Discontinuous gas exchange in Orthoptera – mechanisms and hypotheses

by
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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Date: 16 November 2014

Abstract

Respiratory and ventilatory dynamics in insects are of fundamental importance to understanding evolved variation in gas exchange patterns, such as the discontinuous gas exchange cycle (DGC). However, the evolutionary origin and maintenance of the DGC in tracheate arthropods are poorly understood and highly controversial. Possible reasons for the occurrence of the DGC include water savings (hygric hypothesis) or the prevention of oxygen toxicity (oxidative damage hypothesis). Research presented in this dissertation aimed to examine the variation or modulation of the discontinuous gas exchange pattern, the environmental factors that potentially drive this relationship, and the competing hypotheses by: 1) examining the relative importance of convection *vs* diffusion during the DGC; 2) testing the oxidative damage and hygric hypotheses; 3) investigating ventilatory movements over the entire DGC; 4) examining intratracheal pressure patterns; and 5) investigating prioritisation of abiotic factors in the gas exchange control cascade. To accomplish these aims two model species of Orthoptera (Acrididae) which show discontinuous gas exchange cycles (DGCs) were chosen: the desert locust, *Schistocerca gregaria*, and a grasshopper commonly associated with wetlands, *Paracinema tricolor*. Firstly, I found that in *S. gregaria*, there was no clear intratracheal pressure pattern that accompanies the DGC. This shows that the gas exchange dynamics of Orthoptera differ substantially from those of Lepidoptera, in which a distinctive intratracheal pressure pattern accompanies the DGC, highlighting that the Lepidoptera cannot serve as a general model for all insects showing the DGC. Secondly, I found that in *P. tricolor*, a hierarchy of abiotic factors influence the DGC, rather than only a single hypothesis or factor explaining the occurrence of the DGC. For *S. gregaria*, gas exchange is most efficient at slightly hypoxic to normoxic conditions. At these conditions, diffusive gas exchange dominates, meaning that no body movements are necessary to aid gas exchange, making this mode of gas exchange less energetically costly than active convection. In conclusion, these results confirm that the maintenance of the DGC is affected by multiple abiotic factors. The findings of this dissertation have significant implications for understanding the mechanistic basis and energetic cost of gas exchange in insects.

Opsomming

Asemhalings en ventilasie dinamika in insekte is van fundamentele belang om die ontwikkelde variasie in asemhalingspatrone, soos bv. die diskontinue gaswisseling siklus (DGS), te verstaan. Tog word die evolusionêre oorsprong en instandhouding van die DGS in geleedpotiges wat ‘n trageale asemhalingsisteem besit tans nog nie goed verstaan nie. Moontlike redes vir die voorkoms van die DGS sluit in waterbesparing (waterbesparings hipotese) of die voorkoming van suurstof toksisiteit (oksidatiewe skade hipotese). Navorsing wat in hierdie dissertasie voorgelê word het gemik om die variase en modulasie van die DGS the ondersoek, die omgewingsfaktore wat potensieel hierdie verhouding kan aandryf, asook die kompeterende hipoteses deur: 1) die relatiewe belangrikheid van konveksie teenoor diffusie tydens die DGS te ondersoek; 2) die oksidatiewe skade en waterbesparings hipoteses te ondersoek; 3) ventilasie bewegings tydens die hele DGS te ondersoek; 4) druk patrone in die tragea te ondersoek; en 5) die prioritisering van abiotiese faktore in die gaswisseling hiërargie te ondersoek. Om hierdie doelwitte te bereik is twee model spesies van Orthoptera (Acrididae) wat die DGS toon gekies: die woestyn sprinkaan, *Schistocerca gregaria*, en ‘n sprinkaan wat algemeen in vleilande voorkom, *Paracinema tricolor*. Eerstens het ek gevind dat daar geen duidelike patroon in die druk van die tragea van *S. gregaria* voorkom wat gepaardgaan met die DGS nie. Dit dui daarop dat die gaswisselings dinamika van Orthoptera aansienlik verskil van die van Lepidoptera. In Lepidoptera is daar ‘n duidelike patroon in die druk van die tragea sigbaar wat gepaardgaan met die DGS, wat beklemtoon dat die Lepidoptera nie gebruik kan word as ‘n algemene model vir alle insekte wat die DGS gebruik nie. Tweedens het ek gevind dat eerder as wat slegs ‘n enkele faktor of hipotese die voorkoms van die DGS bepaal, daar ‘n hiërargie van abiotiese faktore is wat die DGS in *P. tricolor* beïnvloed. Vir *S. gregaria* is gaswisseling die doeltreffendste by effens hipoksiese tot normoksiese suurstof kondisies. By hierdie kondisies vind gaswisseling hoofsaaklik deur middel van diffusie plaas, wat beteken dat geen liggaams bewegings nodig is om gaswisseling aan te vul nie. Dit maak hierdie vorm van gaswisseling goedkoper (in terme van energie kostes) as aktiewe konveksie. Ten slotte bevestig hierdie resultate dat die voorkoms en modulasie van die DGS beïnvloed word deur verskeie abiotiese faktore. Die bevindings van hierdie dissertasie hou ook belangrike implikasies in vir die begrip van die meganistiese basis en kostes (in terme van energie) van gaswisseling in insekte.

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Dedications

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Chapter 1: General introduction

1.1. Environmental energy and water availability

Energy and water availability are crucial to life on earth. This energy includes radiation energy (photosynthetically active radiation), thermal energy and chemical energy (Gibbs free energy). The growth, reproduction, responsiveness and internal regulation of an organism require energy in the form of adenosine triphosphate (Raven and Johnson, 2002). The rate at which an organism takes up, transforms and uses energy and materials is called the metabolic rate (Gillooly et al., 2001; Brown et al., 2004). An insect's metabolic rate is dependent on the ambient temperature, as the body temperature of ectotherms is typically close to that of the ambient environment (Hoffmann, 1985; Gillooly et al., 2001; Brown et al., 2004; Algar et al., 2007). An increase in ambient temperature will therefore result in an increase in body temperature of the insect. As the body temperature of the insect increases, so does its metabolic rate, thus ultimately resulting in higher energy uptake per individual (Algar et al., 2007).

A water-rich environment is necessary for organismal growth and reproduction and water is also a vital requirement of cell function, as it is the solvent in which the biochemical reactions releasing energy takes place (Block, 1996; Raven and Johnson, 2002). Desiccation is one of the most important environmental stressors that insects can face in nature. Terrestrial insects are particularly susceptible to dehydration because of their small size and therefore large surface area-to-volume ratio (Kaars, 1981; Hadley, 1994a; Gibbs et al., 1997; Chown and Gaston, 1999). Terrestrial insects face significant metabolic challenges, for example, they have to regulate internal O_2 levels necessary for aerobic metabolism and excrete metabolic by-products (e.g. CO_2), while minimizing water loss (e.g. Chown, 2002; Woods and Smith, 2010). Insects can respond to these simultaneous pressures by means of a range of behavioural and physiological adaptations (reviewed in e.g. Chown et al., 2011), and one such physiological adaptation is to modulate the gas exchange pattern which the insect uses. The mechanisms that are employed are of great interest as insects are the most numerically successful and speciose animals on our planet (Hadley, 1994a; Chown, 2002). It is also important to understand these mechanisms in order to comprehend the degree to which animals can cope with varying environmental conditions at several time-scales, which in turn could help with preserving the biological diversity of earth (Chown and Terblanche, 2007).

1.2. Insect gas exchange

Insects have a tracheal respiratory system which consists of a network of semi-rigid air-filled tubes called the tracheae (Steen, 1971). The tracheae branch throughout the body of the insect and terminate in liquid-filled tracheoles which lie adjacent to cells, or might even penetrate them to lie close to the mitochondria (Wigglesworth, 1930; Wigglesworth, 1953; Miller, 1974; Dejours, 1975). The tracheoles are the main sites at which gas exchange occurs with tissues. The spiracles, which are valve-like structures that occur in the cuticle of the insect, connect the tracheae to the exterior environment. The spiracles can be schematically illustrated as tubes connecting the internal environment of the animal with the external atmosphere (see Figure 1.1) (Schmitz and Wasserthal, 1999; Wobschall and Hetz, 2004; Woods and Smith, 2010).

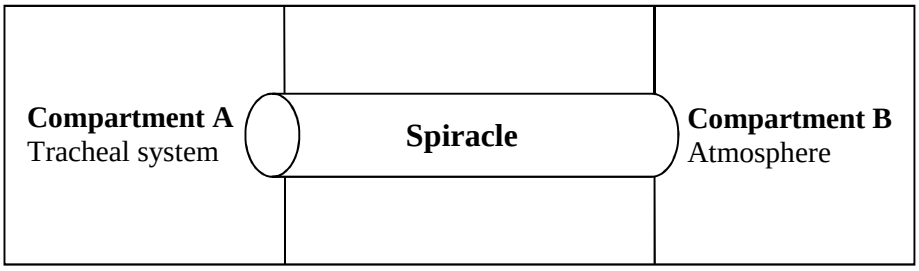

Figure 1.1. Diagram illustrating the spiracle of an insect connecting the internal environment of the insect (tracheal system) with the outside atmosphere.

Insects can manipulate the opening and closing of their spiracles and by doing so can regulate the flow of gases and water vapour into and out of the tracheae (Wigglesworth, 1953; Schneiderman and Williams, 1955; Schneiderman, 1956; Kanwisher, 1966; Dejours, 1975; Hadley, 1994b). This makes it possible to exchange respiratory gases with the environment periodically, and by doing so, perhaps significantly minimizing respiratory water loss (Buck and Keister, 1955; Buck, 1962; Burkett and Schneiderman, 1967; Miller 1974). Oxygen consumption and carbon dioxide production within the insect are continuous, driven by cellular respiration, while the external exchange of gases can be continuous, cyclic or discontinuous (Snyder et al., 1995; Marais et al., 2005). Insects may therefore face a trade-off between the need to support aerobic metabolism and the need to conserve water (Ahearn, 1970; Kaars, 1981; Nicolson and Louw, 1982; Kestler, 1985). This trade-off may play an important role in the determination of ventilation patterns and behaviour in terrestrial insects

(Cloudsley-Thompson, 1975; Lighton and Feener, 1989; Lighton, 1996; Lighton and Fielden, 1996; Danks, 2000; Cloudsley-Thompson, 2001; Gibbs et al., 2003).

1.3. Insect gas exchange patterns

Terrestrial insects can display at least three different patterns of gas exchange when they are at rest (Marais et al., 2005; Quinlan and Gibbs, 2006). These patterns include: 1) continuous gas exchange (Figure 1.2A), in which the spiracles remain open (or if they close, only for brief periods) and gas exchange is relatively constant (Hadley, 1994b; Gibbs and Johnson, 2004); 2) cyclic gas exchange (Figure 1.2B), during which time spiracles may open and close in synchrony, but some gas exchange is always detectable (Shelton and Appel, 2001; Gibbs and Johnson, 2004; Chown et al., 2006); and 3) discontinuous gas exchange (Figure 1.2C), which is a cyclic pattern of gas exchange and includes periods when the spiracles are open, as well as long periods when the spiracles are fully closed, preventing respiratory gas exchange with the atmosphere (Lighton, 1996; Hetz and Bradley, 2005).

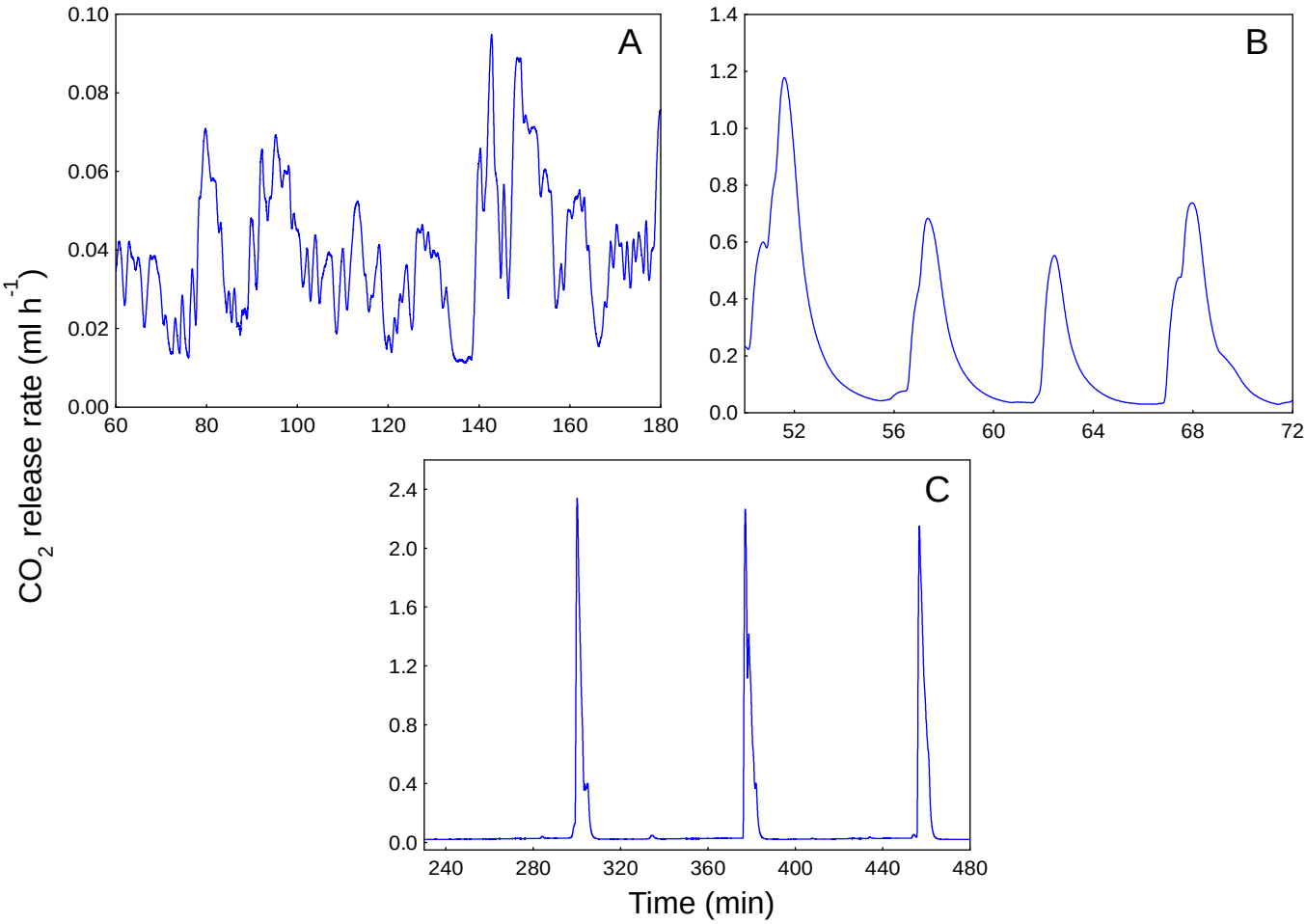

Figure 1.2. Representative continuous, cyclic and discontinuous gas exchange patterns for *Aptera fusca* (Blattodea, Blaberidae) at 15°C and a flow rate of 200 ml min^{-1}. Examples of the different gas exchange patterns: (A) continuous (mass 1.927 g); (B) cyclic (mass 1.861 g); and (C) discontinuous gas exchange (mass 1.892 g).

There is currently renewed interested in understanding how and why such a diversity of gas exchange patterns arose. Even though much research has been undertaken regarding these different gas exchange patterns, several controversies still remain. Many of these controversies are regarding the evolutionary origin, current maintenance and potential fitness advantages of the discontinuous gas exchange cycle (DGC) (Hadley, 1994b; Lighton and Berrigan, 1995; Lighton, 1998; Klok et al., 2002; Marais et al., 2005; Chown et al., 2006; Lighton, 2007). Broad phylogenetic analyses suggest that continuous and cyclic gas exchange are the basal patterns across the Insecta (Marais et al., 2005; Clusella-Trullas and Chown, 2008). By contrast, the DGC evolved independently at least six times in the Insecta within the following groups: Blattodea, Orthoptera, Coleoptera, Lepidoptera, Hymenoptera and Hemiptera (Marais et al., 2005; Contreras and Bradley, 2010). This suggests that the DGC

pattern is not an ancestral trait, but rather that it represents a derived state and is thus probably of some adaptive significance (Marais et al., 2005).

