastic baseplate, covered with acrylic platform; 13. Infrared activity detector. Arrows indicate the direction of airflow.

In vivo hemolymph PCO_2 measurements were recorded every 2 min. Low sampling frequency was necessary to minimize photobleaching over the 2 -day experimental run. *In vivo* hemolymph PO_2 measurements were taken every 30 s using a Microx 4 trace meter (PreSens GmbH). All experiments were conducted inside of an incubator (22 °C, 12L:12D) (Percival Scientific Inc.).

2.2.5 Experimental Protocol

For the first 5 h of experimentation, the cockroaches were exposed to normoxic acapnia (21 kPa PO_2, 0 kPa PCO_2) to recover from the surgery. The next 18 h were split into two, 9 h treatments. Treatments began with either a further 9 h exposure to normoxic acapnia, or 9 h exposure to either hypoxic acapnia (10 kPa PO_2, 0 kPa PCO_2) or normoxic hypercapnia (21 kPa PO_2, 2 kPa PCO_2). The order of the treatments alternated between experiments so that if one experiment started with a 9 h normoxic acapnia treatment, the next experiment began with either the 9 h hypoxia or hypercapnia exposure.

After the first 23 h, the chamber was removed from the incubator, and the cockroach was anaesthetised by a 30-second 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. Once decapitated, the cockroach was returned to the chamber, and the chamber lid was again fixed in place. The chamber was then placed back in the incubator for another 23 h of experimentation. Cockroaches were given 5 h to recover from the surgeries, after which all cockroaches had begun to elicit clear, sustained DGCs. Subsequently, the final 18 h of experiments were again divided into two 9 h blocks, repeating the same modified atmosphere manipulations that were carried out before decapitation.

2.2.6 Data Extraction and Statistical Analysis

2.2.6.1 Data Extraction

Figs. 6 and 7 provide illustrations of raw data traces from cockroaches displaying continuous ventilation and DGCs, displaying how data was sampled from each trace. Measurements of O_2 and CO_2 levels were recorded as % of total gas dissolved in hemolymph. These raw values were converted to partial pressures (PO_2 or PCO_2 in kPa) by dividing each value by 100 then multiplying each value by 101.3 kPa.

Visual analysis of hemolymph pCO_2 fluctuations were used to determine ventilatory patterns, as these measurements were available in cockroaches exposed to all treatments, whereas exhaled CO_2 data was not available in hypercapnia exposed cockroaches. DGCs were identified by large fluctuations in hemolymph PCO_2 resulting from the accumulation of CO_2 during the C-phase and release during the O-phase. Continuous ventilation was assumed if

hemolymph PCO_2 did not visibly deviate from a stable, sustained level, as these conditions coincided with a non-zero $\dot{V}CO_2$ when cockroaches were exposed to normoxic acapnia (Fig. 6). Mean continuous PCO_2 and PO_2 values were taken from the longest sustained period of stable hemolymph PCO_2 in each treatment. When DGCs were displayed, the maximum and minimum PCO_2 and PO_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. This experiment also studied whether exposure to different ambient gas tensions coincided with visibly different patterns of DGCs. Specifically, 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 hemolymph PO_2 trace. The period between when PO_2 began to fall and then rise defined the interburst duration, while the period between when PO_2 began to rise and then fall defined the O-phase duration (Fig. 7). The flutter duration was determined using the ventilation trace and was identified as the period where the cockroach exhibited low frequency and low amplitude ventilatory activity preceding the more frequent and high amplitude period coinciding with the O-phase. The closed phase duration was determined by subracting the F-phase duration from the interburst duration and was obvious in the traces as coinciding with no ventilatory activity and declining hemolymph PO_2. All duration data was averaged between all DGCs in the final 6 h of each treatment gas and compared. The experiment also measured the mean rate of CO_2 release during each DGC in hypoxic and normoxic conditions. Volume of CO_2 recorded using the IRGA was averaged over each DGC providing volume of CO_2 exhaled per second ($\mu l\ s^{-1}$). Rate of CO_2 exhaled during each DGC was averaged for each treatment and compared.

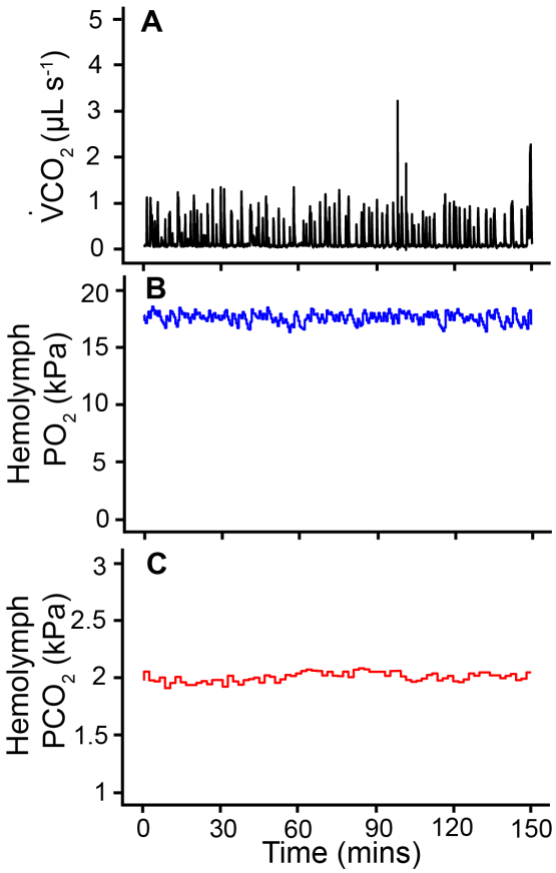

Figure 6. Typical measurements taken from an intact continuously ventilating *G. portentosa*. A) $\dot{V}CO_2$ release (µL s^{-1}); B) Hemolymph PO_2 (kPa); C) Hemolymph PCO_2 (kPa), in an intact individual.

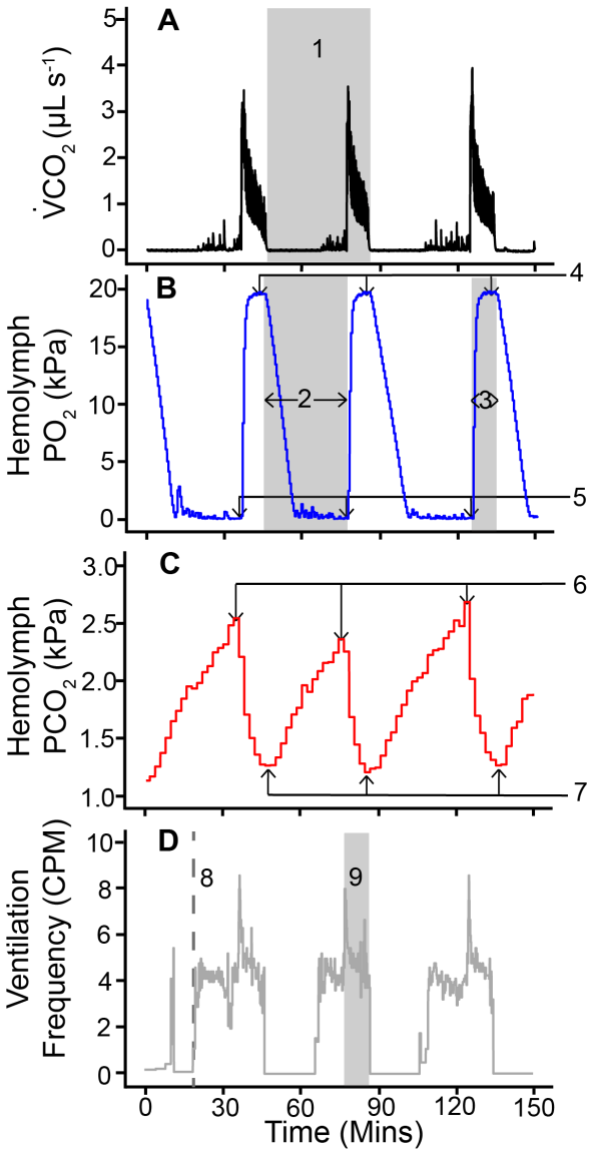

Figure 7. Typical measurements taken from a decapitated *G. portentosa* exhibiting DGCs. A) $\dot{V}CO_2$ release (µL s^{-1}); B) Hemolymph PO_2 (kPa); C) Hemolymph PCO_2 (kPa); D) Ventilation frequency (cyles per minute) (CPM). Numbers illustrate how different data was extracted: 1) Total CO_2 exhaled per cycle (µL); 2) Inter burst duration (s); 3) Open phase duration (s); 4) Average maximum PO_2 (kPa); 5) Average minimum PO_2 (kPa); 6) Average maximum PCO_2 (kPa); 7) Average minimum PCO_2 (kPa); 8) Beginning of abdominal fluttering; 9) Average ventilation frequency (CPM)

2.2.6.2 Statistical Analysis

All metrics were compared between values obtained from cockroaches exposed to normoxic acapnia, and a treatment of either repeated normoxic acapnia (control), hypoxic acapnia (10 kPa PO_2), or normoxic hypercapnia (2 kPa PCO_2). Differences between values obtained in normoxic acapnia exposure and the treatment gas were tested using the paired t-test function on Prism 8 (GraphPad Software Inc, San Diego, CA, USA) with statistical significance being set at $\alpha = 0.05$. Unpaired t-tests with Welch's correction for unequal standard deviations were used to compare changes in measurements between control and manipulation experiments. 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, the low sample sizes lead to an increased likelihood of type II errors.