1.4. Insect metabolic rate

Gas exchange patterns can vary between insect orders, between or within species, or even within a single individual. Several extrinsic and intrinsic factors can influence insect gas exchange patterns (e.g. temperature (through its influence on e.g. metabolic rate and haemolymph buffering capacity), hydration state), however the influence that these factors have on switching between different patterns is typically not well understood. Contreras and Bradley (Contreras and Bradley, 2010) found that gas exchange patterns of *Rhodnius prolixus* and *Gromphadorhina portentosa* vary depending on their metabolic rate. They found that at low metabolic rates discontinuous gas exchange cycles (DGCs) are employed, while as temperature increases (thereby also increasing metabolic demand), this pattern changes to cyclic gas exchange, and eventually to continuous gas exchange at the highest temperatures. Basson and Terblanche (Basson and Terblanche, 2011) also found a shift in the gas exchange pattern used by three different *Glossina* species with increasing temperatures. In these species, increased temperatures lead to significantly fewer individuals exhibiting cyclic gas exchange due to the elevation of metabolic rates through experimentally increased temperatures. At low temperatures, wasps (*Vespula* sp.) exchange gases using burst-interburst (i.e. where no clear flutter-period can be distinguished, only an open (burst) and closed (interburst) spiracle phase) or discontinuous gas exchange patterns. At high temperatures, they exchange gases cyclically (Käfer et al., 2013).

The metabolic rate of insects vary among the different gas exchange patterns (continuous, cyclic, DGC). For the ant *Pogonomyrmex barbatus* metabolic rate is lowest during the DGC, intermediate during cyclic gas exchange and highest during continuous gas exchange (Gibbs and Johnson, 2004). The same is also true for individuals of *Pterostichus niger*, where individuals that use cyclic gas exchange have higher metabolic rates than individuals that use DGCs (Kivimägi et al., 2011). However, Williams et al. (Williams et al., 2010) found that the metabolic rate of *Erynnis propertius* larvae does not differ between the DGC and continuous gas exchange. Marais and Chown (Marais and Chown, 2003) found that a cockroach (*Perisphaeria* sp.) can exhibit four different gas exchange patterns within a single individual,

when at rest (continuous, cyclic, interburst-burst, DGC). They found that the insect's metabolic rate during continuous gas exchange is significantly higher than during the other gas exchange patterns, but that metabolic rates during the other gas exchange patterns do not differ significantly from each other (Marais and Chown, 2003). However, for the Table Mountain cockroach, *Aptera fusca*, no difference in resting metabolic rate between the different gas exchange patterns was found (Groenewald et al., 2013).

1.5. Discontinuous gas exchange

1.5.1. Phases

Discontinuous gas exchange only occurs when insects are at rest (Hamilton, 1964; Kestler, 1985; Quinlan and Hadley, 1993) and it effectively temporally uncouples O_2 uptake from CO_2 emission (Lighton, 1994; Lighton, 1996). A DGC consists of three phases which are described in terms of spiracular behaviour, namely: a closed- (C), flutter- (F), and open- (O) spiracle phase (Figure 1.3). During the C-phase, the spiracles remain tightly shut and gas exchange with the atmosphere is prevented. Pressure within the tracheae declines as O_2 is used by metabolically active tissues, and CO_2 accumulates in the tracheae and dissolves into the haemolymph. When the partial pressure of oxygen within the tracheal system has declined to ~2-5 kPa, the flutter phase is initiated (Levy and Schneiderman, 1966b; also see review by Lighton, 1996). This phase includes the rapid opening and closing of spiracles in order to regulate tracheal oxygen levels (Burkett and Schneiderman, 1967; Miller, 1974; Lighton, 1994; Hetz and Bradley, 2005; Chown et al., 2006), while CO_2 continues to dissolve into the haemolymph. The acidity that this CO_2 produces is then partially buffered by factors in the haemolymph. Once the intratracheal partial pressure of CO_2 reaches a certain threshold, the O-phase is initiated. This threshold may vary depending on the species, e.g. the threshold for *Attacus atlas* pupae is 1-2.5 kPa (Förster and Hetz, 2010), 6.4 kPa for *Hyalophora cecropia* pupae (Levy and Schneiderman, 1966) and for the grasshopper *Taeniopoda eques* the threshold is 2-2.9 kPa (Harrison et al., 1995). During the O-phase the spiracles are fully open and gases are exchanged rapidly through diffusion, but can also be aided by active ventilatory movements (muscle contractions) (Brockway and Schneiderman, 1967; Loveridge, 1968; Lighton, 1988; Hadley, 1994b; Duncan et al., 2010; Harrison et al., 2013). The O-phase continues until the intratracheal partial pressure of CO_2 reaches a certain level, which may vary depending on the species, whereupon spiracles are closed (Levy and

Schneiderman, 1966a; Miller, 1974). This cycle then repeats itself and may continue indefinitely, or until the insect is no longer quiescent.

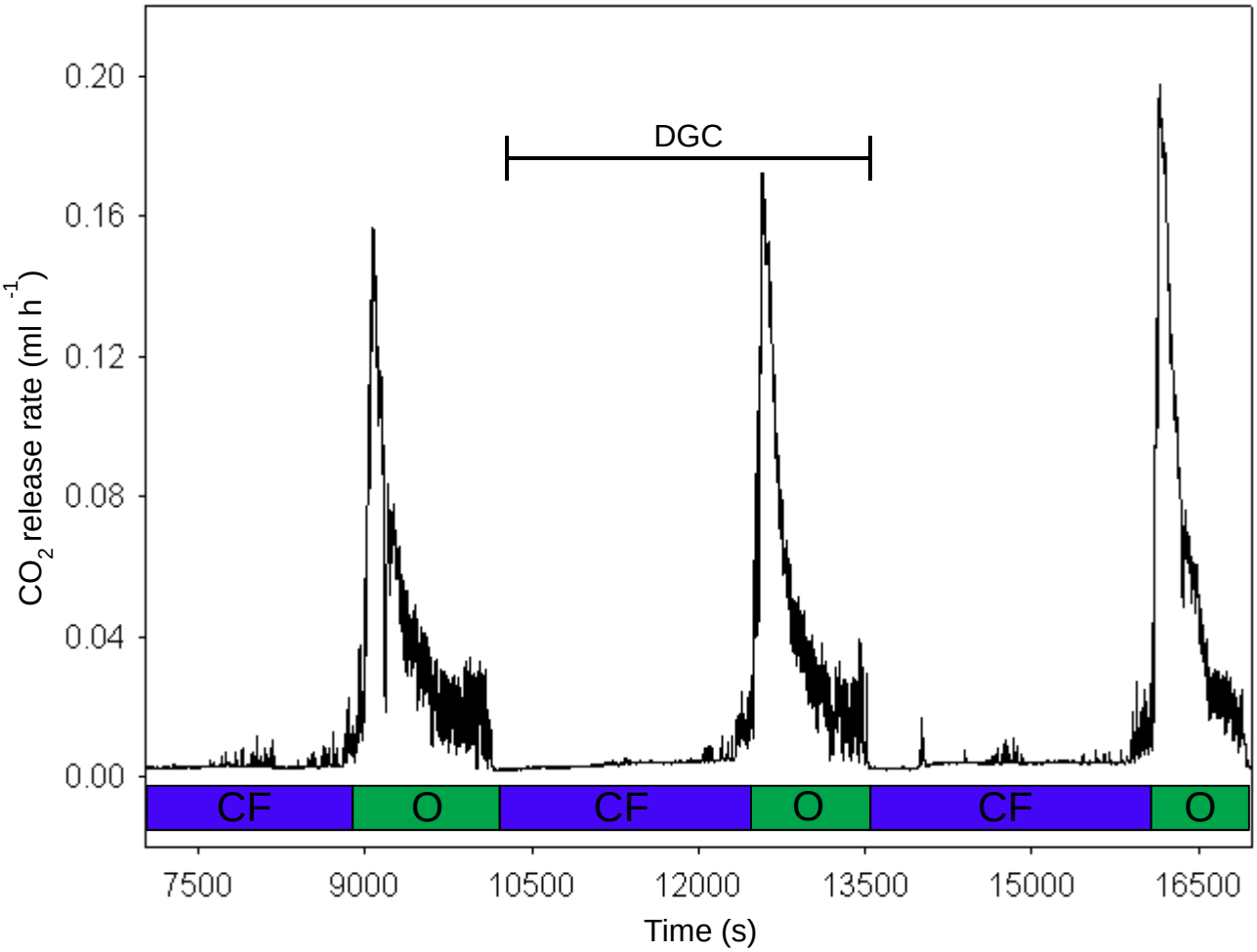

Figure 1.3. Discontinuous gas exchange cycles in a 0.2442 g male *Paracinema tricolor* (Orthoptera, Acrididae) at 15°C and 200 ml min^{-1} flow rate.
The different phases of the discontinuous gas exchange cycle (DGC) are indicated by the different bars (CF- closed/flutter phase; O- open phase).

1.5.2. Hypotheses

It has long been thought that the DGC evolved primarily as a means to reduce respiratory water loss (Hamilton, 1964; Levy and Schneiderman, 1966b; Kestler, 1985; Sláma and Coquillaud, 1992; Hadley and Quinlan, 1993; Lighton et al., 1993b; Hadley, 1994b; Lighton and Berrigan, 1995; Williams et al., 1997; Duncan et al., 2002), because during the C-phase respiratory water loss rates are basically zero (Gibbs and Johnson, 2004; Terblanche et al., 2010), and during the F-phase the outward movement of CO_2 and H_2O is restricted by the inward flow of air (Kestler, 1985; Lighton, 1996; Wobschall and Hetz, 2004). This hypothesis has received much recent support (e.g. Sláma et al., 2007; White et al., 2007; Schimpf et al.,

2009; Williams et al., 2010), but several competing hypotheses have also been proposed to explain the origin and maintenance of the DGC (Lighton, 1996; Chown et al., 2006; Lighton, 2007). These hypotheses are mostly adaptive, but also include non-adaptive explanations (Chown and Holter, 2000).

The adaptive hypotheses are: 1) the hygric hypothesis – the DGC serves to reduce respiratory water loss (Hadley, 1994b; Lighton, 1996); 2) the chthonic hypothesis – the DGC facilitates gas exchange under hypoxic and/or hypercapnic conditions (which are often characteristic of underground environments) (Lighton, 1996; Lighton, 1998); 3) the chthonic-hygric hypothesis – the DGC is an adaptation to reduce respiratory water loss under hypoxic and/or hypercapnic conditions (Lighton and Berrigan, 1995); 4) the oxidative damage hypothesis – the DGC is an adaptation to minimize oxidative damage to tissue, while still providing sufficient gas exchange (Hetz and Bradley, 2005; Chown et al., 2006); and 5) the strolling arthropod hypothesis – the DGC is an adaptation to reduce the risk of parasitic infestation of the tracheae by increasing the frequency of spiracle closure (Miller, 1964; Chown et al., 2006).

The emergent property hypothesis, which is a nonadaptive hypothesis, states that the DGC is the outcome of interactions between O_2 and CO_2 set-points that control the opening and closing of spiracles (Schneiderman, 1960; Burkett and Schneiderman, 1974; Förster and Hetz, 2010; Chown, 2011). There is still no consensus regarding which of these hypotheses is correct. The hygric and oxidative damage hypotheses are probably the most prevalently tested in the modern literature (Lighton, 1998; Shelton and Appel, 2000; Duncan et al., 2002; Chown and Nicolson, 2004; Gibbs and Johnson, 2004; White et al., 2007; Jogar et al., 2008) and much recent support has been found for both of these hypotheses (e.g. Hetz and Bradley, 2005; White et al., 2007; Terblanche et al., 2008; Schimpf et al., 2009; Williams et al., 2010; Schimpf et al., 2011; but see Boardman et al., 2012), suggesting that both may provide mechanistic explanations for the evolution of the DGC.

The oxidative damage hypothesis is supported in moth pupae, which regulate their tracheal partial pressure of O_2 (PO_2) at low levels during the F-phase, even when exposed to hyperoxic conditions (Hetz and Bradley, 2005; see also Terblanche et al., 2008; Boardman et

al., 2012). However, it is not sure whether this hypothesis holds true for other insect orders as well, for example, Matthews et al. (Matthews et al., 2012) found that in *Locusta migratoria* tracheal PO_2 levels are dependent on ambient oxygen levels when exposed to hyperoxic conditions. Schimpf et al. (Schimpf et al., 2009) examined three of the competing hypotheses (hygric, oxidative damage and chthonic) in the cockroach *Nauphoeta cinerea*, and found support only for the hygric hypothesis in this species. They found that this cockroach reduces its O-phase duration after expose to low relative humidity conditions, thereby possibly reducing respiratory water loss. In a different study on this same cockroach species, Schimpf et al. (Schimpf et al., 2011) found that cockroaches that use the DGC have lower respiratory and total water loss rates than cockroaches exhibiting other gas exchange patterns, again lending support to the hygric hypothesis. Support for the hygric hypothesis has also been found in larvae of the moth *Erynnis propertius* which have lower water loss rates during the DGC than during continuous gas exchange, as well as having a lower hygric cost of gas exchange (water loss associated with CO_2 release) during the DGC (Williams et al., 2010). However, Kivimägi et al. (Kivimägi et al., 2011) found no support for the hygric hypothesis in *Pterostichus niger*, as in this species the DGC is present in conditions of both low and high relative humidity.

The underlying mechanistic and evolutionary reasons for variation in patterns of insect gas exchange are receiving renewed attention (White et al., 2007; Terblanche et al., 2008; Chown, 2011; Contreras and Bradley, 2011; Matthews and White, 2011a; Berman et al., 2013), and new hypotheses are continuing to be proposed. One such recently proposed nonadaptive hypothesis is the neural hypothesis, which states that the DGC is the consequence of the down-regulation of brain activity (Matthews and White, 2011b; Matthews and White, 2013). This hypothesis states that the DGC arises when ventilation is not regulated by higher neural centres (i.e. the cephalic ganglion), and the control is given over to the thoracic and abdominal ganglia (Matthews and White, 2011b; Matthews and White, 2013).

Several studies have suggested that the issue is more complex and that several factors work together to influence the expression of the DGC (Chown, 2002; Chown, 2011). It is also possible that due to the global variation of environmental conditions, such as water

availability, temperature, and the partial pressure of O_2 and CO_2, the DGC could be used for a certain need under some circumstances, while under other circumstances it may be used for an entirely different purpose (White et al., 2007). The evolution of the DGC does not have to be explained solely by adaptive hypotheses, but could incorporate a non-adaptive component as well (Chown, 2011). The presence or absence of the DGC under certain circumstances may be influenced by several different environmental factors simultaneously (Chown, 2002; Terblanche et al., 2008). Therefore, the most costly variable in an organism's immediate environment could be the main factor which determines/regulates the expression of the DGC within that specific environment.

1.6. Haemolymph acid-base status

One of the most important aspects for maintaining homeostasis in an organism is the regulation of cellular and tissue acid-base balance (Nation, 2002; Harrison et al., 2012). There are several different physiological systems which insects can use to regulate their haemolymph acid-base status (Harrison, 1994) and one such system is the control of ventilatory CO_2 release through the spiracles. The intratracheal partial pressure of CO_2 (PCO_2) is therefore also regulated by respiratory CO_2 release, with gas exchange through the spiracles playing an important role in regulating tracheal PCO_2. Tracheal PCO_2 affects the haemolymph acid-base status of the insect, as CO_2 reacts with water to form carbonic acid. Results from several different insect species indicate that the ventilatory system may play a role in haemolymph acid-base homeostasis, and that even insects as small as aphids can use the homeostatic control of CO_2 release to regulate respiratory acidaemia (Sláma and Jedlička, 2012), however, results for grasshoppers are variable. For example, Harrison (Harrison, 1989) found that the locust *Schistocerca nitens* increases its ventilation rate in order to regulate haemolymph pH changes, however, Gulinson and Harrison (Gulinson and Harrison, 1996) found that for *Romalea guttata* ventilation rate does not increase with a decrease in haemolymph pH. The DGC could play an important role in the regulation of the acid-base balance of insects, as this cycle consists of closed- as well as open spiracle phases, which will lead to fluctuations in tracheal PCO_2 levels. During the C-phase, the spiracles remain occluded and a large portion of the CO_2 that the insect produces dissolves into the haemolymph, rather than appearing in the tracheal system. The acidity that this CO_2 produces is only partially buffered by factors in the haemolymph, so haemolymph pH declines. During

the O-phase, CO_2 that was dissolved in body fluids can exit the tracheal system through the open spiracles to maintain acid-base homeostasis (Woods and Smith, 2010). It is therefore possible that the DGC can cause significant cyclic variation in haemolymph pH, as was found by Matthews and White (Matthews and White, 2011a) for the cockroach, *Nauphoeta cinerea*, whose haemolymph pH fluctuated by 0.11 units during alternating periods of spiracle opening and closing during the DGC. However, the consequences which the DGC has on the haemolymph pH status of insects are still not well understood.