2.3 Results

2.3.1 Hemolymph PO_2 and PCO_2 During Continuous Breathing

Changes in hemolymph PCO_2 and PO_2 during continuous breathing in control, hypoxia, and hypercapnia treatments are illustrated in fig. 8. Neither mean hemolymph PO_2 nor PCO_2 changed significantly between the first and second 9 h period of exposure to the normoxic acapnic gas mixture ($t(4) = 0.9300$, $p = 0.4050$ and $t(4) = 1.779$, $p = 0.1498$, respectively). Three of six intact cockroaches exposed to hypoxia exhibited sustained DGCs in lieu of continuous breathing. Unfortunately, equipment failure meant that reliable PCO_2 measurements could be obtained from only two of these three individuals. Mean hemolymph PO_2 was significantly lower in continuously ventilating cockroaches exposed to hypoxia ($t(2) = 20.35$, $p = 0.0024$), falling by 7.8 and 10.9 kPa. There was no significant change in hemolymph PCO_2 when cockroaches were exposed to hypoxia ($t(1) = 3.333$, $p = 0.1855$. Following exposure to 2 kPa PCO_2, hemolymph PO_2 was not significantly changed ($t(2) = 1.152$, $p = 0.3684$). There was no significant change in hemolymph PCO_2 when exposed to hypercapnia ($t(3) = 3.069$, $p = 0.0546$), however the observed change in PCO_2 in the hypercapnia exposed cockroaches was significantly higher than that observed in the control group ($t(3) = 3.134$, $p = 0.0273$).

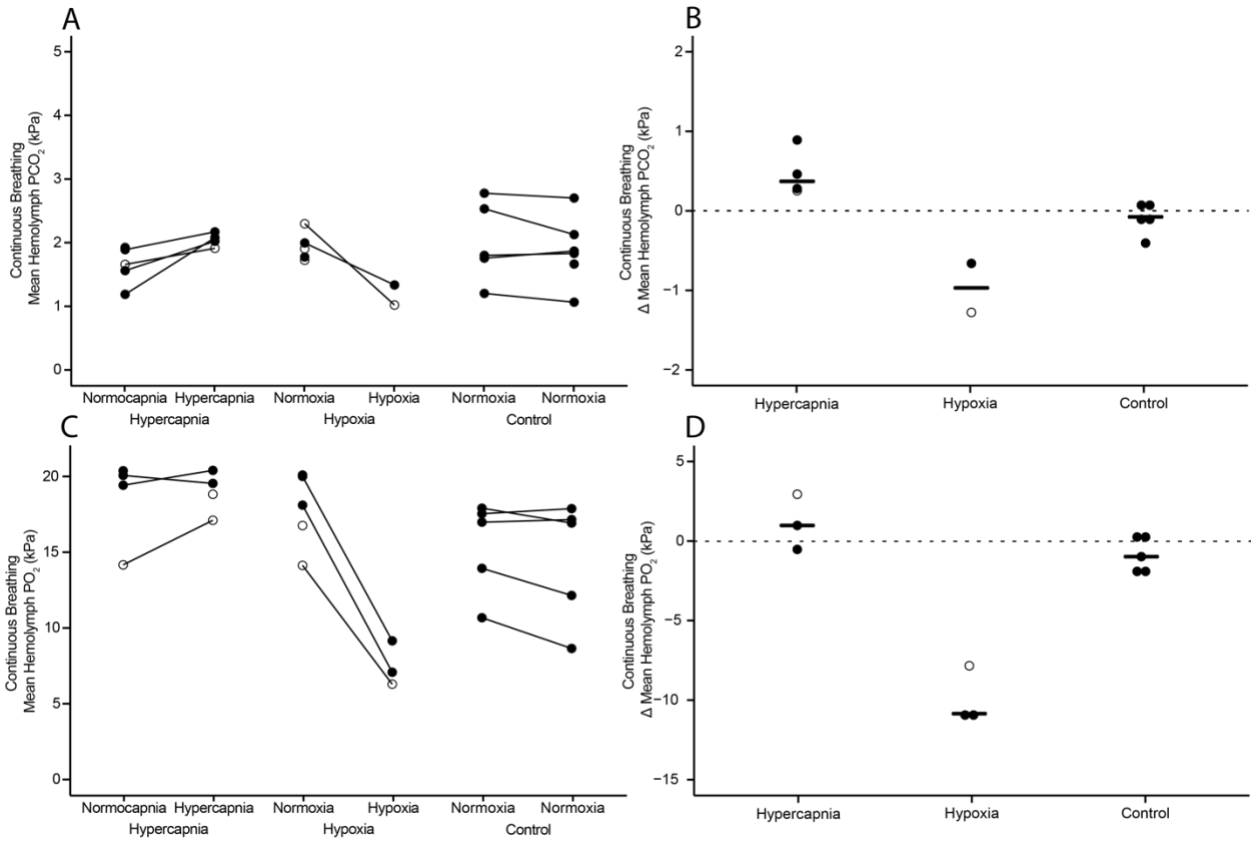

Figure 8. Univariate scatterplots illustrating changes in mean hemolymph PCO_2 and PO_2 in *G. portentosa* exhibiting continuous ventilation when exposed to control, hypoxia, and hypercapnia treatments. A) Mean hemolymph PCO_2 (kPa); B) Mean change in hemolymph PCO_2 (kPa), C) Mean hemolymph PO_2 (kPa); D) Mean change in hemolymph PO_2 (kPa) between control and treatment between control and treatment, during continuous breathing in cockroaches exposed to normoxic acapnia and a treatment of either normoxic hypercapnia, hypoxic acapnia, or normoxic acapnia (control). Lines indicate paired sampling from the same individual. Unfilled symbols represent cockroaches exposed to treatment gas in the first 9 h of experimentation, and filled symbols represent exposure to treatment gas in the second 9 h of experimentation. Horizontal bars in plots B and D indicate median values.

2.3.2 Hemolymph PO_2 and PCO_2 Fluctuations During DGCs

2.3.2.1 DGCs in Intact Cockroaches

Intact *G. portentosa* exposed to normoxic acapnia were observed displaying DGCs in five experiments. Average PCO_2 levels observed during continuous breathing from the same individuals were obtained and subtracted from averaged minimum and maximum PCO_2 values during DGCs. These values are shown in fig. 9, illustrating that intact *G. portentosa* displaying DGCs had maximum and minimum PCO_2 values which fluctuated around the mean values observed during continuous breathing. Average PCO_2 during continuous ventilation was significantly different from both average minimum hemolymph PCO_2 ($t(4) = 2.994$, $p = 0.0402$), and maximum hemolymph PCO_2 ($t(4) = 10.23$, $p = 0.0005$) during DGCs.

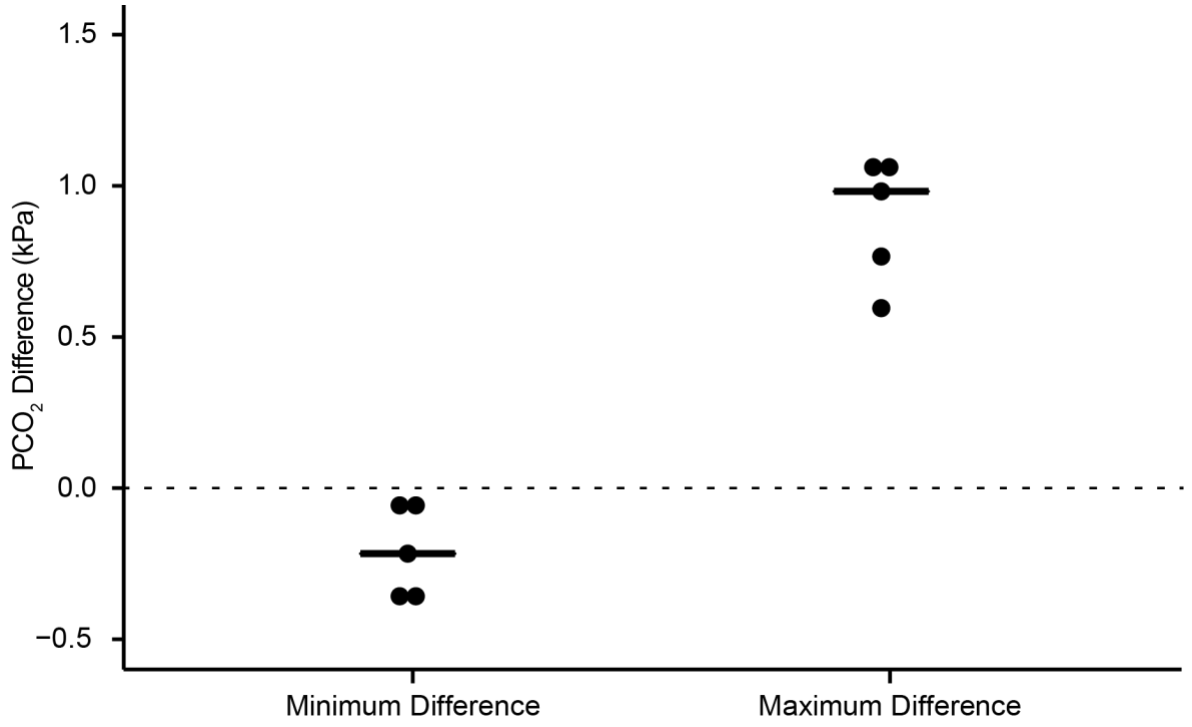

Figure 9. Difference in mean hemolymph PCO_2 (kPa) observed during continuous ventilation, from minimum and maximum PCO_2 (kPa) values observed during DGCs in intact *G. portentosa* exposed to normoxic acapnia. The dotted line indicates no difference in the mean level of PCO_2 during continuous ventilation relative to minimum and maximum values during DGCs. Horizontal bars indicate medium values.

2.3.2.2 DGCs in Decapitated Cockroaches

DGCs were elicited in all decapitated cockroaches. Minimum PO_2 level was consistently observed falling to near 0 kPa during the closed phases in most experiments. Exposure to 10 kPa PO_2 or 2 kPa PCO_2 did not cause any of the decapitated cockroaches to stop displaying DGCs. Changes in minimum and maximum hemolymph PCO_2 during DGCs in decapitated cockroaches exposed to control, hypoxia, and hypercapnia treatments are shown in fig. 10. Sustained exposure to normoxic acapnia did not significantly change either mean maximum PCO_2 ($t(5) = 0.1233$, $p = 0.9067$) or mean minimum PCO_2 ($t(5) = 0.1363$, $p = 0.8969$). However, one of five cockroaches showed increasing, and one of five cockroaches showed decreasing hemolymph PCO_2 over time, despite constant ambient PO_2 and PCO_2 levels. Cockroaches exposed to hypercapnia had significantly increased mean maximum and mean minimum hemolymph PCO_2 ($t(4) = 4.402$, $p = 0.0117$ and $t(4) = 6.517$, $p = 0.0029$, respectively). Conversely, cockroaches exposed to hypoxia had significantly lower mean maximum and minimum hemolymph PCO_2 ($t(4) = 3.844$, $p = 0.0184$ and $t(4) = 2.881$, $p = 0.0450$).

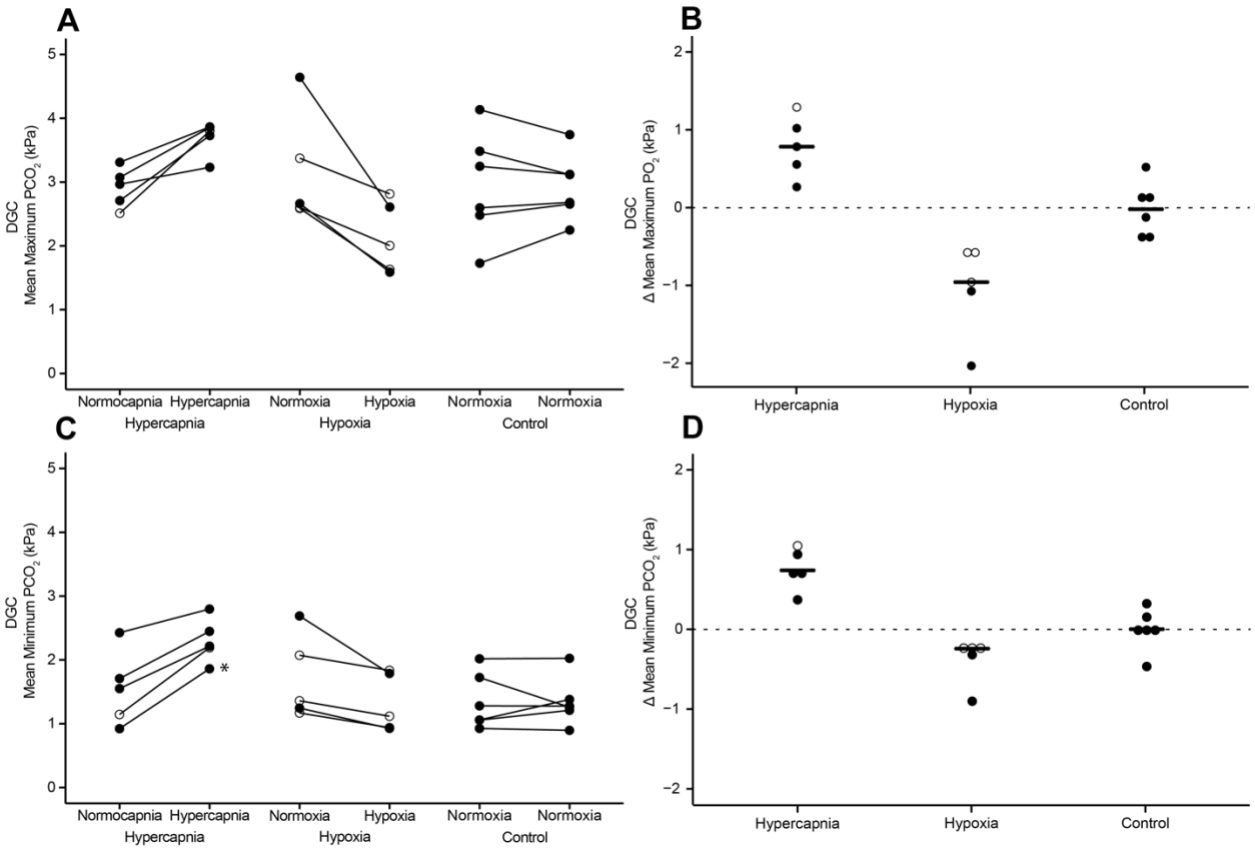

Figure 10. Univariate scatterplots illustrating changes in minimum and maximum hemolymph PCO_2 during DGCs exhibited by decapitated *G. portentosa*. A) Mean maximum hemolymph PCO_2 (kPa); B) Mean change in maximum hemolymph PCO_2 (kPa) between control and treatment; C) Mean minimum hemolymph PCO_2 (kPa), asterisk indicates a PCO_2 value lower than ambient CO_2 level, but was within equipment error limits; D) Mean change in minimum hemolymph PCO_2 (kPa) between control and treatment, during DGCs in cockroaches exposed to normoxic acapnia and a treatment of either normoxic hypercapnia, hypoxic acapnia, or normoxic acapnia (control). Lines indicate paired sampling from the same individual. Unfilled symbols represent cockroaches exposed to treatment gas in the first 9 h of experimentation, and filled symbols represent exposure to treatment gas in the second 9 h of experimentation. Horizontal bars in plots B and D indicate median values.

Changes in minimum and maximum hemolymph PO_2 during DGCs in decapitated cockroaches exposed to control, hypoxia, and hypercapnia treatments are shown in fig. 11. When treated with repeated normoxic acapnia exposures, there was no significant change in either maximum PO_2 ($t(4) = 1.610$, $p = 1.827$) or minimum PO_2 ($t(4) = 1.000$, $p = 0.3739$). When cockroaches were exposed to hypercapnia there was no change in mean minimum PO_2 ($t(4) = 2.236$, $p = 0890$) or mean maximum PO_2 ($t(4) = 1.026$, $p = 0.3628$). Maximum hemolymph PO_2 was significantly reduced in cockroaches exposed to hypoxia ($t(4) = 17.51$, $p < 0.0001$). Minimum hemolymph PO_2 did not change significantly in cockroaches exposed to hypoxia ($t(4) = 1.895$, $p = 0.1310$), although minimum PO_2 was reduced in all cockroaches which did not reach 0 kPa in their normoxic acapnia control treatment.

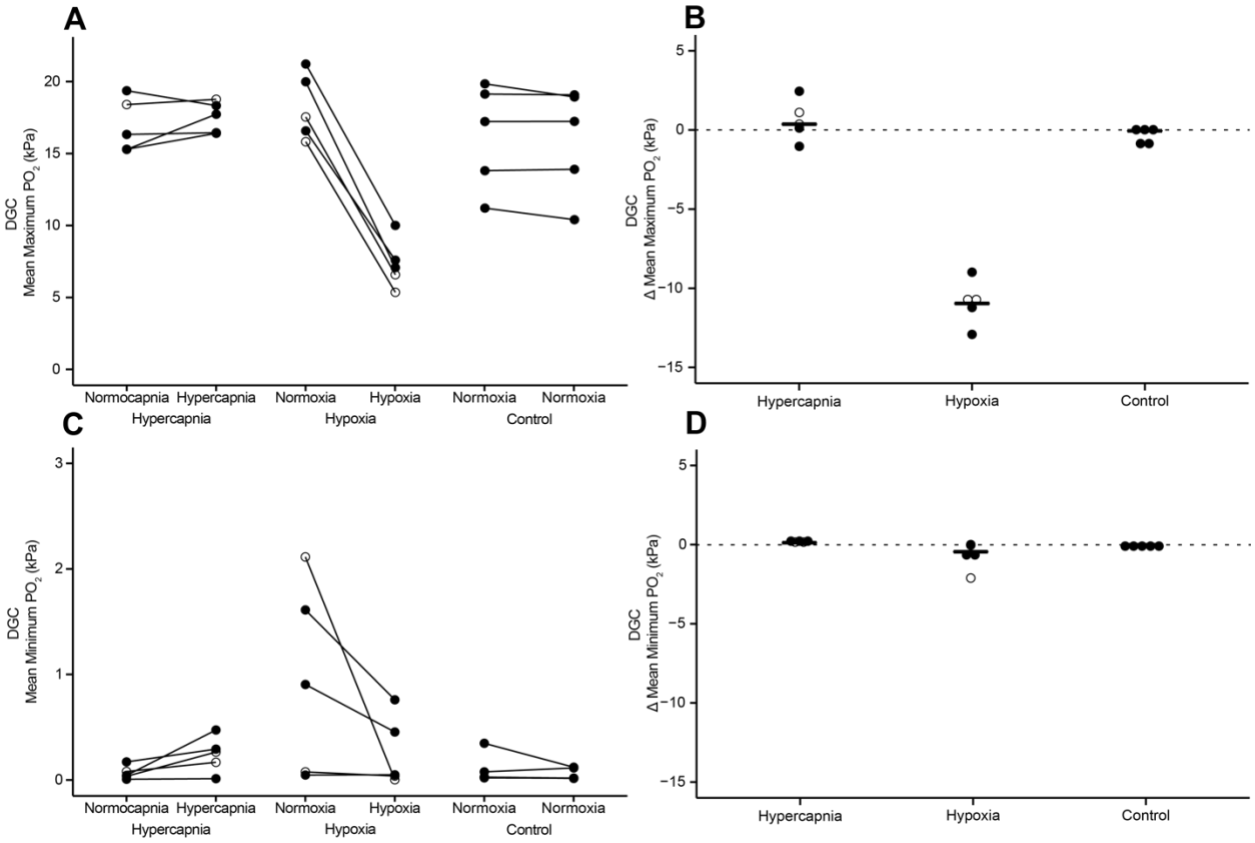

Figure 11. Univariate scatterplots illustrating changes in minimum and maximum hemolymph PO_2 during DGCs exhibited in *G. portentosa* when exposed to control, hypoxia, and hypercapnia treatments. A) Mean maximum hemolymph PO_2 (kPa); B) Mean change in maximum hemolymph PO_2 (kPa) between control and treatment; C) Mean minimum hemolymph PO_2 (kPa); D) Mean change in minimum hemolymph PO_2 (kPa) between control and treatment, during DGCs in cockroaches exposed to normoxic acapnia and a treatment of either normoxic hypercapnia, hypoxic acapnia, or normoxic acapnia (control). Lines indicate paired sampling from the same individual. Unfilled symbols represent cockroaches exposed to treatment gas in the first 9 h of experimentation, and filled symbols represent exposure to treatment gas in the second 9 h of experimentation. Horizontal bars in plots B and D indicate median values.