1.7. Biomechanics of gas exchange – diffusion and convection

Insects vary tremendously in their modes of gas exchange, which is at least partly dependent on the structure of the spiracle and its ability to close (Beckel and Schneiderman, 1957; Miller, 1960b; Burkett and Schneiderman, 1967), or the ability to regulate tracheal cross-sectional area (e.g. through body contractions, Clusella-Trullas and Chown, 2008) (Lighton, 1998; Sláma, 1999; Shelton and Appel, 2000; Chown, 2002). Efficient gas exchange is dependent on the morphological structure of the tracheal system, as well as adequate ventilation. Passive diffusion, or active muscular movements (i.e. convective gas exchange), and the selective opening of the spiracles can be used to modulate whole-animal ventilation patterns (Wigglesworth, 1935; Buck and Keister, 1955; Burkett and Schneiderman, 1967; Miller, 1974; Dejours, 1975; Lighton, 1996; Sláma, 1999; Chown et al., 2006) and may significantly influence the work (energy expended) associated with gas exchange owing to the mechanics involved.

In small insects, or in the inactive stages of larger insects, adequate gas exchange can be obtained by diffusion alone (Krogh, 1941; Weis-Fogh, 1964; Miller, 1974), however, Kestler (Kestler, 1985) found that gas exchange does not take place solely through diffusion, but rather that the predominant mode of gas exchange is diffusion and that it is supplemented with convection. It has recently been found that even insects as small as aphids use ventilatory movements to release CO_2 (Sláma and Jedlička, 2012, pp. 501). In larger or more active insects, diffusion needs to be aided by convection, or gas exchange needs to take place mainly through convection to maintain an adequate supply of O_2 to tissues (Buck, 1962; Sláma, 1988; Sláma et al., 2007; Wasserthal, 2014; also see review by Harrison et al., 2013). All insects depend on liquid diffusion for the final stages of gas exchange between the

tracheoles and mitochondria (Wigglesworth, 1930; Wigglesworth, 1935; Weis-Fogh, 1964; Miller, 1974; Kestler, 1985; Woods et al., 2009).

The most common forms of active ventilation are dorso-ventral and longitudinal abdominal pumping movements (Krogh, 1941; Miller, 1974). Several insect species also utilize auxiliary pumping movements when they are under respiratory stress, for example *Schistocerca gregaria* can use head and prothoracic pumping movements to supplement abdominal pumping (Miller, 1960b). Pumping movements can compress the tracheae and these tracheal compressions sometimes coordinate with spiracle opening and closing and are thought to be used either to mix gases within the tracheal system, to help transport gases from the spiracles to the tracheoles, or to facilitate gas exchange between the tracheal system and the atmosphere (Groenewald et al., 2012; Greenlee et al., 2013; Waters et al., 2013; Huang et al., 2014). When these ventilatory movements are synchronized with spiracle opening and closing, a unidirectional airflow can be produced. Heinrich et al. (Heinrich et al., 2013) found that in the hissing cockroach, *Gromphadorhina portentosa*, synchronized ventilatory and spiracular movements leads to air flowing in through the thoracic spiracles and out through the abdominal spiracles, thereby creating a unidirectional airflow.

1.8. Aims of this study

We don't know why insects exchange gases discontinuously. It's possible they use the DGC to save water or energy, and my work explicitly tests these ideas in a variety of contexts. The outcomes of this work will provide novel information which could help to solve unresolved questions regarding the evolutionary origin, and current maintenance, of the DGC in insects. Findings from this work will also have broad implications for understanding insect responses to changing energy and moisture availability at a range of spatial and temporal scales (e.g. with climate change, evolution to novel habitats, seasonal variation).

The main goal of this dissertation is to examine the variation or modulation of the discontinuous gas exchange pattern and the environmental factors that potentially drive this relationship. Examining these factors will better the understanding of the mechanisms behind

insect gas exchange. To accomplish this goal, the following hypotheses and questions will be addressed:

- Whether the DGC is accompanied by a specific intratracheal pressure pattern and if Lepidoptera serve as a reasonable general model of the DGC across other orders showing the pattern (in this case Orthoptera).
- The oxidative damage and hygric hypothesis will be tested using a full-factorial, experimental approach.
- The effect which different environmental conditions have on insect gas exchange dynamics will be tested by examining insect gas exchange under different abiotic conditions (oxygen, relative humidity).
- Whether different environmental variables (oxygen, relative humidity) have an effect on the insect's haemolymph pH status. Also, the role that gas exchange pattern modulation plays in the acid-base balance of insects will be investigated.
- The abiotic factors that the insect prioritises within the gas exchange control cascade will be examined, with the goal to generate a hierarchy of abiotic factors which influence the DGC.
- The different modes of gas exchange (diffusion and convection) that are used during the DGC will be measured.

Examining these questions and hypotheses will better the understanding of the relative importance of convection *vs* diffusion during insect gas exchange; distinguish whether Lepidoptera can serve as a general model for the DGC; tell us whether the hygric or oxidative damage hypothesis holds true for this insect species; show the effect that different environmental variables have on insect gas exchange, as well as which of the factors the insect regards as the most important priority; and produce a schematic hierarchy of factors that the insect prioritises in the gas exchange control cascade.

Chapter 2: Respiratory dynamics of discontinuous gas exchange in the tracheal system of the desert locust, *Schistocerca gregaria*

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2.1. Introduction

Insects living in terrestrial environments face significant metabolic challenges. For example, they have to regulate internal O_2 levels necessary for aerobic metabolism and excrete metabolic by-products (e.g. CO_2), while minimizing water loss (e.g. Chown, 2002; Woods and Smith, 2010; Matthews and White, 2011a). Insects possess numerous morphological and physiological adaptations for coping with these simultaneous pressures. Among the most controversial of the proposed adaptations is the use of discontinuous gas exchange cycles associated with the possession of occludible spiracles (Lighton, 1994; Chown et al., 2006). Indeed, the underlying mechanistic and evolutionary reasons for variation in patterns of insect gas exchange are receiving much renewed attention (White et al., 2007; Terblanche et al., 2008; Chown, 2011; Contreras and Bradley, 2011; Matthews and White, 2011b). These studies have all argued that a mechanistic understanding of insect respiratory dynamics and gas exchange patterns is central to understanding evolved variation in insect gas exchange patterns.

The respiratory organs of terrestrial insects consist of semi-rigid tracheal tubes which penetrate the body and tissues. These tracheae are air-filled and transport gases between the atmosphere, spiracles, and internal tissues or organs. The tracheae divide into tubes of decreasing diameter and eventually branch into microscopic tubes, or tracheoles. The tracheoles are the main sites of gas exchange with tissues (e.g. Wigglesworth, 1929) (for reviews, see Wigglesworth, 1965; Wigglesworth, 1983); they lie adjacent to cells and might even penetrate them to lie close to mitochondria in some cases (Quinlan and Gibbs, 2006). In most insects the terminal part of the tracheole is filled with fluid (Chapman, 1998; Woods et al., 2009), and the final stages of gas transport between the tracheoles and haemolymph or mitochondria takes place *via* diffusion through a liquid (Miller, 1964). Thus, the spiracles regulate the flow of gases and water vapour into and out of the tracheae (Hadley, 1994b; Chapman, 1998). By closing the spiracles, tracheal partial pressure of O_2 (PO_2) and pressure can be regulated, perhaps minimizing oxidative damage (Hetz and Bradley, 2005), while the loss of water from the internal environment of the insect to the external atmosphere can be limited (e.g. Loveridge, 1968).

In insects, efficient gas exchange is dependent on the morphological structure of the tracheal system, as well as adequate ventilation. Passive diffusion or active muscular movements (i.e. convective gas exchange), and the selective opening of the spiracles can be used to modulate whole-animal ventilation patterns (Miller, 1964; Lighton, 1996; Chown et al., 2006). In small insects and in the inactive stages of some larger species, an adequate rate of O_2 uptake can be obtained predominantly by diffusion, and there is thus little need for respiratory movements (Kestler, 1985). However, in larger or more active insects diffusion has to be supplemented with convection to maintain an adequate O_2 supply (Chown and Nicolson, 2004). These larger insects probably rely on diffusion only for O_2 transport along the finer terminations of the tracheae, while mechanically ventilating the larger tracheal trunks (Wigglesworth, 1972). Although the significance of ventilation is widely acknowledged, the temporal dynamics of gas exchange and variation in pressure associated with such ventilatory movements have only been explored in a limited range of species (mostly Lepidoptera, some Orthoptera and one species of Hymenoptera; see Table 2.1). Most work to date, drawn primarily from the classic gas exchange model of diapausing moth pupae, suggests that the closed (C)-phase of the discontinuous gas exchange cycle (DGC) is typically associated with negative intratracheal pressures, while during the open (O)-phase intratracheal pressure remains at or near atmospheric levels (Table 2.1). Moreover, only a handful of studies combine gas exchange, intratracheal pressure and body movement measurements simultaneously (Table 2.1). Given the diversity of gas exchange patterns among tracheated arthropods, and that the DGC has evolved independently on at least six occasions within the Insecta (Marais et al., 2005; Contreras and Bradley, 2010), further investigation of patterns and mechanisms underlying gas exchange is required, perhaps encompassing a wider range of species.

Here, I therefore simultaneously measured intratracheal pressure, rate of CO_2 release ($\dot{V}CO_2$) and body movements (i.e. pumping body movements) of the desert locust *Schistocerca gregaria*. This study aimed to assess the role of ventilatory movements over the entire gas exchange cycle and the relative importance of convection *vs* diffusion in this species. Intratracheal pressure was determined for the different phases of the DGC, as well as for the start of each of the phases of the DGC. Specifically, I aimed to test whether the DGC is accompanied by a specific intratracheal pressure pattern. I hypothesized that the C-phase of the DGC is accompanied by a gradual decrease in intratracheal pressure, followed by rising

pressure during the flutter (F)-phase, until the pressure reaches and stabilizes at or near atmospheric levels during the O-phase.

Table 2.1. Summary of literature measuring insect gas exchange, intratracheal pressure and body movements.

					Measurements			
Family	**Species**	**Reference**	**Method**	**Cannulated spiracle**	**Gas exchange**	**Pressure**	**Body movements**	**Findings**
Acrididae	*Dissosteira carolina*	McCutcheon, 1940	Manometer	Metathoracic	No	Yes	Yes	Mean pressure 266.6 Pa
Acrididae	*Melanoplus differentialis; Schistocerca americana*	Krolikowski and Harrison, 1996	Differential pressure transducer	Metathoracic	No	Yes	Yes	Pressure range 300-3500 Pa No subatmospheric pressures
Formicidae	*Cataglyphis bicolor*	Lighton et al., 1993	Differential pressure transducer	Mesothoracic	Yes	Yes	No	O-phase pressure ~0 Pa C-phase pressure falls to -50 Pa Pressure pulses of up to 40 Pa during C and F phases
Saturniidae	*Attacus atlas*	Hetz et al., 1993	Differential pressure transducer	2nd or 3rd abdominal spiracle	Yes	Yes	No	O-phase pressure ~0 Pa C-phase pressure -250 to -750 Pa
Saturniidae	*Attacus atlas*	Hetz and Bradley, 2005	Differential pressure transducer	?	Yes	Yes	Yes	O-phase pressure ~0 Pa C-phase pressure below atmospheric

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Saturniidae	*Attacus atlas*	Wobschall and Hetz, 2004	Differential pressure transducer	2nd or 3rd thoracic spiracle	Yes	Yes	Yes	O-phase pressure ~0 Pa C-phase pressure falls
Saturniidae	*Hyalophora cecropia*	Levy and Schneiderman, 1966a	Manometer	3rd abdominal spiracle	Yes	Yes	No	O-phase pressure within 0.7 Pa of atmospheric C-phase pressure -466.6 Pa **
Saturniidae	*Hyalophora cecropia*	Schneiderman and Schechter, 1966	Electronic pressure transducer	?	Yes	Yes	Yes	Min. pressure -546.6 Pa C-phase pressure falls O-phase pressure ~0 Pa *
Saturniidae	*Hyalophora cecropia*	Brockway and Schneiderman, 1967	Strain-gauge transducer	3rd or 4th abdominal spiracle	No	Yes	Yes	O-phase pressure ~0 Pa C-phase pressure min. -667 Pa During about 95% of the DGC pressure is below atmospheric *
Saturniidae	*Hyalophora cecropia*	Burkett and Schneiderman, 1974	Strain-gauge transducer	?	No	Yes	No	C-phase mean pressure -313.3 Pa *
Saturniidae	*Samia cynthia*	Terblanche et al., 2008	Differential pressure transducer	2nd or 3rd abdominal spiracle	Yes	Yes	No	O-phase pressure ~0 Pa C-phase pressure falls Some positive peaks observed during C- and O-phases (max. peaks ±50 Pa & ±100 Pa respectively)

continued on next page…

Saturniidae, Sphingidae	*Actias selene; Sphinx Ligustri; Hyalophora cecropia; Manduca sexta*	Sláma, 1988	Anemometric transducer	?	Yes	Yes	No	*Hyalophora cecropia*: C-phase pressure below 0 Pa
Scarabaeidae	*Circellium bacchus*	Duncan et al., 2010	Differential pressure transducer	2nd abdominal spiracle	Yes	Yes	Yes	Positive and subatmospheric pressure pulses

C-phase, closed phase; O-phase, open phase; DGC, discontinuous gas exchange cycle; max., maximum; min., minimum.
*Because of the large dead air space (tracheal system, cannula and transducer), the recorded pressure changes were smaller than the actual pressure changes that occur in the tracheal system.
**The first direct and repeated measurements of the barometric pressure within an insect's tracheal system.

2.2. Materials and methods

2.2.1. Animals

Adult *S. gregaria* (Forskål 1775) (Orthoptera: Acrididae) were supplied by Grillenzucht Hildner (Gerhardshofen, Germany). Individuals were kept separated from each other in plastic containers. Animals were fed fresh lettuce every second day and were kept at ambient temperature (18-22°C), relative humidity and air conditions. Both male and female adult grasshoppers were used. Animals were fasted for at least 8 h before respirometry commenced.

2.2.2. Respirometry

Twelve individuals were recorded for 8 h each at 15°C. Rate of CO_2 release ($\dot{V}CO_2$), body movements (i.e. activity) and intratracheal pressure were recorded with 8 samples s^{-1}. Carbon dioxide release was measured in a custom-made flow through respirometer, with a length of 60 mm and a volume of ~10 ml. The temperature of the cuvette was controlled by a custom-made Peltier-cooling unit with a computer-controlled feedback (accuracy ±0.1°C). Ambient air (from outside the lab) was scrubbed of CO_2 and water vapour by passing it through two columns containing a 5 mol l^{-1} NaOH solution. The scrubbed air was then driven through two 200 ml columns filled with distilled water, which were kept at a constant temperature of 8°C, to keep the water vapor pressure constant. The flow rate of the air was regulated at 250 ml min^{-1} by a mass flow controller (MKS 1179, MKS Instruments, Wilmington, MA, USA). The air was then fed through the reference cell of a pressure compensated differential infrared gas analyser (URAS 14, range 200 p.p.m., ABB Analytical, Frankfurt, Germany), whereafter it flowed through the respirometry cuvette, and then through the analysing cell of the gas analyser, to measure the CO_2 output of the locust. During a trial, the body movements of each individual were recorded with three different infrared sensors, located dorsally above the animal. These consisted of reflective interrupters (Osram SFH 9201, Osram, Germany) that sent infrared light (wavelength 850-950 nm) to the animal and measured the reflected changes in radiation due to body movements. Data were recorded to a computer hard disk using a customized recording program in TurboLab 4.03 (Bressner Technology, Gröbenzell, Germany).

2.2.3. Intratracheal pressure

Each individual was weighed before and after spiracle intubation, as well as after each trial. Body movements, $\dot{V}CO_2$ and intratracheal pressure were recorded following methods outlined elsewhere (Wobschall and Hetz, 2004; Moerbitz and Hetz, 2010). To measure intratracheal pressure, the animal's mesothoracic spiracle was cannulated. Before intubation of the spiracle commenced, animals were restrained by binding the mesothoracic and metathoracic legs to the abdomen with adhesive tape (Figure 2.1). The restraints did not confine the abdomen or physically impede abdominal ventilatory movements. These measures were taken to ensure that the tubing could not be removed from the cannulated spiracle by the animal's leg movements.