Simultaneously recorded values of PO_2 and PCO_2 were plotted against each other on an x/y scatter plot, PCO_2 on the ordinate and PO_2 on the abscissa, to illustrate how hemolymph PO_2/PCO_2 typically fluctuate throughout each DGC in decapitated cockroaches exposed to different ambient PO_2/PCO_2 gas tensions (Fig. 12). These fluctuating levels contrast with the model for DGC regulation illustrated in Fig. 4, as proposed by Förster and Hetz (2010). Decapitated cockroaches exposed to hypoxia had significantly decreased minimum and maximum PCO_2, and decreased maximum PO_2 (Fig. 12.A). Decapitated cockroaches exposed to hypercapnia had significantly increased minimum and maximum PCO_2 (Fig. 12.B). Decapitated cockroaches exposed to normoxic acapnia generally did not change minimum or maximum PCO_2 over the course of the experiment (Fig. 12.C). However, minimum and maximum PCO_2 did drift in two of five cockroaches, with one experiment showing hemolymph PCO_2 increasing, and the other showing hemolymph PCO_2 decreasing, over time. Fig. 12.D illustrates the example where hemolymph PCO_2 levels increased over time during exposure to normoxic acapnia.

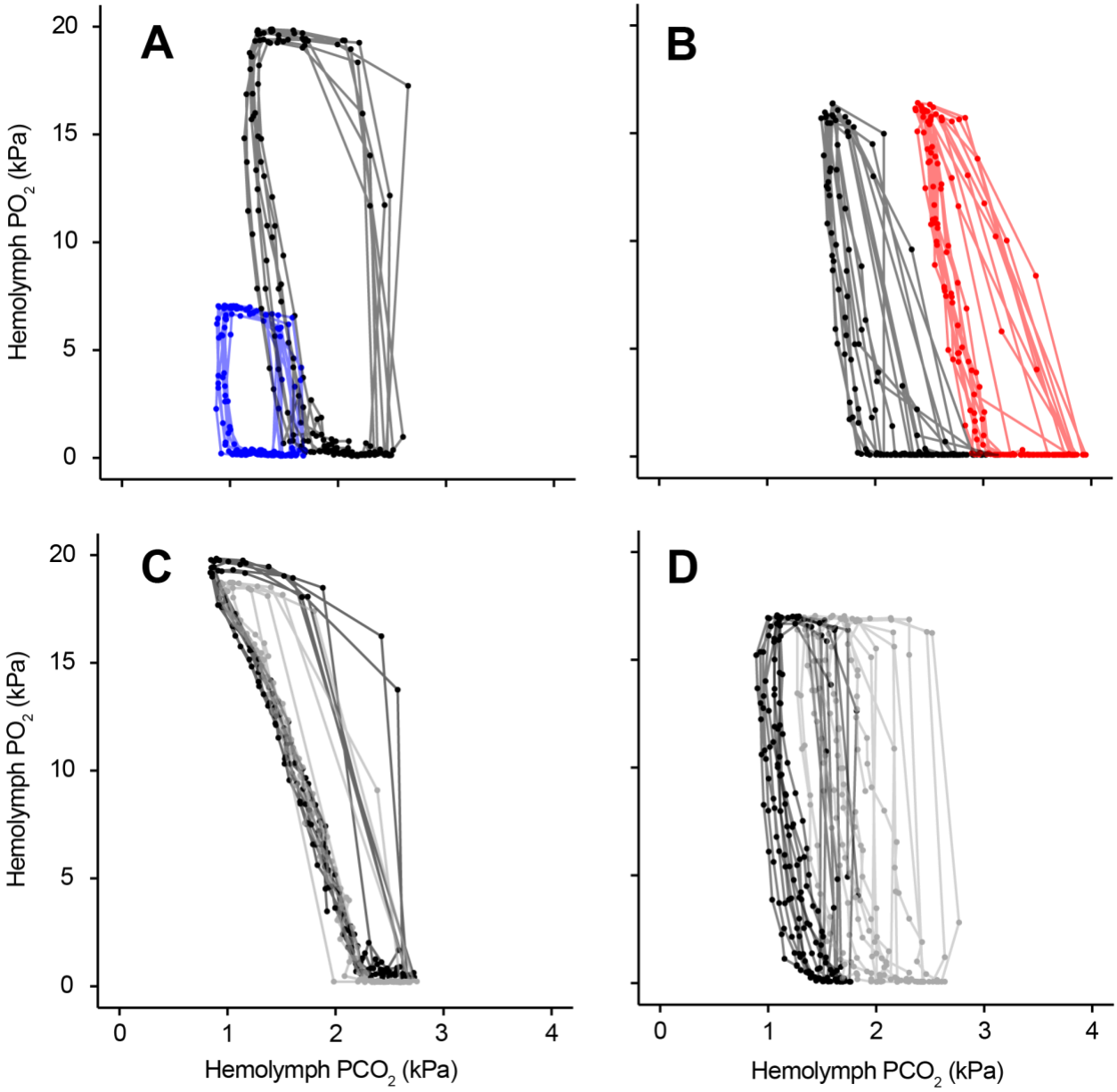

Figure 12. Scatterplot with lines connecting consecutive datapoints illustrating simultaneous changes in PCO_2 and PO_2 (kPa) during DGCs exhibited in *G. portentosa* when exposed to control, hypoxia, and hypercapnia treatments. Data was recorded from DGCs observed in the final 6h exposure to normoxic acapnia (black, all plots) and the final 6h exposure to either: hypoxic acapnia (blue, plot A); normoxic hypercapnia (red, plot B); or a control treatment of normoxic acapnia (grey, plots C and D). Plot C illustrates PCO_2 and PO_2 fluctuations which remained similar throughout the experiment, plot D shows PCO_2 and PO_2 fluctuations which shifted over time despite constant exposure to the same ambient PO_2 and PCO_2 levels.

2.3.3 DGC Phase Durations

Changes in cycle duration, interburst duration, and C-phase, F-phase and O-phase durations during DGCs in decapitated cockroaches exposed to control, hypoxia, and hypercapnia treatments are shown in figs. 13 and 14. Cockroaches exposed to repeated normoxic acapnia exposure showed no significant change in O-phase duration ($t(5) = 2.328$, $p = 0.0673$), F-phase duration ($t(3) = 2.977$, $p = 0.0588$) or interburst duration ($t(5) = 2.216$, $p = 0.0775$). However, mean total cycle duration significantly increased ($t(5) = 4.652$, $p = 0.0056$) resulting from a significant increase in C-phase duration ($t(3) = 3.744$, $p = 0.0332$). In cockroaches exposed to hypercapnia there was no significant change in mean total cycle duration ($t(5) = 2.501$, $p = 0.0544$), F- phase duration ($t(4) = 2.406$, $p = 0.0739$) or C-phase duration ($t(4) = 0.5380$, $p = 0.6191$). Hypercapnia exposed individuals showed significantly decreased mean interburst durations ($t(4) = 3.122$, $p = 0.0262$) and increased mean O-phase durations ($t(5) = 4.380$, $p = 0.0072$). Cockroaches exposed to hypoxia showed no significant change in C-phase duration ($t(3) = 1.690$, $p = 0.1896$), F-phase duration ($t(3) = 0.4430$, $p = 0.6878$) or O-phase duration ($t(5) = 1.230$, $p = 0.2736$). However, cockroaches exposed to hypoxia showed significantly decreased mean total cycle duration ($t(5) = 4.493$, $p = 0.0064$) and mean interburst duration ($t(5) = 5.947$, $p = 0.0019$).

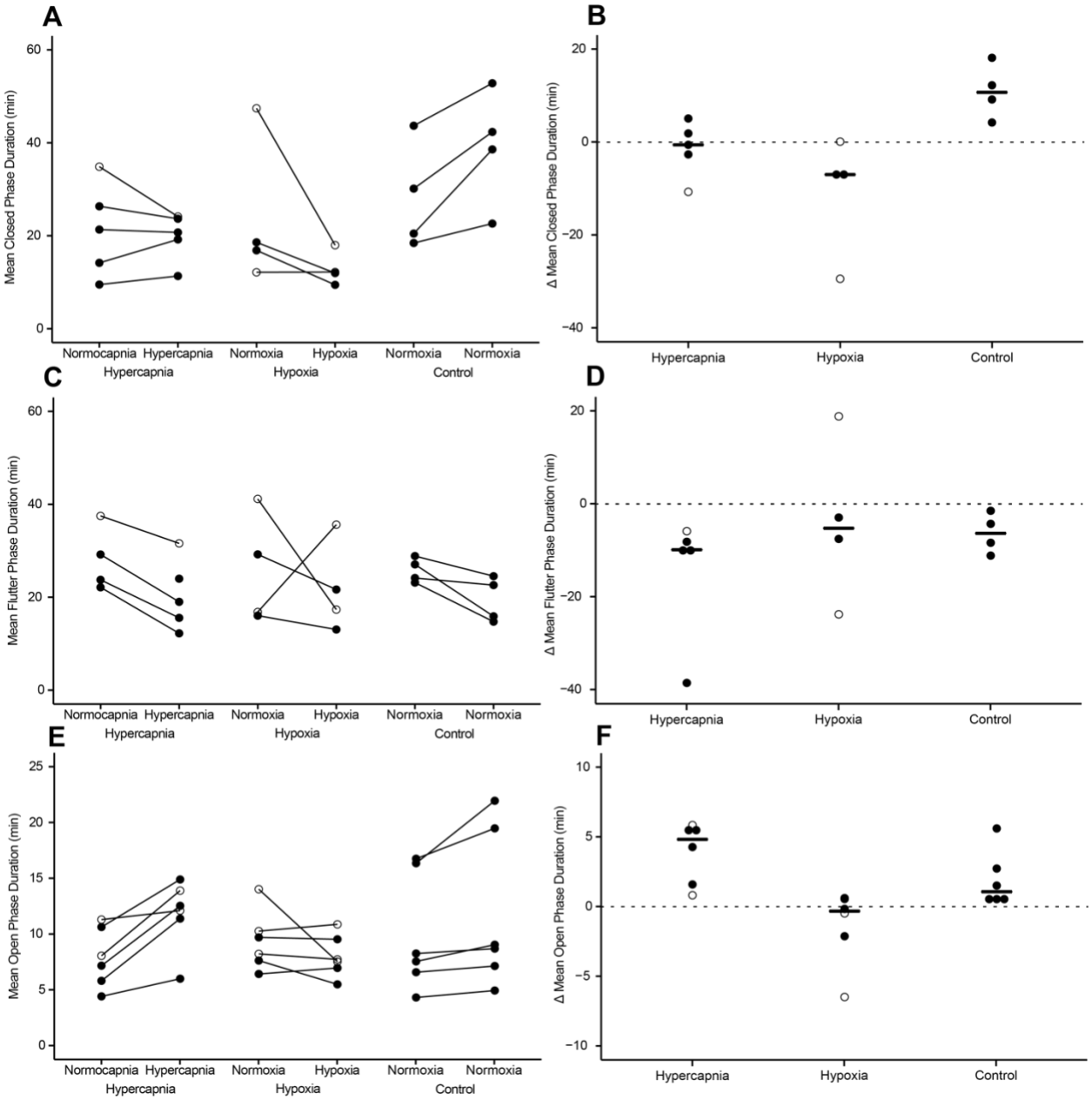

Figure 13. Univariate scatterplots illustrating changes in mean closed phase, flutter phase, and open phase durations during DGCs exhibited in *G. portentosa* when exposed to control, hypoxia, and hypercapnia treatments. A) Mean C-Phase duration (min) B) Mean change in C-phase duration between control and treatment, C) Mean F-phase duration (min); D) Mean change in F-phase duration between control and treatment; E) Mean O-phase duration (min); F) Mean change in O-phase duration between control and treatment, during DGCs in cockroaches exposed to normoxic acapnia and a treatment of either normoxic hypercapnia, hypoxic acapnia, or normoxic acapnia (control). Lines indicate paired sampling from the same individual. Unfilled symbols represent cockroaches exposed to treatment gas in the first 9 h of experimentation, and filled symbols represent exposure to treatment gas in the second 9 h of experimentation. Horizontal bars on plots B, D and F indicate median values.