Figure 2.1. *Schistocerca gregaria* individual with a steel cannula inserted into a mesothoracic spiracle; the area around the spiracle was sealed with dental wax.
Mesothoracic and metathoracic legs were restrained with adhesive tape. The short piece of polyethylene tubing, with the thin steel cannula (0.25 mm i.d.) at the end, was placed underneath the adhesive tape, as well as being secured with wax to the tape, in order to stabilize the cannulated tubing.

For the cannulation, a short piece of polyethylene tubing (PE10) was modified to include a thin steel tube (0.25 mm i.d.) at the end, which was inserted into the animal's spiracle. The surrounding of the spiracle was then sealed with dental wax. The tubing was connected to a precision micro-pressure transducer (SenSym SDXL010, SensorTechnics, Pucheim, Germany). The other port of the transducer opened into the respirometry chamber to measure

pressure differences between the chamber and tracheal system. The pressure sensor electrically consisted of a Wheatstone bridge that was driven with a precision voltage reference of 6.000 V (REF02, Burr Brown, Texas Instruments, Dallas, TX, USA) buffered with a precision operational amplifier (OPA177, Burr Brown). Differential voltage output of the pressure sensor bridge was amplified with a differential amplifier (INA131, Burr Brown).

After intubation, animals were allowed 20-30 min to recover from the handling stress of the intubation procedure, before recording of their CO_2 release commenced. After this period animals no longer struggled and appeared to ventilate normally.

2.2.4. Data extraction and analysis

The data for each individual were imported into ExpeData Version 1.1.25 (Sable Systems, Las Vegas, NV, USA) data acquisition and analysis software. The data from the first hour after an individual was placed into the respirometer were discarded. For each individual the first three consecutive discontinuous gas exchange cycles (DGCs) were selected for data analysis. An individual's breathing pattern was defined as a DGC when, according to the CO_2 trace, a burst (O-phase) and interburst [closed/flutter (CF)-phase] period were clearly discernible. The C-phase and F-phase were combined to form the interburst period because the F-phase could not be separated from the C-phase by using the rate of CO_2 release. In addition, there was no obvious pattern in the intratracheal pressure trace that could be used to distinguish these two phases. The beginning of the interburst period was used as the onset of a DGC. To examine the relationships between the different variables (phase duration, pressure, rate of CO_2 release, volume of CO_2 released) and to assess whether any pattern of intratracheal pressure exists that accompanies the DGC, correlation analysis was performed using STATISTICA v.10 (StatSoft, Tulsa, OK, USA). Data collected from the three different infrared sensors were converted to *z*-scores (standard scores) before being used in correlation analysis, as the baselines of the channels from each individual differed. The data were converted using the following formula:

$$z = \frac{x - u}{s.d.}, \qquad (1)$$

where z is the standardised body movement score, x is the observed body movement score (raw signal from the activity detector), u is the mean body movements, and s.d. is the standard deviation of the body movement channel.

2.3. Results

All 12 individuals exhibited DGCs when recorded at 15°C (mean±s.d. body mass 1.761±0.325 g). There was no significant relationship between body mass and any DGC phase duration, $\dot{V}CO_2$ during the O-phase and interburst period, or volume of CO_2 released during the O-phase ($p>0.39$ in all cases). The mean duration of the interburst period increased with the mean DGC duration ($r=0.922$, $n=12$, $p<0.0001$). Mean interburst $\dot{V}CO_2$ was positively correlated with mean interburst duration ($r=0.608$, $n=12$, $p=0.0358$). Mean O-phase volume was positively related to mean interburst duration ($r=0.734$, $n=12$, $p=0.0066$). Moreover, the interburst period of a DGC commenced at any intratracheal pressure and was not restricted to sub-atmospheric pressures (Figure 2.2, Table 2.2).

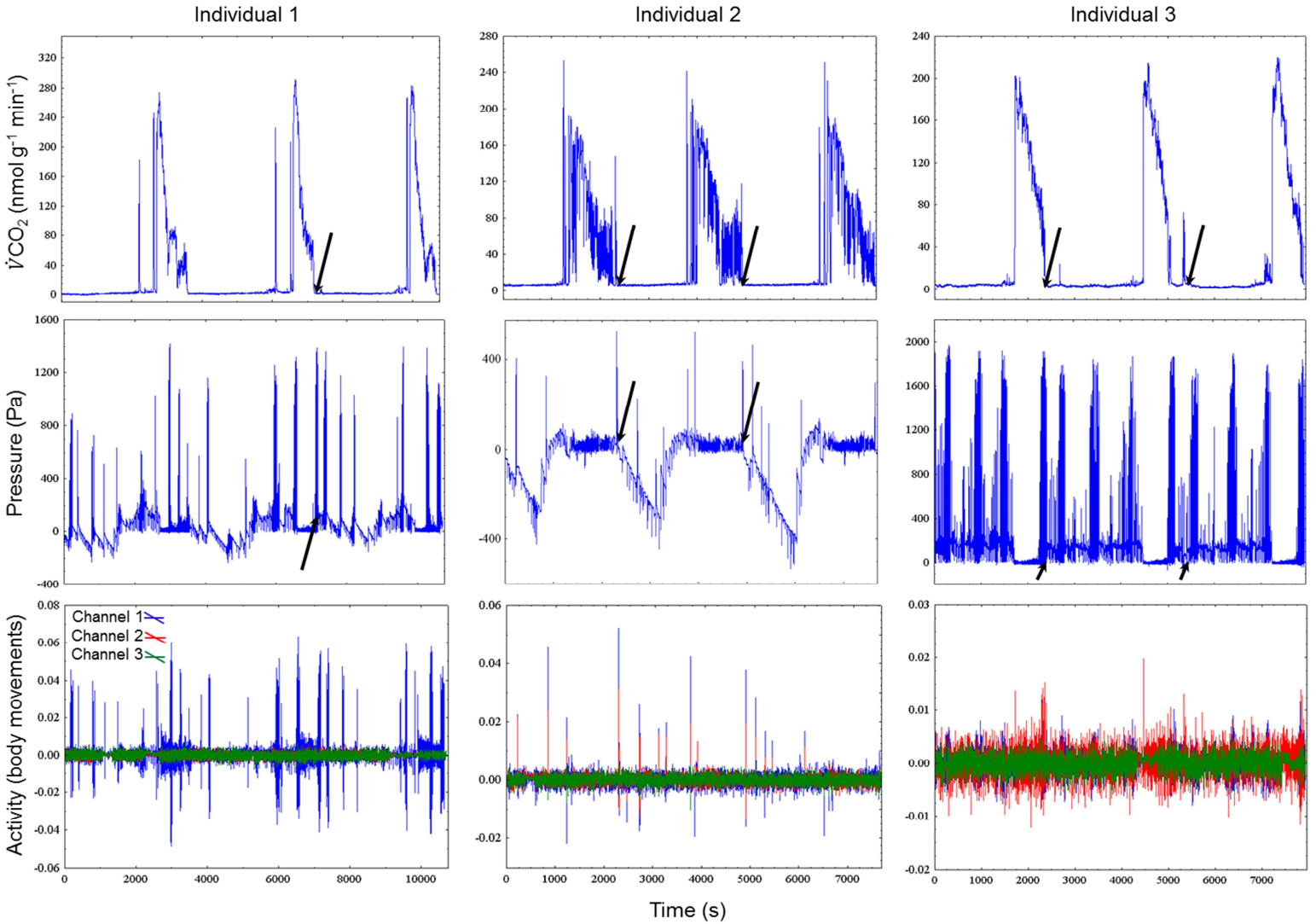

Figure 2.2. Recordings of CO_2 release rate ($\dot{V}CO_2$, nmol g^{-1} min^{-1}), intratracheal pressure (Pa) and body movements for three different *Schistocerca gregaria* individuals (mass 2.2869 g, 1.4529 g and 2.2971 g, respectively).

All individuals were recorded at 15°C, 250 ml min^{-1} flow rate, and sampling frequency of 1 s for $\dot{V}CO_2$ and 0.125 s for body movements and pressure. Note that axes scaling varies for the different parameters between individuals for clarity. Arrows indicate the start of the interburst period where the intratracheal pressure is not sub-atmospheric.

Table 2.2. Intratracheal pressure data at the start of interburst periods and Open phases for three consecutive discontinuous gas exchange cycles per individual, for each of the 12 recorded *Schistocerca gregaria* individuals.

Individual	Cycle	Pressure at start of interburst period (Pa)	Pressure at start of O-phase (Pa)
1	1	49.9	18.7
	2	18.9	-4.4
	3	70.9	41.3
2	1	404.2	170.6
	2	124.6	213.2
	3	73.5	160.5
3	1	-1.3	-2.6
	2	-4.9	0.9
	3	3.1	-2.5
4	1	159.3	170.6
	2	34.8	209.3
	3	344.5	404.9
5	1	-11.9	11.2
	2	11.4	74.4
	3	4.8	29.7
6	1	-26.3	56.3
	2	-16.7	72.0
	3	-32.3	85.7
7	1	-19.9	18.5
	2	44.3	32.1
	3	51.5	40.8
8	1	149.5	105.5
	2	118.7	196.1
	3	161.3	174.9
9	1	48.7	80.4
	2	113.2	234.1
	3	93.5	121.2
10	1	178.2	115.1
	2	98.1	90.4
	3	172.8	179.6
11	1	371.9	38.7
	2	40.0	68.9
	3	179.7	182.3
12	1	-40.2	256.8
	2	38.3	246.5
	3	124.9	97.1

O-phase, open phase.

Mean O-phase $\dot{V}CO_2$ was negatively, but not significantly, related to mean O-phase duration (r=-0.524, n=12, p=0.0806). Mean O-phase volume was positively correlated with mean O-phase duration (r=0.726, n=12, p=0.0075). To examine whether any relationship exists between the duration of the different phases of a DGC and the intratracheal pressure pattern of an individual, correlations between the variables were assessed. No significant relationship existed between mean O-phase duration and mean pressure during an O-phase within those same DGCs (r=-0.509, n=12, p=0.0914), or between mean interburst duration and mean pressure during that same interburst period (r=-0.098, n=12, p=0.7615).

There were no characteristic patterns for intratracheal pressure changes that were common among all individuals, but rather intratracheal pressure traces showed high among-individual variability. In *S. gregaria*, the O-phase could include periods of negative intratracheal pressure, while the interburst period sometimes showed high positive pressure peaks (Table 2.3).

The mean intratracheal pressure of individual *S. gregaria* during the interburst period ranged from -112.1 to 224.6 Pa, with four individuals having a negative mean intratracheal pressure during the interburst period and eight individuals having a positive mean intratracheal pressure. Mean $\dot{V}CO_2$ during the interburst period ranged from 1.49 to 14.67 nmol g^{-1} min^{-1}. In eight individuals, $\dot{V}CO_2$ during the interburst period was statistically greater than 0, regardless of whether the mean intratracheal pressure was negative or positive. Four of the 12 individuals recorded had a negative mean intratracheal pressure during the interburst period, and in three out of these four individuals $\dot{V}CO_2$ during the interburst period was statistically greater than the infrared gas analyser baseline (see e.g. Figure 2.2, individual 2).

Examples of DGCs, together with intratracheal pressure and body movements, are presented in Figure 2.2. These traces clearly show that *S. gregaria* employs two of the four different ventilatory movements described by Miller for resting locusts (Miller, 1960b). Dorso-ventral muscles are contracted at a high rate, while longitudinal muscles are used rarely, but coincide with higher positive pressure pulses (Figure 2.2, individual 1 and individual 2). Also, strong pumping actions do not necessarily lead to CO_2 release, and large outputs of CO_2 are not always associated with abdominal pulsation body movements (Figure 2.2, individual 3).

Table 2.3. Summary statistics for different components of the discontinuous gas exchange cycle in *Schistocerca gregaria* for all individuals.

	Mean±s.d.
Mass (g)	1.761±0.325
DGC duration (s)	3189±953
Interburst duration (s)	1968±772
O-phase duration (s)	1233±382
DGC $\dot{V}CO_2$ (nmol g^{-1} min^{-1})	36.10±4.52
Interburst $\dot{V}CO_2$ (nmol g^{-1} min^{-1})	6.25±4.16
O-phase $\dot{V}CO_2$ (nmol g^{-1} min^{-1})	84.83±19.18
O-phase area (nmol g^{-1} CO_2)	27.82±7.35
Mean DGC pressure (Pa)	56.8±63.3
Mean DGC pressure min. (Pa)	-166.7±132.5
Mean DGC pressure max. (Pa)	1166.5±399.2
Mean interburst pressure (Pa)	46.2±82.8
Mean interburst pressure min. (Pa)	-159.9±137.2
Mean interburst pressure max. (Pa)	1068.5±443.7
Mean O-phase pressure (Pa)	71.1±43.0
Mean O-phase pressure min. (Pa)	-66.3±65.1
Mean O-phase pressure max. (Pa)	1128.4±404.9

O-phase, open phase; DGC, discontinuous gas exchange cycle; max., maximum; min., minimum; $\dot{V}CO_2$, rate of CO_2 release. n=12 in all cases.

2.4. Discussion

Several authors have demonstrated that a comprehensive understanding of gas exchange dynamics in insects should include measurements of intratracheal pressure, as these measurements can provide novel information about both the kinetics of gas exchange and insect respiration in general (e.g. Buck, 1958; Levy and Schneiderman, 1966a, Terblanche et al., 2008). This was certainly the case here, where I found for the first time that for *S. gregaria* the O-phase can include periods of negative intratracheal pressure, as well as pressures well above atmospheric levels (Table 2.3), the interburst period can start at any pressure amplitude (Table 2.2) and can include periods of high intratracheal pressures (Table 2.3; Figure 2.2). Rather than the patterns found here being unusual forms of the DGC, several aspects of the DGC recorded conformed well with established patterns for Orthoptera and

other arthropod taxa. For example, the positive relationship observed between O-phase duration and DGC duration is similar to that reported previously for a variety of dung beetles (Davis et al., 1999) and for *Garypus californicus* (Lighton and Joos, 2002) (for review, see Chown and Nicolson, 2004).

The pumping movements of an insect's abdomen lead to volume changes of the tracheal system, which in turn lead to pressure fluctuations within the internal body (detected as intratracheal or haemolymph pressure changes) of the insect (Buck, 1962). In locusts, the pumping movement of the abdomen is generally thought to ventilate the tracheae and larger tracheal trunks (Wigglesworth, 1972), and both inspiration and expiration are active processes (Miller, 1964). In an actively ventilating orthopteran, the first four pairs of spiracles open during the inspiratory phase of abdominal ventilation, while the remaining pairs open during the expiratory phase (Weis-Fogh, 1967; Harrison, 1997), leading to an anterior to posterior (unidirectional) flow of air through the insect (Henderson et al., 1998). However, the path of this unidirectional flow of air is subject to variation, and occasionally any of the spiracles might serve for both inspiration and expiration (Steen, 1971). If there is a unidirectional flow of air through the insect, it could be possible for the insect to have a negative pressure in one part of its tracheal system, while a positive pressure is present in another part, depending on coordinated spiracle and ventilation movements. This could explain the periods of negative intratracheal pressure during the O-phase (Table 2.3).

The pressure patterns I recorded in *S. gregaria* are similar to those reported for other grasshoppers [e.g. *Melanoplus differentialis* (Krolikowski and Harrison, 1996)] in which tracheal pressures varied substantially among individuals, although this is unlike the situation for lepidopteran pupae (e.g. Levy and Schneiderman, 1966a; Wobschall and Hetz, 2004; Terblanche et al., 2008). Moreover, the negative intratracheal pressure during the O-phase is unexpected as the spiracles typically remain open, indicating that the intratracheal pressure should be close to atmospheric levels. This latter result also differs from findings in lepidopteran pupae in which intratracheal pressure during the O-phase was maintained at levels similar to atmospheric pressure, and intratracheal pressure never rose much above atmospheric levels (e.g. Wobschall and Hetz, 2004; Terblanche et al., 2008). By contrast, in *M. differentialis* grasshoppers, intratracheal pressure measured in the thoracic trachea never reached atmospheric levels when these individuals were at rest (Krolikowski and Harrison,

1996). In lepidopteran pupae, intratracheal pressure generally falls during the C-phase, rises during the F-phase and remains constant near atmospheric levels during the O-phase (Schneiderman and Schechter, 1966; Hetz et al., 1993). However, for *S. gregaria*, the C-phase can start at any intratracheal pressure (above or below atmospheric pressure) (Table 2.2) and can include periods of high intratracheal pressure (Table 2.3; Figure 2.2). Therefore, in *S. gregaria* the respiratory cycle is not accompanied by a clearly distinguishable sub-atmospheric intratracheal pressure cycle, as is the case with lepidopteran pupae.