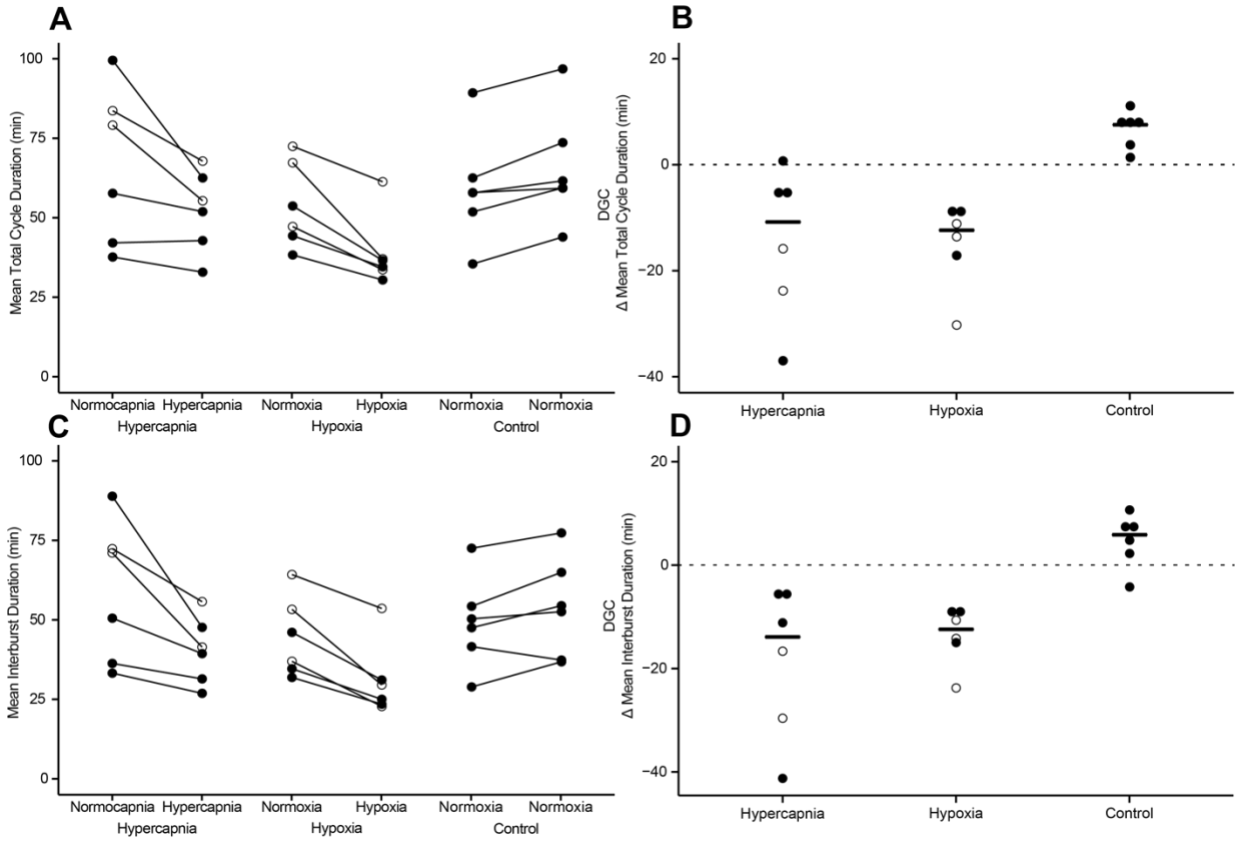

Figure 14. Univariate scatterplots illustrating changes in mean total cycle durations interburst durations during DGCs exhibited in *G. portentosa* when exposed to control, hypoxia, and hypercapnia treatments. A) Mean total cycle duration (min) B) Mean change in total cycle duration between control and treatment; C) Mean interburst duration (min); D) Mean change in interburst duration between control, during DGCs in cockroaches exposed to normoxic acapnia and a treatment of either normoxic hypercapnia, hypoxic acapnia, or normoxic acapnia (control). Lines indicate paired sampling from the same individual. Unfilled symbols represent cockroaches exposed to treatment gas in the first 9 h of experimentation, and filled symbols represent exposure to treatment gas in the second 9 h of experimentation. Horizontal bars on plots B and D indicate median values.

2.3.4 Ventilatory Patterns Summarised

Median changes in PO_2 and PCO_2 during continuous ventilation and discontinuous gas exchange cycles, as well as median changes in phase durations are summarised in Table 1.

Table 1.
Changes in median PCO_2 and PO_2 levels during continuous ventilation and DGCs, and changes in DGC phase durations in response to normoxic acapnia (control), hypoxic acapnia, and normoxic hypercapnia treatments

	Norm./Norm.				Norm./Hypox.				Norm./Hypercap.			
DGC Phase duration	**Chng.**	**ΔMED (mins)**	**Sig.**	**N**	**Chng.**	**ΔMED (mins)**	**Sig.**	**N**	**Chng.**	**ΔMED (mins)**	**Sig.**	**N**
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
Gas kPa continuous	**Chng.**	**ΔMED (kPa)**	**Sig.**	**N**	**Chng.**	**ΔMED (kPa)**	**Sig.**	**N**	**Chng.**	**ΔMED (kPa)**	**Sig.**	**N**
PO_2	=	-0.9		5	↓	-9.35	*	3	=	0.2		4
PCO_2	=	-0.1		4	↓	-1	*	2	=	0.35		4
Gas kPa DGC												
PO_2 min.	=	0		5	=	-0.4		5	=	0.1		5
PO_2 max.	=	3.5		5	↓	-10.9	*	5	=	0.55		5
PCO_2 max.	=	0.15		6	↓	-1	*	4	↑	0.8	*	5
PCO_2 min.	=	0.05		6	↓	-0.3	*	4	↑	0.7	*	5

2.3.6 DGC Ventilation Frequency

Changes in ventilation frequency (f_v: cycles per minute) during the O-phase of DGCs in decapitated cockroaches exposed to control, hypoxia, and hypercapnia treatments are shown in fig. 15. Cockroaches treated with repeated exposures of normoxic acapnia showed f_v decreased significantly over time (t (4) = 4.586, p = 0.010). Changes in f_v when exposed to either hypercapnia or hypoxia were not significant (t (3) = 1.870, p = 0.1582 and t (4) = 0.9863, p = 0.3798, respectively).

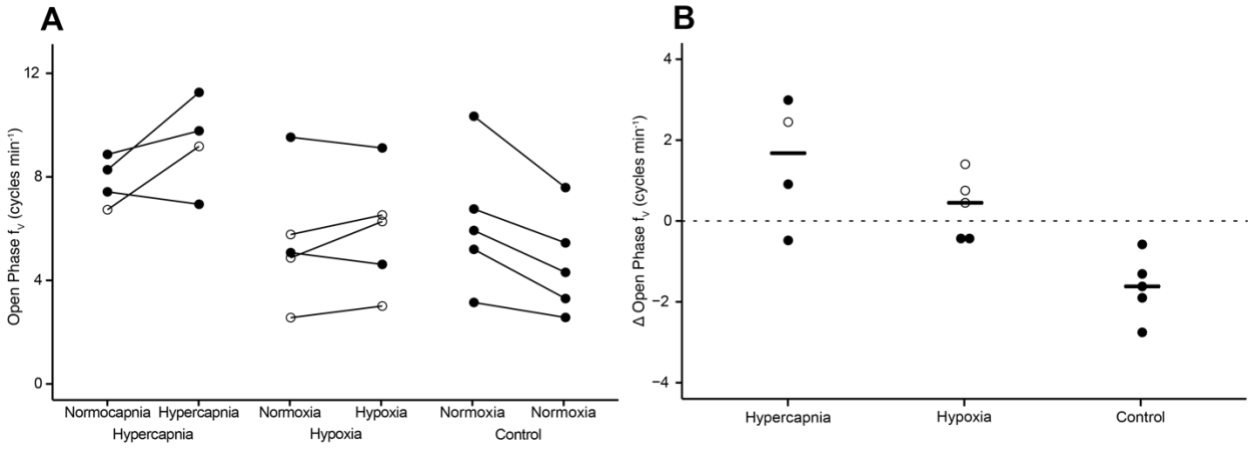

Figure 15. Univariate scatterplots illustrating changes in mean O-phase ventilation frequency during DGCs exhibited in *G. portentosa* when exposed to control, hypoxia, and hypercapnia treatments - A) mean open phase ventilation frequency (f_v) (cycles per minute); and B) Mean change in open phase f_v between control and treatment (cycles per minute), during DGCs in cockroaches exposed to normoxic acapnia and a treatment of either normoxic hypercapnia, hypoxic acapnia, or normoxic acapnia (control). Lines indicate paired sampling from the same individual. Unfilled symbols represent cockroaches exposed to treatment gas in the first 9 h of experimentation, and filled symbols represent exposure to treatment gas in the second 9 h of experimentation. Horizontal bars in plots A and B indicate median values.

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

Changes in mean $\dot{V}CO_2$ (μL min^{-1}) in decapitated cockroaches exposed to control and hypoxia treatments are shown in fig. 16. Mean $\dot{V}CO_2$ was not significantly different in the control group when exposed to repeated normoxic acapnia ($t(5) = 1.177$, $p = 0.2920$), or in the hypoxia group between control and treatment ($t(5) = 0.9283$, $p = 0.3959$). As $\dot{V}CO_2$ could not be measured in a hypercapnic atmosphere, any change in $\dot{V}CO_2$ associated with this treatment could not be determined.

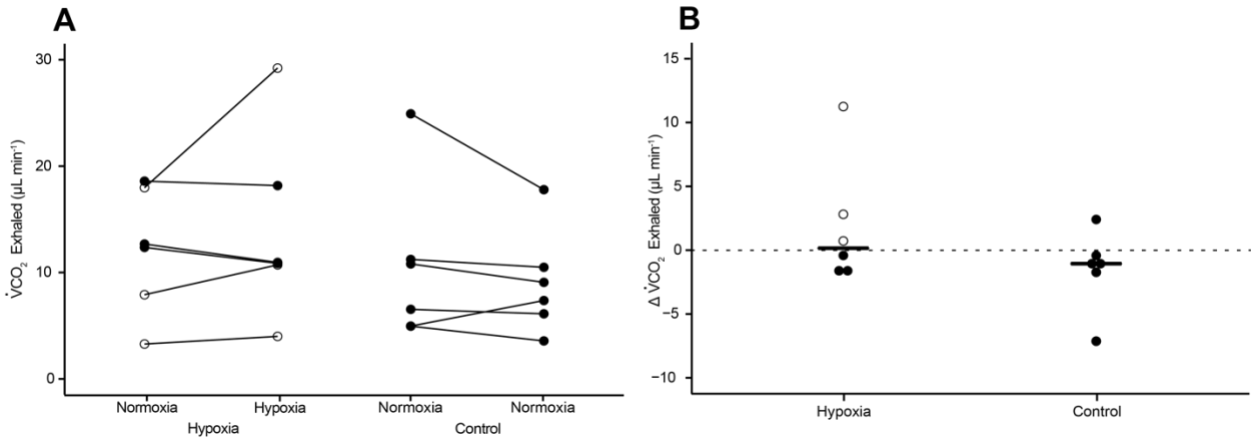

Figure 16. Univariate scatterplots illustrating changes in mean $\dot{V}CO_2$ release during each cycle of DGCs exhibited in *G. portentosa* when exposed to control and hypoxia treatments. A) mean $\dot{V}CO_2$ during each cycle of the DGC (μL) B) Mean change in $\dot{V}CO_2$ released during each cycle of the DGC (μL) between control and treatment, during DGCs in cockroaches exposed to normoxic acapnia and either normoxic acapnia (control) or hypoxic acapnia. Unfilled dots illustrate exposure to treatment gas in the first 9 h of experimentation, and filled dots illustrate exposure to treatment gas in the second 9 h of experimentation. Horizontal bars on plot B indicate median values.

2.4 Discussion

The ultimate goal of this thesis is to test the hypothesis that DGCs arise as a result of internal PCO_2 oscillating around a hypercapnic ventilatory threshold. Results from this thesis suggest that continuously ventilating *G. portentosa* exposed to normoxic acapnia control continuous ventilation around a PCO_2 ventilatory threshold. It was predicted that exposure to hypercapnia above the level which stimulated hyperventilation in continuously ventilating cockroaches would terminate DGCs by fixing hemolymph PCO_2 above the ventilatory threshold, locking them in the O-phase. However, DGCs were sustained in *G. portentosa* despite hypercapnia exposure significantly increasing hemolymph PCO_2 beyond perceived ventilatory threshold levels. Additionally, DGCs persisted despite hypoxia exposure significantly decreasing hemolymph PCO_2 below percieved threshold levels. Therefore, the results of this thesis do not suggest that PCO_2 hysteresis around a CO_2 ventilatory threshold is responsible for the production of DGCs. Despite strict PO_2 and PCO_2 levels not being associated with DGC phase transitions, hypercapnia and hypoxia were observed altering ventilatory patterns. These results are all discussed in context with previous findings in the rest of this discussion.

2.4.1 Cockroach Gas Exchange Regulation

2.4.1.1 Regulation of Continuous Ventilation

G. portentosa displaying continuous rhythmic ventilation in normoxic acapnia maintained hemolymph PO_2 and PCO_2 at stable levels of 17.1 ± 3.3 kPa and 1.9 ± 0.4 kPa, respectively (Fig. 8). These values were similar to those observed in another species of cockroach, the speckled feeder cockroach (*Nauphoeta cinerea*), which maintained tracheal PO_2 and PCO_2 close to 18 kPa and between 1 and 2 kPa respectively (Matthews and White, 2011b). Additionally, Förster and Hetz (2010) found that the spiracles of diapausing *A. atlas* pupae were fixed open when their tracheal systems were perfused with air containing a PCO_2 of 1.5 kPa and above. Similarities between our data and those from other species suggest that, while displaying continuous gas exchange in atmospheric air, *G. portentosa* maintain hemolymph PCO_2 and PO_2 at levels similar to other insects. This also suggests that in normoxic conditions, this ~1.9 kPa hemolymph PCO_2 drives continuous ventilation, as hemolymph PO_2 remains close to ambient levels.

It is likely that 10 kPa PO_2 exposure elicited hyperventilation in these cockroaches, as median hemolymph PCO_2 decreased during hypoxia exposure (Fig. 8). The observed decrease in hemolymph PCO_2 was not significant using a paired t-test, likely due to the low sample size (n=2). However, hyperventilation in hypoxia, and the associated decrease in hemolymph PCO_2, has been shown in other species of terrestrial insects. For example, *N. cinerea* exposed to ≤ 10 kPa PO_2 displayed hyperventilation, resulting in a reduced internal CO_2 level (Matthews and White, 2011b). It is likely that *G. portentosa* exhibit this same hyperventilatory response. Exposure to 2 kPa PCO_2 increased median hemolymph PCO_2 in continuously breathing *G. portentosa*. Our measurements indicate that 2 kPa PCO_2 exposure was associated with a significant increase in hemolymph PCO_2 relative to the control group. Increasing ambient PCO_2 from 0 to 2 kPa did not result in a 2 kPa increase in hemolymph PCO_2, further supporting a role for hyperventilation in maintaining hemolymph PCO_2 at the level observed during continuous breathing in normoxic acapnia (~1.9 kPa).

2.4.1.2 Spontaneous DGC Regulation

As hemolymph PO_2 remains near ambient levels in continuous ventilation, DGCs are unlikely to be regulated by hemolymph PO_2. The average hemolymph PCO_2 observed during continuous ventilation in each cockroach (~ 1.9 kPa) was assumed to be the hypercapnic ventilatory threshold level, a PCO_2 sufficient to stimulate continuous ventilation, with any increase above this driving hyperventilation. During spontaneous DGCs in intact cockroaches exposed to normoxic acapnia, hemolymph PCO_2 fluctuated significantly above and below the mean hemolymph PCO_2 observed during continuous ventilation (Fig 9.). These results were consistent with our initial hypothesis that DGCs resulted from hysteresis around a PCO_2 threshold level (Fig. 4). However, as decapitated cockroaches maintained DGCs when ambient PCO_2 was elevated above this perceived threshold, it is unlikely that oscillation around a PCO_2 threshold is necessary to generate DGCs. Instead, it appears that the observed oscillations are the result of DGCs themselves, and are not causative.

2.4.2 Elicited DGC Regulation and Manipulation

2.4.2.1 Hemolymph PO_2 and PCO_2 Fluctuations During DGCs in Normoxic Acapnia

Decapitation triggered sustained DGCs in all cockroaches. During DGCs in normoxic acapnia, ventilation during the O-phase caused hemolymph PO_2 to rise to the same near-ambient levels as observed in continuously breathing individuals, but during the C-phase minimum hemolymph PO_2 consistently decreased to ~0 kPa (Fig. 11). It is worth noting that in many experiments the O-phase was not triggered as soon as hemolymph PO_2 (as recorded at the location of the sensor) fell to ~0 kPa. Instead, hemolymph often remained near anoxia for a substantial period of time before O-phase ventilation was initiated. These results are comparable to those of a previous experiment on the migratory locust (*Locusta migratoria)* which found that although PO_2 within tracheal air sacs never reached 0 kPa during DGCs in normoxic acapnia, tracheal PO_2 fell to near 0 kPa during exposure to hypoxia (Matthews et al., 2012). Results from *L. migratoria* suggested that the minimum O_2 level reached during a DGC changed based on the volume of O_2 in the insect's tracheal system at the beginning of the closed phase, and thus its depletion to some threshold level was not responsible for the onset of ventilation. It appears that insects are capable of maintaining DGCs independent of feedback from O_2 chemoreceptors, and that haemolymph PO_2 fluctuations follow expected trends based on ventilatory activity, rising during ventilation and falling during apnoea. Similar to the observations from intact cockroaches, mean hemolymph PCO_2 preceding ventilation (maximum PCO_2) during DGCs from decapitated cockroaches was higher than PCO_2 observed during continuous ventilation. During each control experiment spiracles were generally observed opening at the same PCO_2 levels each DGC. However, in two of the six control experiments it was observed that minimum and maximum hemolymph PCO_2 drifted over time. In the first of these experiments, minimum and maximum hemolymph PCO_2 decreased over time and in the latter experiment, they increased. The observed drift in hemolymph PCO_2 is not believed to result from degrading probes, as in both cases PCO_2 only began to drift following the initiation of DGCs in decapitated cockroaches. These two experiments indicate that strict PCO_2 thresholds may not be responsible for maintaining DGCs, as the O and C-phases were observed to occur across a range of PCO_2. Exposing decapitated cockroaches displaying DGCs to hypoxia and hypercapnia further tests if thresholds are required for producing DGCs.