The body movements accompanying gas exchange at rest are similar to those reported previously for this species. Indeed, it was established more than 50 years ago through visual observation and mechanical recordings that dorso-ventral contractions and longitudinal telescoping movements of the abdomen occur in grasshoppers at rest (e.g. McCutcheon, 1940; Weis-Fogh, 1967; Lewis et al., 1973). More recent work that included gas exchange recordings showed that gas exchange may also take place when no abdominal pumping occurs, suggesting that gas exchange is primarily diffusive (although some micro-ventilation pumping actions can be employed, are barely visible to the naked eye and may aid convection) (e.g. Harrison, 1997; Greenlee and Harrison, 1998). However, my study is unique because I recorded body movement patterns, gas exchange and intratracheal pressure simultaneously, which has never previously been done for any Orthoptera (Table 2.1).

Hamilton (Hamilton, 1964) found that in adult *Schistocerca* bursts of CO_2 coincided with ventilation, and postulated this as being a means to conserve respiratory water. Kestler (Kestler, 1978) also explained these coordinated ventilatory movements (involving both diffusive and convective exchanges) as being a means to reduce respiratory water loss. By compressing the abdomen and closing the spiracles, pressure inside the abdomen can be increased, thereby also compressing the tracheal system and gases within the tracheae. But what could the advantage of such high pressures achieved during the interburst period be? One potential explanation is that this might slow down evaporation of water. The pressure cycle theory suggests that changes in intratracheal pressure could help with the regulation of the influx and efflux of water vapour, by making it possible for the animal to regulate the hydrostatic pressure within the tracheae (Corbet, 1988). Within the tracheal system, respiratory gases occur at a vapour-liquid interface between the air-filled tracheae and insect

body fluids. Water can move from the body fluids to the trachea and *vice versa*, and by regulating intratracheal pressure (and thereby also hydrostatic pressure) it should thus be possible to reduce the rate or reverse the direction of water movement. Water evaporates into subsaturated air, but if the vapour pressure within the air exceeds the supersaturation ratio, water will condense. An increase in intratracheal pressure will lead to increased hydrostatic pressure within the tracheal system, and an increase above the supersaturation ratio will cause condensation. The tracheae are lined with cuticle that is impermeable to water, but the tracheoles are liquid filled and have permeable walls, making it possible for water and respiratory gases to move through these walls (Krogh, 1941; Wigglesworth, 1965; Kerkut and Gilbert, 1985, Mill, 1985). However, several studies have suggested that the cuticle lining is not impermeable and that liquids can pass through the tracheal walls (Keister and Buck, 1949; Wigglesworth, 1953; Woods et al., 2009). Therefore, increasing the hydrostatic pressure in the tracheal system above the supersaturation ratio will lead to the condensation of water and the movement of these water droplets from the tracheae into the tracheoles (as suggested by Beament, 1964), and possibly also through the tracheal walls into the haemolymph. The remarkably high intratracheal pressure pulses observed during the interburst period in *S. gregaria* could be high enough to meet, or perhaps even exceed, the supersaturation ratio for water vapour, thus reversing the direction of water movement. According to Henry's law, at a given temperature the partial pressures of gases, like water vapour, within a gas mixture are elevated when the total pressure is elevated. In contrast, evaporation from a surface (like the tracheoles) occurs faster if the pressure is lower. The osmolality of the fluid within the tracheoles should set the equilibrium water partial pressure within the tracheal system.

If the osmolality of the tracheole fluid is assumed to be 300 mosmol l^{-1} - which is equivalent to a water chemical activity (a_w) of 0.995, or 99.5% relative humidity, and approximates values for insect haemolymph (see Edney, 1977) - at this high relative humidity tracheal pressure can be directly interpreted as water vapour pressure, assuming the system is in equilibrium. Therefore, the highest pressure pulses within an individual of around 1971 Pa are equivalent to water vapour pressure of 14.78 mmHg. At 15°C the saturation partial pressure of distilled water is 12.76 mmHg. This partial pressure exceeds the saturation pressure of water vapour, and air in the tracheal system is therefore likely to be oversaturated during the interburst phase of the DGC. Another possibility is that these high pressure pulses

could be used to ensure effective mixing of gases in the closed tracheal system of the insect, which could perhaps allow the insect to increase the duration of the interburst period as a result of more efficient distribution of O_2. The insect could also use these high pressure pulses to drive O_2 into portions of the tracheal system that need it, which could include for example the head, which has a high metabolic rate due to the combination of neurons and eyes. Another possibility is that these high pressure pulsations are the unintentional result of muscular contractions of the body walls to ensure adequate mixing of the haemolymph.

Terrestrial insects display at least three different patterns of gas exchange when at rest (for reviews, see Chown et al., 2006; Quinlan and Gibbs, 2006; Bradley, 2007). A DGC consists of three phases, which are typically described in terms of spiracular behaviour; namely a C, F and O spiracle phase (Schneiderman, 1960). In some of the recorded individuals, although a true C-phase of the DGC could be identified by means of subatmospheric intratracheal pressure recordings, some CO_2 continued to be released (e.g. Figure 2.2, individual 2). This observed CO_2 release during the C-phase could be trans-cuticular CO_2 release, as has been postulated by Shelton and Appel (Shelton and Appel, 2000; Shelton and Appel, 2001) for the termites *Reticulitermes flavipes* and *Zootermopsis nevadensis*, whose CO_2 release never drops below baseline levels. This particularly novel finding has important implications for the identification of patterns solely on the basis of $\dot{V}CO_2$ traces, although to date discussion has mostly focussed on the use of appropriate flow rates or temperatures for pattern identification (e.g. Gray and Bradley, 2006; Terblanche and Chown, 2010; Contreras and Bradley, 2011).

In conclusion, this study highlights the importance of using multiple parameters when examining the mechanisms of gas exchange and suggests that much is still to be learned about the fundamentals of insect respiration.

Chapter 3: A diffusion-convection switch during hypoxia in insect discontinuous gas exchange

This chapter is in preparation as “A diffusion-convection switch during hypoxia in insect gas exchange” by Berlizé Groenewald, Stefan K. Hetz, Steven L. Chown and John S. Terblanche.

3.1. Introduction

Terrestrial insects have a complex respiratory system that consists of a network of air-filled tubes (tracheae) that branch throughout the insect's body. The tracheae divide and subdivide into tubes of decreasing diameter and eventually divide into microscopic tubes (tracheoles). The internal environment of the insect is connected to the external atmosphere by the spiracles, which are valve-like structures embedded into the insect's cuticle. The spiracles regulate the flow of respiratory gases (primarily O_2, CO_2 and H_2O) into and out of the tracheal system. Although it has been suggested that insects can maintain adequate gas exchange through diffusion alone (Krogh, 1920; Weis-Fogh, 1964), several studies have shown that diffusion is aided by convection when at rest, or that the main mode of gas transport in many insect species is convection (Buck, 1962; Sláma, 1988; Wobschall and Hetz, 2004; Sláma et al., 2007; Wasserthal, 2014; also see review by Harrison et al., 2013). However, the relative importance of convection and diffusion remains controversial in insect gas exchange (Socha et al., 2008).

Insects display at least three different gas exchange patterns when they are at rest, and of these patterns the discontinuous gas exchange cycle (DGC) has enjoyed most attention to date in respiratory and comparative physiology (e.g. Schneiderman, 1956; White et al., 2007). It is thought that discontinuous gas exchange cycles (DGCs) have evolved on at least six independent occasions (Marais et al., 2005; Contreras and Bradley, 2010), and is largely regarded as the textbook mode of gas exchange in insects and tracheate arthropods (Chapman, 1998). The DGC is typically described on the basis of spiracle behaviour and includes a closed (C)-phase, flutter (F)-phase and open (O)-phase. During the C-phase no external gas exchange occurs, intratracheal CO_2 builds up and O_2 is consumed while cellular respiration proceeds uninterrupted. The F-phase consists of small, high frequency bursts of CO_2, while intratracheal partial pressure of O_2 (PO_2) remains relatively constant (Förster and Hetz, 2010). The F-phase is followed by the O-phase, during which spiracles are held widely open and respiratory gases are exchanged freely with the external atmosphere by means of either diffusion, convection or both (e.g. Wobschall and Hetz, 2004; Duncan et al., 2010). There is however still much debate about the modes of gas exchange (diffusion *vs* convection) that dominate during these different phases of the DGC (e.g. Lighton, 1988; Chown and Holter, 2000; Wobschall and Hetz, 2004).

Several different forms of convection are present in insects and include: 1) active convection, which includes active body movements (muscular contractions) which compress the tracheae and/or air sacs (Sláma, 1988); 2) passive convection, during which gas exchange is made possible through pressure differences between the internal environment of the insect and the external atmosphere (Miller, 1974) - this includes passive suction ventilation, i.e. a sub-atmospheric pressure is created in the tracheae (due to the consumption of O_2 and CO_2 dissolving into the haemolymph) and air is then sucked in through the spiracles due to the pressure difference between the tracheal system and the external atmosphere (Miller, 1974; Kestler, 1985; Wobschall and Hetz, 2004); and 3) cardiogenic ventilatory mechanisms (i.e. haemolymph circulation), whereby the net flow of haemolymph to a certain part of the body will result in a decrease in air sac volume in that part of the body. By reversing the flow of haemolymph, air sac volume in different body parts (posterior *vs* anterior) can be regulated, thereby creating respiratory convection (Kestler, 1985; Corbet, 1988; Socha et al., 2010; Wasserthal, 2014).

Tracheae and air sacs are semi-rigid structures and can therefore be deformed by applying pressure, or when pressure is released allowing elastic recoil to partly aid in convective gas exchange (Förster and Hetz, 2008). Locusts, for example, use dorso-ventral contractions of the abdomen to drive air through the tracheal system, with inspiration occurring through the first four pairs of anterior spiracles, and expiration through the last six pairs (McCutcheon, 1940; Buck, 1962; Henderson et al., 1998). However, several variations of active ventilation exist and the desert locust, *Schistocerca gregaria*, has been found to use at least four different types: 1) dorso-ventral abdominal movements; 2) abdominal longitudinal telescoping movements; 3) neck ventilation; and 4) prothoracic ventilation (Miller, 1960b). Groenewald et al. (Groenewald et al., 2012) found that both dorso-ventral and longitudinal movements are employed in the desert locust, but that dorso-ventral movements are used more regularly, with longitudinal movements only rarely being used. Tracheal compressions sometimes coordinate with spiracle opening and closing and are thought to be used to mix gases within the tracheal system, to help transport gases from the spiracles to the tracheoles, or to facilitate gas exchange with the atmosphere (Groenewald et al., 2012; Waters et al., 2013; Huang et al., 2014).

Active convection is a less efficient (in terms of energy) mode of gas transfer than diffusion, as there are significant energetic costs associated with active pumping movements (muscle contractions). Therefore, diffusion-dominated gas exchange could play an important role in gas exchange and oxygen uptake during periods when the insect is under least demand and tries to decrease its energy expenditure. However, it remains a significant challenge to distinguish among the relative roles of diffusion and convection in insect gas exchange (Harrison et al., 2013). The aims of this study were to investigate the relative roles of convection and diffusion during the DGC at differing ambient oxygen conditions, and also to investigate the energetic costs associated with diffusive and convective based gas exchange. To accomplish these aims the rate of CO_2 release ($\dot{V}CO_2$), intratracheal pressure and individual body movements in three axes (*x*, *y*, *z*) (Figure 3.1) were measured simultaneously using the desert locust, *S. gregaria*. These variables were recorded under a range of oxygen partial pressures, both above and below present-day atmospheric oxygen levels (5, 8, 13, 21 and 35 kPa O_2). Previous experiments on the desert locust indicated that gas exchange in this species is likely not a purely diffusive process, as respiratory movements are present during gas exchange (Fraenkel, 1932; Krogh, 1941; Hamilton, 1959; Miller, 1960b). I hypothesized that at ambient oxygen and slightly hypoxic conditions, the mode of gas exchange will be mainly diffusive, while as oxygen partial pressures decreases to more hypoxic conditions, the mode will change to mainly convective gas exchange.

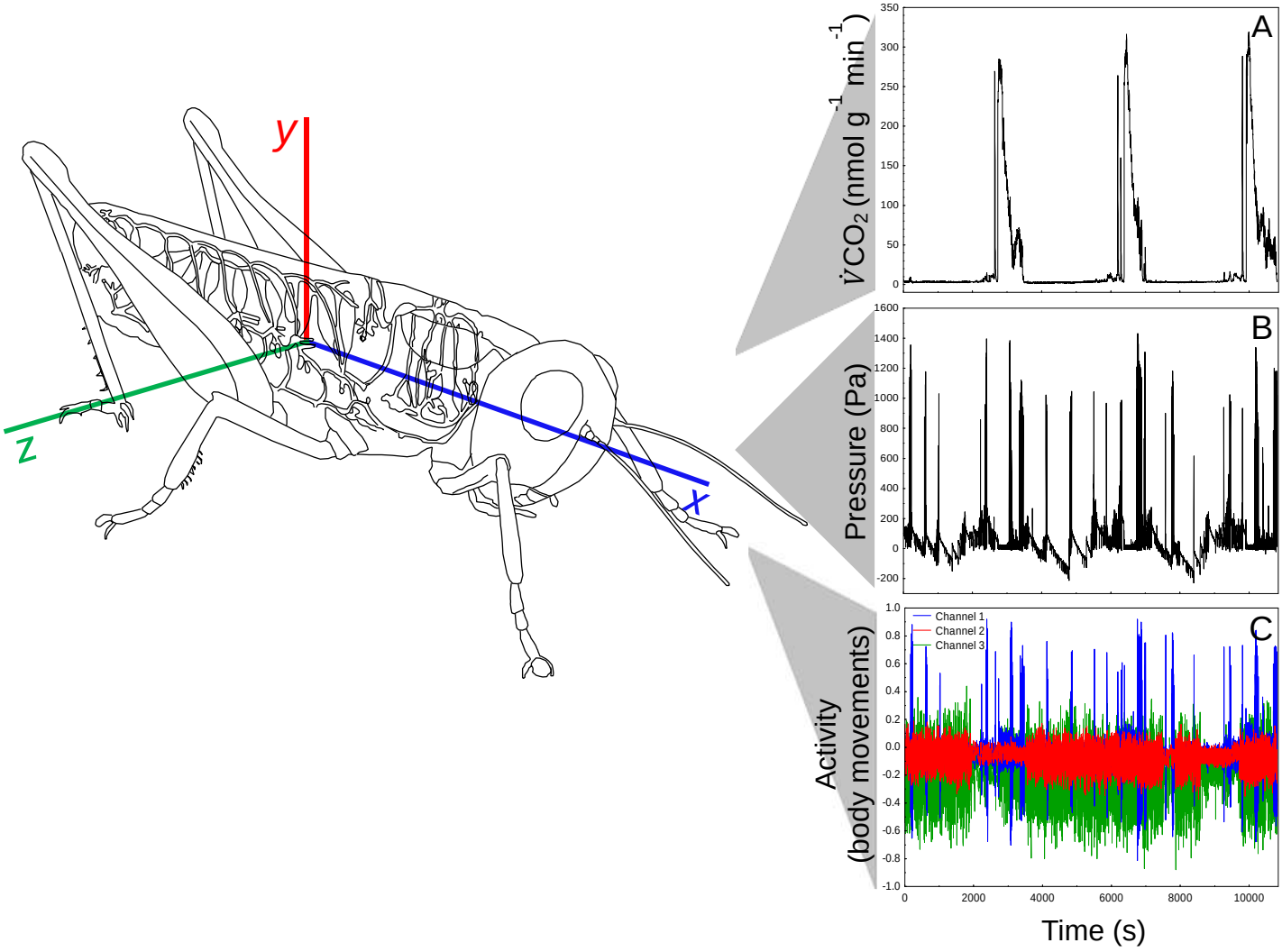

Figure 3.1. Schematic illustration indicating the three different axes in which body movements were measured.
Graphs for the different variables that were measured simultaneously are also indicated: (A) rate of CO_2 release ($\dot{V}CO_2$); (B) intratracheal pressure; and (C) body movements in three directions. The recording was done at 15°C, a flow rate of 250 ml min^{-1} and a sampling frequency of 1 s.

3.2. Materials and methods

3.2.1. Animals

Adult *Schistocerca gregaria* (Forskål 1775) (Orthoptera: Acrididae) were supplied by Grillenzucht Hildner (Gerhardshofen, Germany). Individuals were kept in separate plastic containers and fed fresh lettuce every second day. Containers were kept in the lab at ambient

room temperature, relative humidity and light: dark conditions. Both male and female adult grasshoppers were used and grasshoppers were fasted for at least 8 h prior to a trial.

3.2.2. Respirometry and treatment conditions

To investigate gas exchange parameters for the different modes of gas exchange (diffusion *vs* active convection) at different oxygen partial pressures, rate of CO_2 release ($\dot{V}CO_2$), body movements (i.e. activity) and intratracheal pressure were recorded simultaneously for a total of 13 individuals (mean±s.d. body mass 1.722±0.282 g) at a rate of 1 sample s^{-1}. Each individual was measured for a total of 36 hours at 15°C. These 36 hours consisted of three 12 hour periods of differing oxygen partial pressures, of which one period was always normoxia. The other oxygen partial pressures that were used were 5, 8, 13 and 35 kPa O_2. The sequence of oxygen partial pressures were randomised between trials.

Ambient air (from outside the lab) was passed through two columns containing a 5 mol l^{-1} NaOH solution and Drierite to remove CO_2 and water from the airstream. The scrubbed air was then driven through two 200 ml columns filled with distilled water, which were kept at a constant temperature of 8°C, to keep the water vapour partial pressure constant at 1.06 kPa (Magnus, 1844). The flow rate of the air was regulated at 250 ml min^{-1} by a mass flow controller (MKS 1179, MKS Instruments, Wilmington, MA, USA). An aquarium pump (WISA 300, Gardner Denver Thomas GmbH, Puchheim, Germany) was used to mix normoxic air with gas flowing from pressurised gas cylinders (Air Liquide Deutschland GmbH, Berlin) and controlled at a constant flow rate of 250 ml min^{-1} by a flow control valve (the 'master valve' (MKS 1179, MKS Instruments, Wilmington, MA, USA)), connected to a custom made mass flow controlling unit. Gas cylinders, containing either pure nitrogen or pure oxygen, were used to obtain the desired oxygen partial pressures. The flow rate of gas from the cylinder depended on the desired oxygen partial pressure and was controlled at the desired rate (see equation 2 in Terblanche et al., 2008) by a flow control valve (the 'slave valve' (MKS 1179, MKS Instruments, Wilmington, MA, USA)), connected to a custom made mass flow controlling unit. To ensure that the oxygen partial pressure was at the desired level, an oxygen analyser (S-3AIII, AMETEK, Berwyn, Pennsylvania, USA) was used to confirm the oxygen partial pressure in the airstream. After the gases have been mixed, the airstream was fed through the reference cell of a pressure compensated differential infrared

gas analyser (URAS 14, range 200 p.p.m., ABB Analytical, Frankfurt, Germany). Thereafter the airstream flowed through a custom-made respirometry cuvette (length 60 mm; volume ~10 ml) and then through the analysing cell of the gas analyser. A custom-made Peltier-cooling unit with a computer-controlled feedback regulated the temperature of the cuvette (accuracy±0.1°C, resolution 0.02°C, measured by a custom made thermometer with the sensor SEM833ET (Hygrosens Instruments, Löffingen, Germany)). During a trial the body movements of each individual were measured with three different infrared sensors, located laterally, at the tip of the abdomen, and dorsally above the animal's abdomen. These consisted of reflective interrupters (Osram SFH 9201, Osram, Germany) that sent infrared light (wavelength 850–950 nm) to the animal and measured the reflected changes in radiation due to body movements. Data were recorded to a computer hard disk using a customized recording program in TurboLab 4.03 (Bressner Technology, Gröbenzell, Germany).

3.2.3. Intratracheal pressure

For the measurement of intratracheal pressure, an animal's mesothoracic spiracle was cannulated. To immobilize animals prior to the cannulation, their mesothoracic and metathoracic legs were bound to their abdomen with adhesive tape. The restraints did not confine the abdomen or physically impede abdominal ventilatory movements. These measures were taken to ensure that the tubing could not be removed from the cannulated spiracle by the animal's leg movements.

For the cannulation of the spiracle, a short piece of polyethylene tubing (PE10) was modified to include a thin steel tube (0.25 mm i.d.) at the end. This steel tube was then inserted into the animal's spiracle and the surrounding of the spiracle was sealed with dental wax. The tubing was then connected to a precision micro-pressure transducer (SenSym SDXL010, SensorTechnics, Pucheim, Germany). The other port of the transducer opened into the respirometry chamber to measure pressure differences between the chamber and tracheal system. The pressure sensor electrically consisted of a Wheatstone bridge that was driven with a precision voltage reference of 6.000 V (REF02, Burr Brown, Texas Instruments, Dallas, TX, USA) buffered with a precision operational amplifier (OPA177, Burr Brown). Differential voltage output of the pressure sensor bridge was amplified with a differential amplifier (INA131, Burr Brown). Animals were given a 20-30 min recovery period after the

intubation procedure to recover from the handling stress before the recording of their CO_2 release commenced. Each individual was weighed to 0.1 mg with a digital microbalance (Sartorius 1201 MP2, Sartorius AG, Göttingen, Germany) prior to and after spiracle intubation, as well as at the end of each trial.

3.2.4. Data extraction and analysis

Respirometry data were drift corrected by using ExpeData data acquisition and analysis software (Version 1.1.25, Sable Systems, Las Vegas, NV, USA). Data from 2-5 consecutive DGCs per individual were extracted in ExpeData. The gas exchange pattern was defined as being discontinuous when a clear O-phase and closed/flutter (CF)-phase was visible in the $\dot{V}CO_2$ trace.

A DGC was measured from the onset of the CF-phase until the end of the O-phase. Closed and F-phases were combined due to the difficulty of differentiating the F-phase in all individuals by using the rate of CO_2 release (see Wobschall and Hetz, 2004; Groenewald et al., 2012). Terblanche et al. (Terblanche et al., 2008) found that intratracheal pressure traces can serve as a good indicator to distinguish between the different phases of the DGC. However, I found no obvious pattern in the intratracheal pressure traces for *S. gregaria* that could be used to differentiate between the DGC phases. Gas exchange patterns of individual grasshoppers were assessed based on their $\dot{V}CO_2$. For each individual at each oxygen partial pressure, where a DGC was present, mean $\dot{V}CO_2$, mean duration and mean intratracheal pressure of the entire cycle, as well as for each of the different phases, were extracted. The volume of CO_2 released during the CF-phase and O-phase, as well as the cycle frequency was also extracted. The relationships between these parameters and the different oxygen partial pressures were investigated by using simple linear regressions, as well as general linear-mixed effects models (LMEM). For the LMEM, oxygen treatment was used as a fixed factor and individual as a random factor. The degrees of freedom were calculated using Satterthwaite's method.

To distinguish between the different modes of gas exchange (diffusion *vs* active convection) at the different oxygen partial pressures, the body movements recorded by the activity

detectors (i.e. activity data) during two to three consecutive DGCs per oxygen partial pressure per individual were extracted. The coefficient of variation (CV) was then calculated for the activity data, whereafter the data were used to draw a scatterplot with distance weighted least squares fit. The CV was calculated as the standard deviation of activity divided by the mean (following e.g. Lighton and Ottesen, 2005). The degree of spiracular control exhibited at each oxygen partial pressure was determined by calculating the CV of $\dot{V}CO_2$ and intratracheal pressure. The calculated values were then used to draw a scatterplot with distance weighted least squares fit. All statistical analyses were performed using STATISTICA v.12 software (StatSoft, Tulsa, OK, USA), except linear-mixed effects models, which were performed using SAS v.9.4 (SAS Institute Inc., NC, USA). Piece-wise, breakpoint regression was used to determine the threshold PO_2 for transitions in these relationships.

3.3. Results

Data were obtained from 13 individuals (mean±s.d. body mass 1.722±0.282 g). Each individual was measured under three different oxygen partial pressures selected from the range of 5, 8, 13, 21 and 35 kPa O_2, with one of these three selected partial pressures always being normoxia. However, DGCs were not always present at all three oxygen treatments within an individual. A typical recording of $\dot{V}CO_2$ of a single individual at three different oxygen partial pressures is shown in Figure 3.2. The presence of the DGC did not differ significantly between the different oxygen treatments (p>0.05 in all cases; Table 3.1). There was a significant negative relationship between mean O-phase duration and experimental oxygen partial pressure, while mean O-phase $\dot{V}CO_2$ had a significant positive relationship with oxygen partial pressure (p=0.0057 and p=0.0076, respectively; Table 3.3; see also Table 3.2). Linear-mixed effects models also indicated that there was a significant relationship between mean CF-phase $\dot{V}CO_2$ and oxygen partial pressure (p=0.0005; Table 3.3). Changes in DGC parameters (mean DGC, CF-phase and O-phase $\dot{V}CO_2$ and duration) at the different oxygen treatments (5, 8, 13, 21 and 35 kPa O_2) per individual, as well as the mean across all individuals, are shown in Figure 3.3. This figure shows that gas exchange parameters between individuals varied substantially.

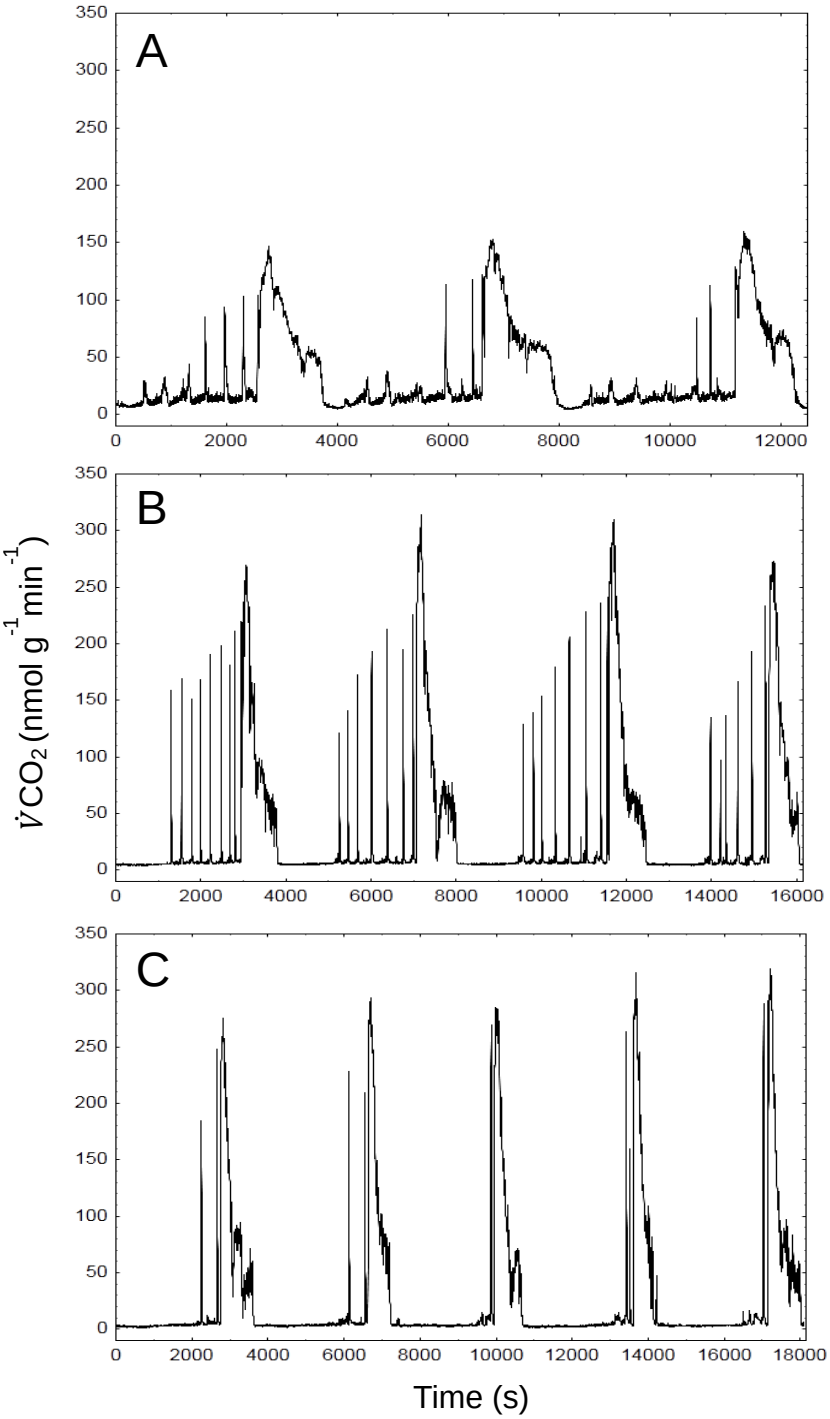

Figure 3.2. Recording of CO_2 release rate ($\dot{V}CO_2$) for a single *Schistocerca gregaria* individual (mass 2.2869 g) at three different oxygen partial pressures: (A) 8 kPa O_2; (B) 13 kPa O_2; and (C) 21 kPa O_2.
The recording was done at 15°C, a flow rate of 250 ml min^{-1} and a sampling frequency of 1 s. Note that the *x*-axis scaling varies for the different oxygen partial pressures.

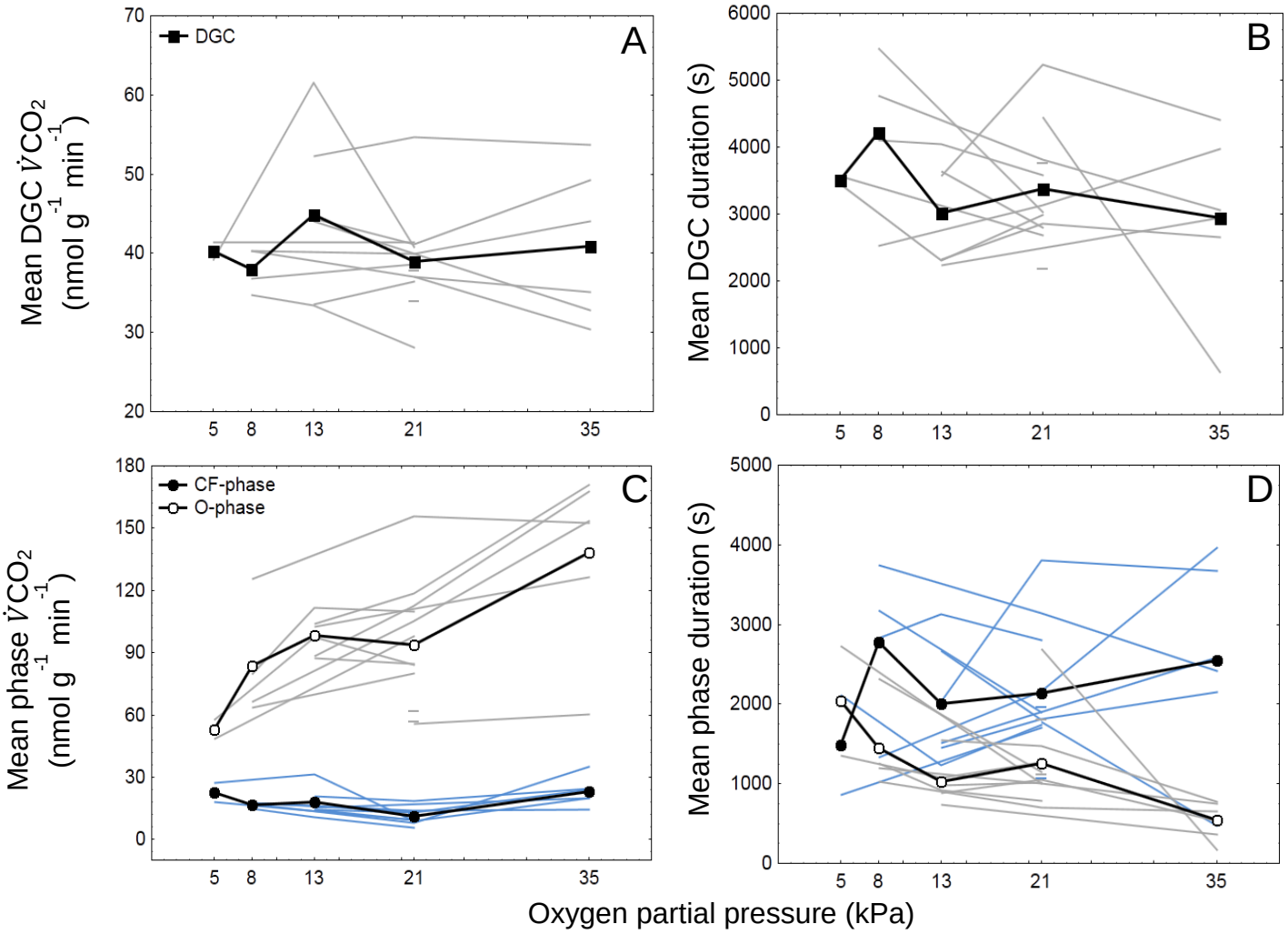

Figure 3.3. Changes in discontinuous gas exchange parameters (CO_2 release rate ($\dot{V}CO_2$) and duration) for the different oxygen partial pressures (5, 8, 13, 21 and 35 kPa O_2).