2.4.2.2 Hemolymph PO_2 and PCO_2 Fluctuations During DGCs in Hypoxia

We did not predict that exposure to 10 kPa PO_2 would terminate DGCs in *G. portentosa* as this level is higher than that previously shown to eliminate DGCs in other species of cockroach. For example, Matthews and White (2011b) observed that exposure to 5 kPa PO_2 was necessary to terminate DGCs in *N. cinerea*, while Woodman et al. (2008) showed that the American cockroach (*Periplaneta americana)* abandoned DGCs at a PO_2 of 2 kPa. Instead, exposure to mild hypoxia, as in our experiments, should reveal whether O_2 chemoreception is involved in modulating the phases of the DGC. DGCs were maintained in all decapitated *G. portentosa* exposed to hypoxia and, as internal PO_2 increased to near-ambient levels during O-phase ventilation, all showed significantly reduced maximum hemolymph pO_2 relative to normoxia. There was no difference in minimum hemolymph PO_2 between normoxic or hypoxia exposure, as internal PO_2 fell to near anoxic levels in both.

DGCs were maintained despite hypoxia exposed *G. portentosa* showing significantly decreased minimum and maximum hemolymph PCO_2 (Fig. 10). This trend was also observed in the DGCs displayed by decapitated *N. cinerea* (Matthews and White, 2011b), indicating that either hypoxia increases sensitivity to CO_2, leading to the initiation of the O-phase at a lower PCO_2 threshold, 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 hemolymph PO_2 reached ~0 kPa in both the control and hypoxia treatments at the same time that the maximum hemolymph PCO_2 level was recorded. Two different levels of PCO_2 were therefore associated with the initiation of the O-phase at the same low PO_2. Thus, neither a hypoxia-induced change in PCO_2 sensitivity, nor crossing a strict PCO_2 ventilatory threshold, are required to trigger the O-phase during DGCs. However, DGCs are not completely insensitive to hypoxia. The decreased hemolymph PCO_2 resulting from exposure to 10 kPa O_2 indicates that hypoxia stimulates increased ventilation, resulting in increased CO_2 clearance. Interburst and total DGC cycle duration also became significantly shorter when cockroaches were exposed to hypoxia (Fig. 13 and 14). This indicates that hypoxia is still sensed by decapitated *G. portentosa* and that chemoreception of pO_2 plays a role in the control of abdominal ventilation during the O-phase of the DGC.

2.4.2.3 Hemolymph PO_2 and PCO_2 Fluctuations During DGCs in Hypercapnia

G. portentosa were exposed to an ambient PCO_2 of 2 kPa, as this was higher than the mean level which was observed in the hemolymph of continuously ventilating *G. portentosa* breathing normoxic normocapnia air. Thus, assuming insects regulate internal PCO_2 around this level, under these hypercapnic conditions the cockroaches' internal PCO_2 should remain at, or above, this putative PCO_2 threshold level, stimulating them to breathe continuously. However, exposing decapitated *G. portentosa* to 2 kPa ambient CO_2 did not stop them breathing discontinuously, despite this same level of ambient CO_2 inducing hyperventilation in continuously breathing intact cockroaches (fig. 8). This demonstrates that oscillations in hemolymph PCO_2 around a CO_2 chemosensory threshold do not account for the appearance of DGCs: During hypercapnia exposure, both minimum and maximum hemolymph PCO_2 increased significantly compared to levels in normoxic normocapnia, while internal PO_2 level continued to vary between ~0 and near ambient. These results indicate that between the acapnia and hypercapnia treatments, the transitions between the F→O and O→C phases occurred at significantly different hemolymph PCO_2 but comparable PO_2. It is clear that decapitated cockroaches displaying DGCs are still sensitive to CO_2, as exposure to 2 kPa PCO_2 caused both minimum and maximum hemolymph CO_2 level to increase by < 1 kPa, indicating hyperventilation (Fig. 10).

To further investigate whether *G. portentosa* DGCs could persist despite significantly elevated minimum and maximum CO_2, a single decapitated cockroach was exposed to 3 kPa PCO_2 (Fig. 17). This cockroach continued to display DGCs despite its minimum PCO_2 values exceeding the maximum hemolymph PCO_2 values previously recorded in acapnia (Fig. 17). However, slightly increased maximum and minimum hemolymph PO_2 levels appear to indicate that hypercapnia is stimulating an increase in ventilation during the gas exchange portions of the cycle, while not abolishing the cycle itself. Although previous research has shown that hypercapnia exposure terminates DGCs in insects (Harrison et al., 1995, Terblanche et al., 2008), our research indicates that in these cockroaches, significantly higher levels of PCO_2 than used here are likely required to abolish DGCs and stimulate continuous ventilation. An insensitivity to hypercapnia has been shown for at least one burrowing species of cockroach, with (Miller, 1981) reporting that quiescent burrowing cockroaches (*Blaberus craniifer*) abolish DGCs for continuous breathing only when exposed to > 5-10 kPa PCO_2. It appears that although

cockroaches alter ventilation during the O-phase in response to chemoreception of PCO_2, the alternating ventilation and apnoea typical of the DGC does not result from chemoreceptive feedback around a ventilatory PCO_2 threshold.

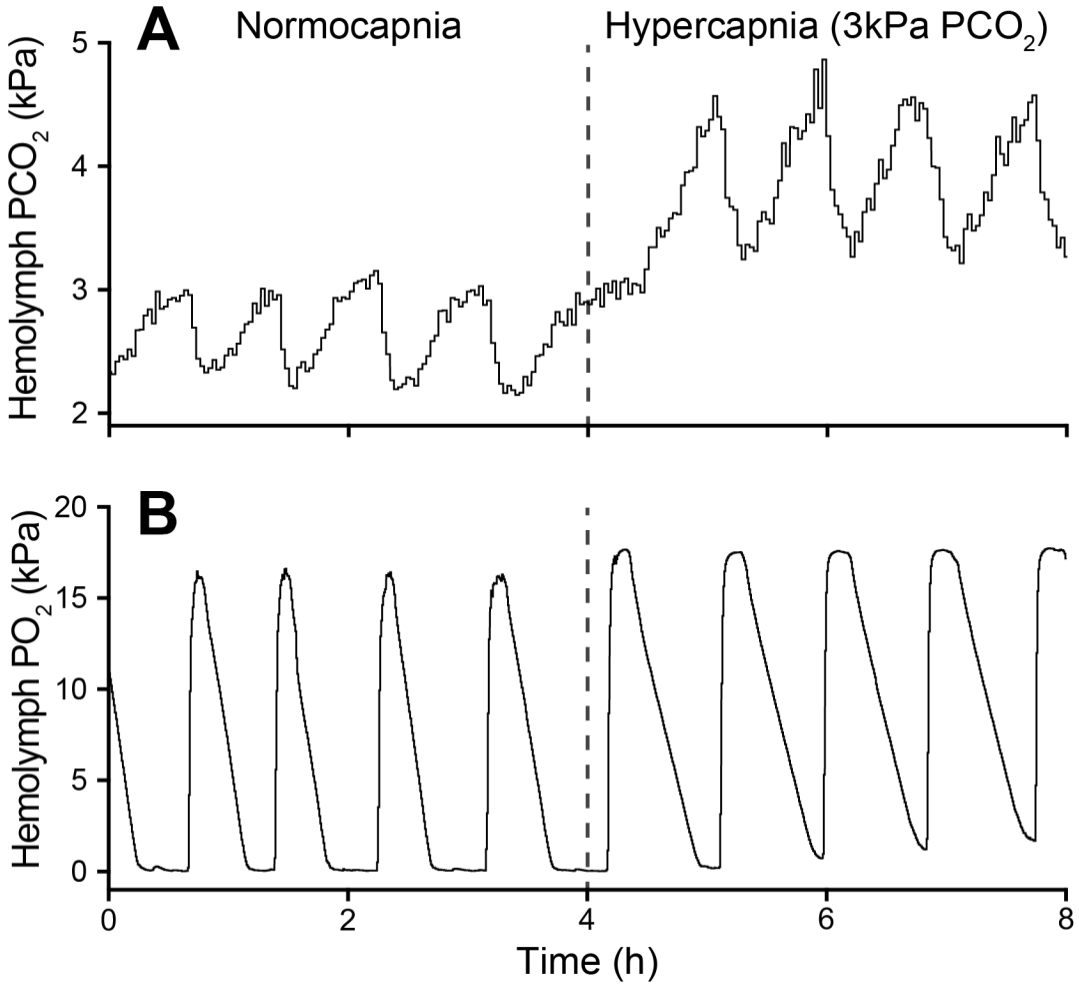

Figure 17. Line charts illustrating hemolymph PCO_2 (kPa) and PO_2 (kPa) fluctuations recorded from a single decapitated *G. portentosa* during exposure to 3 kPa PCO_2. A) indicates hemolymph PCO_2 (kPa) fluctuations, B) indicates hemolymph PO_2 (kPa) fluctuations when exposed to normoxic acapnia (first 4h) and normoxic hypercapnia (second 4h). The dashed vertical lines indicate the point at which hypercapnia exposure began.

2.4.3 Effects of Hypoxia and Hypercapnia on DGC Patterns

2.4.3.1 Ventilatory Frequency

G. portentosa exposed to 10 kPa PO_2 did not show significantly changing mean ventilation frequency during the O-phase (Fig. 15). Similarly, the findings of Matthews and White (2011b) show that exposure to hypoxia does not affect ventilatory frequency during gas exchange in *N. cinerea*, except in cases of extreme hypoxia (<5 kPa PO_2). Median ventilation frequency increased in *G. portentosa* exposed to hypercapnia, although the change in mean ventilation frequency was not significant (Fig. 15). (Matthews and White, 2011b) found that *N. cinerea* did modulate their ventilatory pattern in response to hypercapnia, stating that while abdominal ventilation frequency declined over the course of a ventilatory burst in normoxic normocapnia, in hypercapnia it increased again towards the end of the burst. Qualitatively, it appeared that hypercapnia affected ventilation pattern during the O- phase, resulting in more erratic and exaggerated ventilatory movements. However, the phototransistor sensor could not be used to determine quantitative changes in the amplitude of abdominal ventilatory movements.