For (A) changes in $\dot{V}CO_2$ and for (B) changes in the duration of an entire discontinuous gas exchange cycle (DGC) are indicated. Each grey line represents data from an individual grasshopper and squares indicate averages for all grasshoppers at specific O_2 partial pressures. For (C) changes in $\dot{V}CO_2$ and for (D) changes in the duration for both the closed/flutter (CF)-phase and the open (O)-phase are indicated. Grey lines indicate O-phase data for individual grasshoppers and blue lines indicate CF-phase data for individual grasshoppers. Open circles indicate mean O-phase values and filled circles indicate mean CF-phase values for all grasshoppers at a specific O_2 partial pressure.

Table 3.1. Summary of discontinuous gas exchange parameters (rate of CO_2 release, duration, volume, cycle frequency) at the different oxygen partial pressures (5, 8, 13, 21 and 35 kPa O_2).

	DGC		CF-phase		O-phase							
Oxygen partial pressure (kPa)	**Duration (s)**	$\dot{V}CO_2$ nmol g^{-1} min^{-1}	**Duration (s)**	$\dot{V}CO_2$ nmol g^{-1} min^{-1}	**Duration**	$\dot{V}CO_2$ nmol g^{-1} min^{-1}	**Volume** nmol g^{-1}	**Frequency** µHz	**DGC present**	χ^2	**d.f.**	***p***
5	3506	40.239	1482	22.635	2042	52.929	29.115	285.3	2(6)	0.42	5	0.9946
8	4217	38.021	2772	16.740	1448	83.610	31.387	213.2	4(6)	0.22	5	0.9988
13	3017	44.898	2005	17.812	1021	98.496	27.100	347.4	6(7)	1.46	6	0.9624
21	3379	38.908	2140	11.156	1255	93.572	29.633	272.2	12(12)	5.12	11	0.9254
35	2943	40.889	2543	22.958	540	138.424	22.654	958.9	6(7)	1.46	6	0.9624

DGC, discontinuous gas exchange cycle; $\dot{V}CO_2$, rate of CO_2 release; CF-phase, closed/flutter phase; O-phase, open phase; d.f., degrees of freedom.
Numbers in brackets indicate the total number of individuals recorded for the specific treatment.
Chi-square (χ^2) values were calculated with Pearson's Chi squared test (see Crawley (2007) pp. 303-304) for individuals that showed a DGC.

Table 3.2. Summary of linear regression relationships between gas exchange parameters and different oxygen treatments.

Regression	Intercept±SE	Slope±SE	Multiple r^2	Adjusted r^2	*F*	d.f.	*p*
Mean DGC phase duration (s) *vs* oxygen treatment (kPa)	3839.092±413.677	-25.751±19.176	0.06	0.03	1.803	1, 28	0.1901
Mean DGC phase $\dot{V}CO_2$ (nmol g^{-1} min^{-1}) *vs* oxygen treatment (kPa)	40.781±3.218	-0.016±0.149	0.00	-0.04	0.011	1, 28	0.9159
Mean CF-phase duration (s) *vs* oxygen treatment (kPa)	1994.458±383.140	12.352±17.760	0.02	-0.02	0.484	1, 28	0.4925
Mean CF-phase $\dot{V}CO_2$ (nmol g^{-1} min^{-1}) *vs* oxygen treatment (kPa)	14.441±2.887	0.099±0.134	0.02	-0.02	0.545	1, 28	0.4665
Mean O-phase duration (s) *vs* oxygen treatment (kPa)	**1793.229±212.769**	**-33.492±9.863**	**0.29**	**0.27**	**11.532**	**1, 28**	**0.0021**
Mean O-phase $\dot{V}CO_2$ (nmol g^{-1} min^{-1}) *vs* oxygen treatment (kPa)	**58.573±12.200**	**2.109±0.566**	**0.33**	**0.31**	**13.909**	**1, 28**	**0.0009**
Mean O-phase volume (nmol g^{-1}) *vs* oxygen treatment (kPa)	32.441±3.509	-0.233±0.163	0.07	0.03	2.043	1, 28	0.164
Mean cycle frequency (µHz) *vs* oxygen treatment (kPa)	200.734±98.450	7.797±4.564	0.09	0.06	2.919	1, 28	0.0986

DGC, discontinuous gas exchange cycle; $\dot{V}CO_2$, rate of CO_2 release; CF-phase, closed/flutter phase; O-phase, open phase; d.f., degrees of freedom.
Bold indicates significant effects ($p<0.05$).

Table 3.3. Summary of linear-mixed effects models relationships between gas exchange parameters and different oxygen treatments.

Effect	Num d.f.	Den d.f.	F	*p*
Mean DGC duration (s) *vs* oxygen treatment (kPa)	4	25	1.2	0.3343
Mean DGC $\dot{V}CO_2$ (nmol g^{-1} min^{-1}) *vs* oxygen treatment (kPa)	4	24.02	1.16	0.3532
Mean CF-phase duration (s) *vs* oxygen treatment (kPa)	4	25	0.99	0.4312
Mean CF-phase $\dot{V}CO_2$ (nmol g^{-1} min^{-1}) *vs* oxygen treatment (kPa)	4	24.05	7.31	**0.0005**
Mean CF-phase volume (nmol g^{-1}) *vs* oxygen treatment (kPa)	4	24.15	2.15	0.105
Mean O-phase duration (s) *vs* oxygen treatment (kPa)	4	25	4.71	**0.0057**
Mean O-phase $\dot{V}CO_2$ (nmol g^{-1} min^{-1}) *vs* oxygen treatment (kPa)	4	25	4.44	**0.0076**
Mean O-phase volume (nmol g^{-1}) *vs* oxygen treatment (kPa)	4	24.07	1.1	0.38
Mean DGC pressure (Pa) *vs* oxygen treatment (kPa)	4	25	0.88	0.4927
Mean CF-phase pressure (Pa) *vs* oxygen treatment (kPa)	4	25	1.18	0.345
Mean O-phase pressure (Pa) *vs* oxygen treatment (kPa)	4	25	0.25	0.9088
Cycle frequency (Hz) *vs* oxygen treatment (kPa)	4	25	0.95	0.4528

DGC, discontinuous gas exchange cycle; $\dot{V}CO_2$, rate of CO_2 release; CF-phase, closed/flutter phase; O-phase, open phase; d.f., degrees of freedom.
Bold indicates significant effects ($p<0.05$).

Box-plots of the different DGC parameters (mean $\dot{V}CO_2$ and duration for an entire DGC, CF-phase and O-phase, as well as O-phase volume) at the different oxygen treatments show that the mean $\dot{V}CO_2$ during an O-phase is significantly higher at 35 kPa O_2 than at 5 and 21 kPa O_2 (Figure 3.4E; Tukey HSD: $p=0.011$ and $p=0.035$, respectively). Also, the mean O-phase duration is significantly shorter at 35 kPa O_2 than at 5 and 21 kPa O_2 (Figure 3.4F; Tukey HSD: $p=0.007$ and $p=0.048$, respectively).

To investigate whether any relationships exist between the mean duration or $\dot{V}CO_2$ of the different phases of the DGC and the mean intratracheal pressure during the corresponding phases, correlations between these variables were assessed over all oxygen treatments. There was no significant relationship between mean O-phase duration or $\dot{V}CO_2$ and mean pressure during the corresponding O-phases (r=0.276, n=30, p=0.1405 and r=-0.233, n=30, p=0.2164, respectively). There was also no significant relationship between mean CF-phase duration or $\dot{V}CO_2$ and mean pressure during the corresponding CF-phases (r=-0.025, n=30, p=0.896 and r=0.173, n=30, p=0.3612, respectively). No clear characteristic intratracheal pressure pattern existed that was common among all individuals and intratracheal pressures varied highly among individuals. Across all individuals from all oxygen treatments the mean intratracheal pressure during the O-phase was 68.7 Pa (min. -170.9 Pa – max. 1864.7 Pa) and the mean pressure during the CF-phase was 58.7 Pa (min. -597.4 Pa – max. 1751.3 Pa).

The degree of spiracular control increases with increasing oxygen partial pressure (see Figure 3.5A). By knowing the CV of activity (i.e. the amount of body movements exhibited by an individual), it was possible to create an activity index which describes the different modes of gas exchange (diffusion and convection). For this index, it is assumed that individuals displaying fewer body movements, which would have been detected as activity in the detectors, utilize mainly diffusion to exchange gases, as there are fewer active body movements (pumping movements) which could aid active convection. Thus, individuals displaying more activity (pumping movements) per DGC are assumed to be using mainly active convection to exchange respiratory gases. The CV of activity decreases between the treatments of 5 to 13 kPa O_2, whereafter there is an increase in activity between 21 and 35 kPa O_2 (Figure 3.5B). For the CV of both $\dot{V}CO_2$ and intratracheal pressure, as well as for the CV of activity, a breakpoint is present at 19.4 kPa O_2 (R=0.826, R=0.837 and R=0.856, respectively). For the activity data, a breakpoint was also present at a CV of 0.27 (R=0.747), after which the insect switches to a more active mode of gas exchange.

To examine whether there is a metabolic (energetic) cost associated with gas exchange at the different oxygen partial pressures, 3D contour plots with a distance weighted least squares fit were drawn with the CV of activity on the z-axis; the mean O-phase volume (nmol g^{-1}) on the x-axis; and the mean DGC $\dot{V}CO_2$ (nmol g^{-1} min^{-1}) on the y-axis (see Figure 3.6). These graphs

were drawn for each of the O_2 partial pressures separately to enable comparison of the metabolic cost associated with gas exchange between the different O_2 treatments relative to normoxia. Data for individuals from the 5 and 8 kPa O_2 partial pressures were pooled due to low samples sizes.

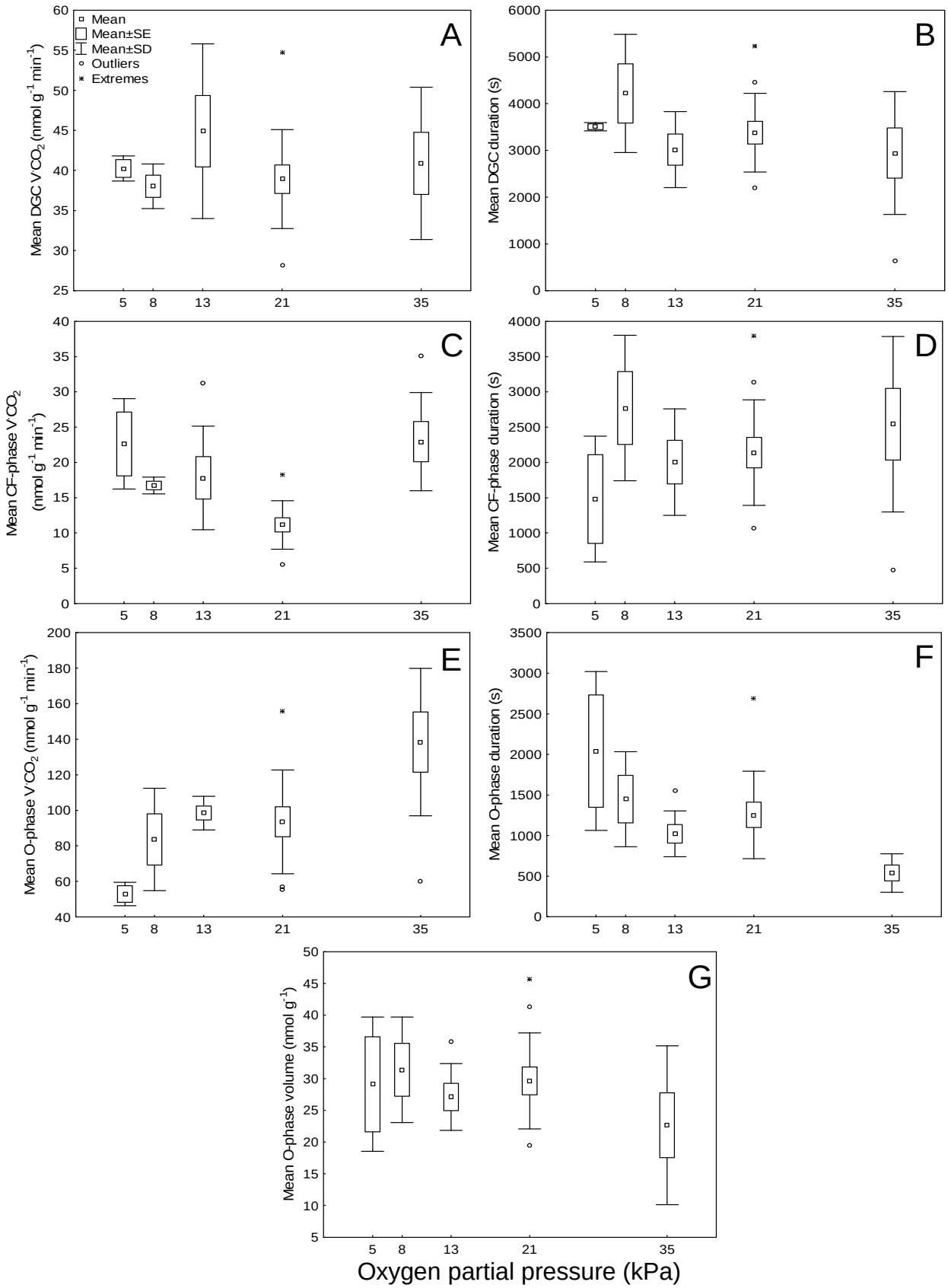

Figure 3.4. Box-plots showing changes in (A) mean discontinuous gas exchange cycle (DGC) rate of CO_2 release ($\dot{V}CO_2$); (B) mean DGC phase duration; (C) mean closed/flutter (CF)-phase $\dot{V}CO_2$; (D) mean CF-phase duration; (E) mean open (O)-phase $\dot{V}CO_2$; (F) mean O-phase duration; and (G) mean O-phase emission volume, with changes in oxygen partial pressure.