2.4.3.2 Durations of DGC phases

Hypoxia exposure was not predicted to affect total interburst duration or open phase duration, as the transitions between apnoeic and ventilatory periods were assumed to be mediated by the CO_2 threshold. Contrary to our predictions, hypoxia was correlated with a significantly decreased interburst duration which contributed to a significantly reduced cycle duration and an increased DGC frequency (Fig. 14). Generally, reduced median C+F-phase durations contributed to the decreased median interburst duration, although hypoxia did not result in significant changes in either phase overall (Fig. 13). Decreased interburst duration in hypoxia is consistent with the findings of Chown and Holter (2000) in which both C- and F-phase duration in the scarabid beetle *Aphodius fossor* decreased in response to hypoxia, resulting in an increased frequency of ventilatory periods. Somewhat contradictory to these findings, research on *L. migratoria* (Snelling et al., 2011) found that although hypoxia reduced the C-phase duration, it increased the F-phase duration, resulting in no net change to interburst duration. In addition, where our research found no significant effect of hypoxia on O-phase duration, varying effects have also previously been described in other insect species. For examples, the O-phase duration in response to hypoxia exposure has been shown to greatly reduce in *A. fossor* (Chown and Holter, 2000), increase in *L. migratoria*, and, in agreement with our findings, have no substantial effect in the carpenter ant *Camponotus vicinus* (Lighton and Garrigan, 1995). There is clearly significant variation in the effects of hypoxia exposure on specific phase durations in different species, and so unsurprisingly, the effects of hypoxia on *G. portentosa* did not align with our predictions. The considerable interspecific variation in effects of hypoxia on DGC phase durations is further evidence that DGCs are not simply resulting from predictable ventilatory responses to oscillating gas tensions. Instead, results from this thesis suggests that although patterns of DGCs are modulated by hypoxia detection, PO_2 fluctuations are not required to trigger phase transitions.

Despite initial predictions, hypercapnia exposure did not terminate DGCs in decapitated cockroaches, but did significantly alter specific aspects of the DGC phases. Hypercapnia exposure correlated with a significantly increased O-phase duration (Fig. 13) and decreased interburst duration, which overall culminated in a significantly reduced cycle duration and increased DGC frequency (Fig. 14). A longer O-phase may increase total ventilation, as there is more time available for gas exchange between the cockroach and its external environment. This

increased O-phase duration may be another way in which DGCs can be modulated to increase total gas exchange in hypercapnia, while maintaining DGCs. Additionally, the decreased interburst duration resulting from hypercapnia exposure indicates how hypercapnia could still stimulate increased ventilation in decapitated *G. portentosa* while DGCs were maintained, resulting in elevated CO_2 exhalation. However, hypercapnia is often associated with the termination of DGCs in intact insects (Miller, 1981, Harrison et al., 1995, Terblanche et al., 2008), whereas our observations come from decapitated cockroaches. Our findings therefore primarily indicate that PCO_2/pH chemoreception is modulated to affect DGC patterns, but DGCs patterns do not necessarily emerge to facilitate enhanced ventilation in hypercapnic environments. Although the evidence suggesting that phase transitions are not dependent oscillating PCO_2 chemoreceptive feedback, our results clearly demonstrate that ventilatory patterns, primarily the duration of the apnoeic periods, may be modulated by PCO_2 chemoreception.

2.4.4 Conclusions for Cockroach Ventilatory Control

1. Continuously ventilating cockroaches in normoxic acapnia appear to regulate breathing in response to internal PCO_2/pH, corroborating previous findings in other insect taxa.
2. DGC phase transitions were observed occurring across a range of PCO_2, which is inconsistent with the hypothesis that DGCs emerge due to hemolymph PCO_2 fluctuating around a ventilatory threshold.
3. Hypoxia and hypercapnia exposure appeared to affect DGC phase durations and ventilatory patterns in *G. portentosa*, suggesting that chemoreception of PO_2 and PCO_2/pH still modulates DGC patterns.

Chapter 3: General Conclusions and Future Directions

3.1 Ventilatory Control in *G. portentosa*

During continuous ventilation, mean hemolymph PO_2 generally remained only a few kPa below ambient levels, whereas mean hemolymph PCO_2 from all our experiments was observed at a stable level somewhere between ~1-3 kPa. It was assumed that these stable PCO_2 levels were the PCO_2 ventilatory thresholds, whereby ventilation was increased or decreased to maintain homeostasis around these setpoints. The initial hypothesis for the production of DGCs was that they are driven by instability in the ventilatory control system, resulting from a delayed response to PCO_2 chemoreception around this PCO_2 ventilatory threshold. However, contrary to the proposed hypothesis, this thesis found that decapitated *G. portentosa* exposed to hypoxia and hypercapnia did not terminate DGCs despite ambient gas mixtures significantly altering hemolymph PO_2 and PCO_2 away from perceived ventilatory thresholds. This refutes the hypothesis that DGCs emerge merely as ventilatory behaviours which are turned on or off due to chemoreceptive feedback and oscillations around O_2 and CO_2 thresholds. Although DGCs appeared to operate independently of defined PCO_2 or PO_2 thresholds, it was clear that hypoxia and hypercapnia exposure affected many parameters of the DGCs. Predominantly, hypoxia and hypercapnia reduced interburst duration, therefore increasing the frequency of ventilatory periods. Additionally, although it could not be quantified, hypercapnia appeared to affect ventilatory movements during the O-phase. These results show that although the DGCs in *G. portentosa* may operate independently of chemosensory feedback, ventilatory patterns during DGCs may still be modulated by hypoxia and hypercapnia exposure.

3.2 Significance and Implications

This work is the first to successfully report hemolymph PO_2 and PCO_2 recorded simultaneously *in vivo* in a ventilating insect. The results presented in this thesis demonstrate that the phase transitions observed during DGCs of *G.* portentosa are not dependent on chemosensory feedback triggered by crossing PO_2 or PCO_2 thresholds. However, as DGCs have been suggested to have evolved independently in at least five insect taxa (Marais et al., 2005), it is conceivable that different insect taxa control DGCs via different mechanisms. It is therefore imperative that

these types of experiment are conducted on insects across a range of taxa in order to determine if evolutionarily distant insects share similar mechanisms for the control of DGCs. Specifically, further research on previous insect species which have provided support for the threshold model, such as *A. atlas* (Förster and Hetz, 2010).

No evidence is provided in this thesis for testing other mechanisms of DGC regulation. However, previous research has found evidence for insect episodic breathing regulated by rhythm generators. Potential rhythm generators have already been identified in cockroaches and other insects. For example, Myers and Retzlaff (1963) isolated the CNS of the Cuban burrowing cockroach (*Bysotia fumigata*) and showed that deafferented ganglia fired episodically with similar frequency to episodic ventilatory movements. Additionally, deafferented ganglia in the distantly related *L. migratoria* have been observed firing discontinuously in a pattern corresponding with discontinuous ventilatory patterns (Bustami and Hustert, 2000). With this thesis finding no clear evidence to support the hysteresis hypothesis, it is suggested that future research could focus on identifying rhythm generators which control DGCs independently of strict chemosensory feedback.

3.3 Limitations

The finite supply of PCO_2 probes restricted the final sample size available for data analysis, causing a higher likelihood that the statistical analysis will result in type II errors. For this reason, if median changes in measurements noticeably differed between exposures, these differences are occasionally discussed in context with previous research, even in cases where t-tests suggest non-significance at $\alpha = 0.05$. Additionally, with the limited number of experiments available, acute exposures to hypoxia and hypercapnia were chosen in order to obtain the largest number of data points in a single treatment. However, research in other insects has shown that ventilatory systems do not respond linearly to PO_2 and PCO_2 chemoreception, and so measuring PO_2 and PCO_2 control over a range of ambient hypoxic and hypercapnic tensions would be useful in further determining the effects of ambient gas manipulations on DGC patterns.

It is possible that the photochemical tips of each PO_2 and PCO_2 optode succumbed to degrees of photobleaching over the course of the experiments. However, photobleaching was not believed to have any substantial effect on the measurements recorded over the course of the experiments as PO_2 and PCO_2 measurements did not show observable signal drift over time. The

effects of photobleaching were reduced by low sampling frequencies: once per 30 s by the PO_2 optodes and once per 2 min by the PCO_2 optodes. Measurements of PO_2 taken at 30 s intervals were able to capture changes in PO_2. However, this sampling frequency may have been too low to capture any small, fast changes in hemolymph O_2 during the flutter phase. Additionally, slight changes in hemolymph PO_2 during the flutter phase may not have been observed as the PO_2 optodes were designed to measure PO_2 over a large range (0-100 kPa), so their ability to resolve small changes is limited to differences of around 0.01 kPa in an atmosphere of 1 kPa PO_2. For future study of O_2 fluctuations during specifically the flutter phase, researchers may choose to use O_2 probes designed for a smaller range of O_2 measurements, at higher sampling frequencies. Individual PCO_2 measurements taken at 2-min intervals appeared to be sufficient for capturing changes in PCO_2, despite PCO_2 probes taking longer to equilibrate at each measurement than the PO_2 probes. However, it should be noted that as a result of the slow equilibration to changing PCO_2 levels, the probes would have been unable to provide exact minimum and maximum PCO_2 measurements. Instead as the PCO_2 probes will not have had time to equilibrate PCO_2 levels before the true levels changed due to opening or closing of spiracles, recorded values would have fallen slightly within the true range of minimum and maximum PCO_2 fluctuations.

Although these experiments found no substantial effect of hypoxia or hypercapnia on ventilatory frequency, qualitatively it appeared that these conditions were affecting ventilatory movements of decapitated *G. portentosa*. Where our research was unable to quantify these observations, future research may choose to study other ventilatory parameters in conjunction with ventilatory frequency, such as tracheal tidal volume changes, and how these parameters change in response to hypoxia or hypercapnia. Another limitation in our study of ventilatory response comes from the assumption that abdominal movements are directly associated with spiracle and ventilatory activity. High physical activity in intact cockroaches made it impossible to parse out ventilatory frequency measurements in continuously breathing intact *G. portentosa*. Additionally, it may have been incorrect to assume that the low amplitude abdominal movements preceding the O-phase corresponded with the F-phase, as spiracle behaviour was not observed at all. As our predictions about F-phase response to hypoxia and hypercapnia were originally predicated on data taken solely from spiracle behaviour in the atlas moth pupae (Förster and Hetz, 2010), and not ventilatory movements, the assumptions may have led to incorrect comparisons.

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