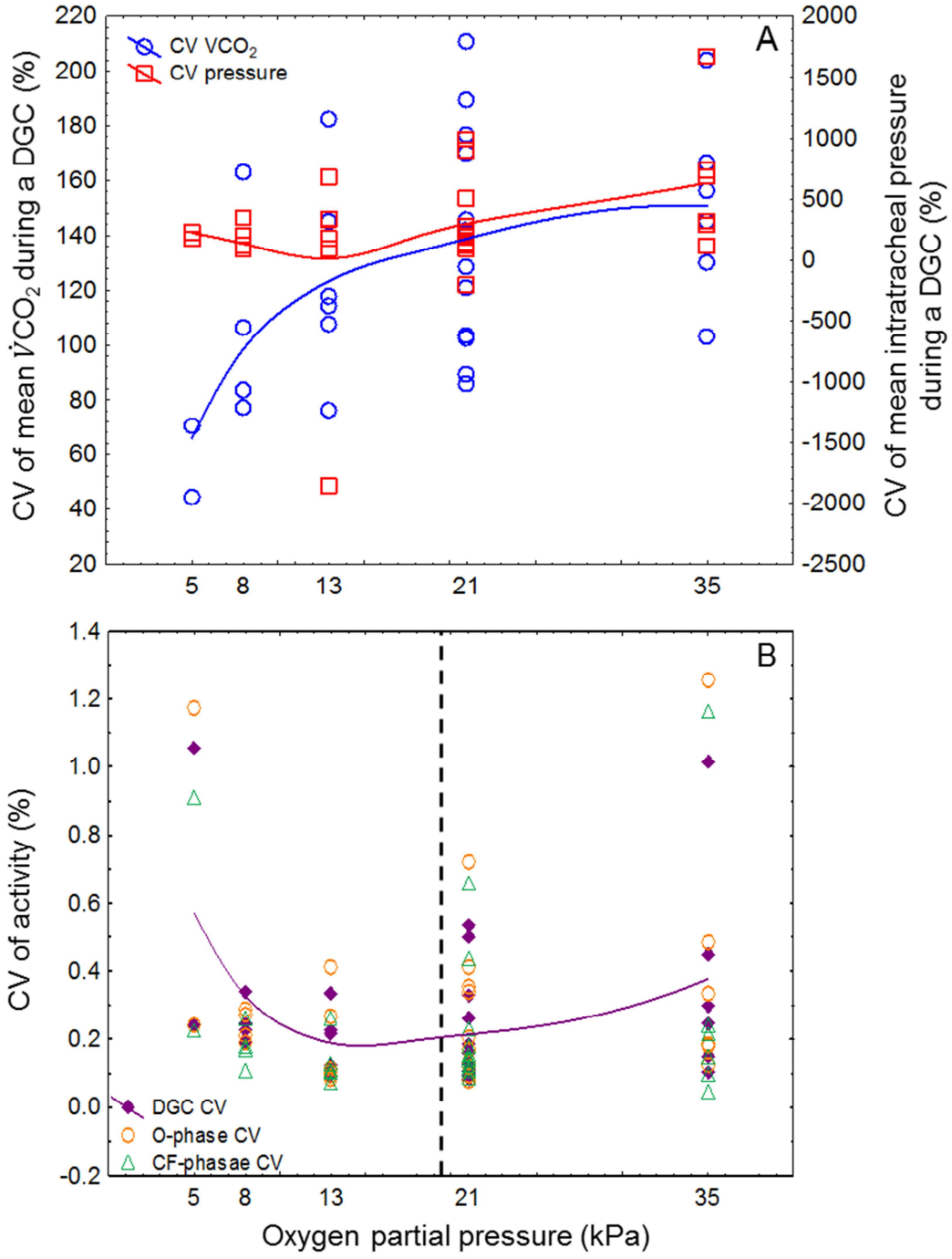

Figure 3.5. Scatterplots indicating (A) the coefficient of variation (CV) for the mean rate of CO_2 release ($\dot{V}CO_2$) and mean intratracheal pressure during a discontinuous gas exchange cycle (DGC), at each of the oxygen treatments (5, 8, 13, 21 and 35 kPa O_2) and; (B) the CV of activity at each of the oxygen treatments during a discontinuous gas exchange cycle (DGC), as well as during a closed/flutter (CF) and open (O) phase.
Fitted lines are distance weighted least squares. The stippled line indicates a breakpoint at 19.4 kPa O_2 derived from piece-wise regression.

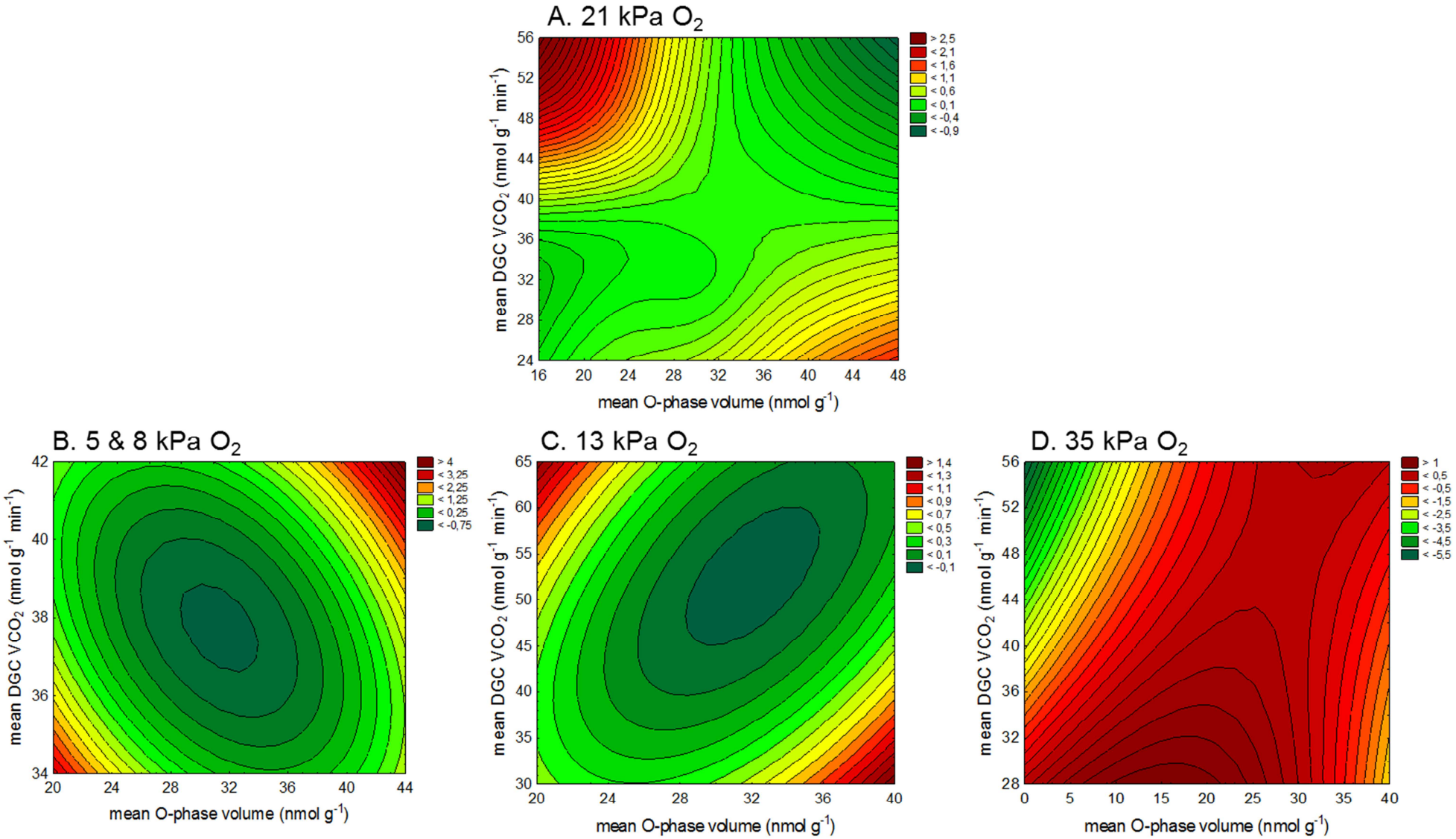

Figure 3.6. Contour plots with distance weighted least squares fit for the different oxygen treatments: A) 21 kPa O_2; B) 5 and 8 kPa O_2; C) 13 kPa O_2; and D) 35 kPa O_2.

Data for the 5 and 8 kPa O_2 treatments were pooled due to low sample sizes. Green indicates less activity (smaller coefficient of variation (CV) of activity) while red indicates more activity (larger CV of activity). Note the different scale values for each of the panels; note also that axes scaling differs.

The relationship between mean O-phase volume (nmol g^{-1}), mean DGC $\dot{V}CO_2$ (nmol g^{-1} h^{-1}) and cycle frequency (cycles h^{-1}) at the different O_2 treatments (5, 8, 13, 21 and 35 kPa O_2) is indicated in Figure 3.7. This figure shows that for the 35 kPa O_2 treatment, smaller O-phase volumes (as compared to O-phase volumes from the other O_2 treatments) could provide sufficient gas exchange. Arbitrary ventilatory frequency isopleths were plotted according to Scott and Milsom (Scott and Milsom, 2007) and indicate the frequency that the volume needs to be released at to create ventilation ($\dot{V}CO_2$). The isopleths represent hypothetical cycle frequencies, as recorded ventilatory frequencies are too slow and the values therefore too small (observed frequency range: min. 0.7 - max. 5.7 cycles h^{-1}) to be included on the graph. For drawing the graph, individual values were binned into different groups based on the volume of CO_2 released (bin groups: 2-9; 9-16; 16-23; 23-30; 30-37; 37-44; and 44-51 nmol g^{-1}). Within each of these bins the mean O-phase volume (i.e. the mean O-phase volume for several individuals) was calculated for each O_2 partial pressure that occurred in that bin. The graph shows that *S. gregaria* individuals are capable of exchanging gases at a very low frequency relative to the volume of the O-phase and DGC $\dot{V}CO_2$.

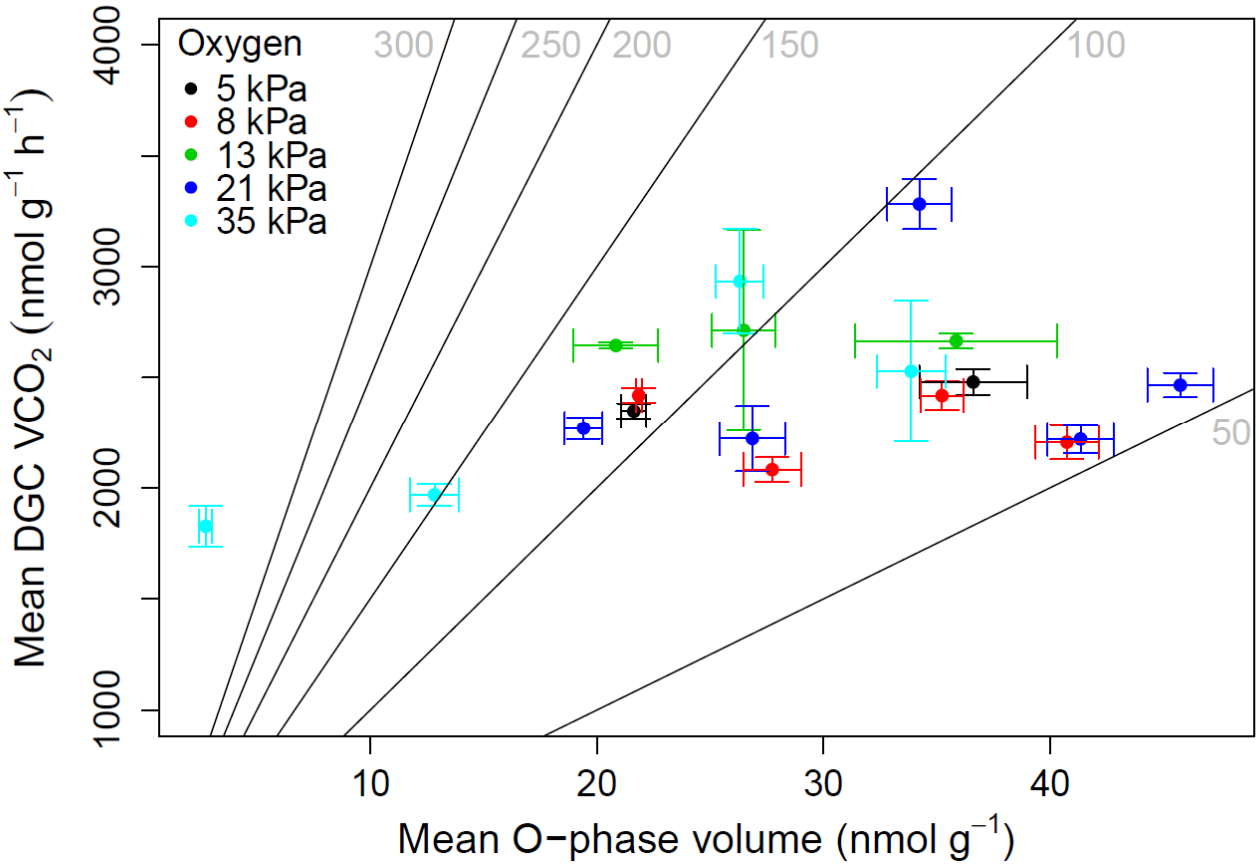

Figure 3.7. Ventilatory response of *Schistocerca gregaria* under different oxygen partial pressures.
Diagonal lines within the plot represent hypothetical ventilatory frequency isopleths (discontinuous gas exchange cycle (DGC) frequency as cycles h^{-1}) indicating the frequency that the volume of CO_2 needs to be released at to create ventilation ($\dot{V}CO_2$). The actual recorded ventilatory frequencies are too slow and the values therefore too small to be included on the graph, therefore only the hypothetical isopleths are plotted. The different colours represent the different O_2 partial pressures. Note that *y*-axis and isopleth units are per hour.

3.4. Discussion

Conditions of hypoxia or increased temperatures lead to an increase in metabolic demand and can result in the expression of ventilatory movements (Miller, 1960b, Weis-Fogh, 1967; Harrison, 1989; Greenlee and Harrison, 1998) if diffusion alone is no longer an adequate means for maintaining oxygen supply. This increased oxygen demand would therefore lead to a switch in the mode of gas exchange from diffusive to convective gas exchange to increase oxygen supply to tissues. However, active convection comes at a higher energetic cost to the animal than diffusive gas exchange and with an increased metabolic rate (i.e. increased oxygen demand) the animal would also need to prioritise saving energy. Therefore, if

diffusive gas exchange would ensure adequate oxygen uptake the animal would favour this mode of gas exchange. The top priority to the animal would however be to ensure adequate O_2 uptake to maintain aerobic metabolism, the next priority would be to regulate haemolymph acid-base status by ensuring sufficient CO_2 release (Groenewald et al., in press; Chapter 4), and only thereafter to minimize energetic costs associated with gas exchange.

The observed increase in ventilatory movements under hypoxic conditions (Figure 3.5B) could be due to the animal trying to meet its cellular oxygen demand (the top priority). In the honeybee (*Apis mellifera ligustica*) there is a temperature-induced switch in the mode of gas exchange from diffusive and continuous to convective and discontinuous. At low temperatures gas exchange occurs through diffusion, while above 12°C the mode of gas exchange switches to convection (Lighton and Lovegrove, 1990). In the locust, *Schistocerca americana*, hypoxic conditions stimulate and hyperoxic conditions suppress (however only weakly) ventilation (see Harrison et al., 2006). For *Schistocerca gregaria*, conditions of both hypoxia and hyperoxia stimulate ventilatory movements (Figure 3.5B), but hyperoxic conditions to a lesser degree. This unexpected finding of increased ventilatory movements in hyperoxia could be due to an imbalance of CO_2 to O_2 in the tracheal system – conditions of hyperoxia should decrease ventilation, however, if ventilation is decreased (by an increase in the CF-phase duration and/or a decrease in the O-phase duration), then this decreased spiracle opening will lead to a build-up of CO_2 in the tracheal system (which dissolves into the haemolymph, leading to higher haemolymph acidity). This CO_2 build-up will then necessitate an increase in ventilation to regulate the haemolymph acid-base status of the insect. This could perhaps explain my finding that in *S. gregaria* hyperoxic conditions stimulate ventilatory movements (Figure 3.5B).

I created an activity index (based on the coefficient of variation (CV) of activity) which describes whether diffusion or active convection is used as the main mode of gas exchange. This index assumes that more activity (i.e. body/pumping movements) indicates that active convection is the main mode of gas exchange, while less or no activity indicates that diffusion is the main mode of gas exchange. A breakpoint is present in this index at a CV of activity of 0.27%, above which the mode of gas exchange will be mainly active convection, while below this breakpoint the mode of gas exchange will by mainly diffusion. Oxygen

partial pressures of 5, 8 and 35 kPa result in active convection being the main mode of gas exchange, while at 13 and 21 kPa O_2 diffusion is mainly used to exchange respiratory gases (Figure 3.5B). The degree of spiracular control exhibited by an insect can be expressed as a relative index by calculating the CV of $\dot{V}CO_2$ (a method developed by Lighton and Lovegrove, 1990; Lighton and Ottesen, 2005). A low CV value indicates that gas exchange is continuous and no cycling is evident, while a high CV value is indicative of the discontinuous gas exchange cycle (DGC) (Lighton and Lovegrove, 1990). For both the CV of $\dot{V}CO_2$ and intratracheal pressure, there is a breakpoint in the O_2 partial pressure at 19.4 kPa O_2 whereafter the individual controls its spiracular opening and closing more tightly (Figure 3.5B). This indicates that in slightly hypoxic conditions spiracles are less tightly controlled than at higher oxygen partial pressures. A possible explanation for this increase in spiracle control under hyperoxic conditions could be that the animal has to intensify the regulation of spiracle opening and closing to regulate acid-base balance, as more CO_2 build up occurs at hyperoxic conditions. It has previously been identified that high variability exists in intratracheal pressure traces of *S. gregaria* individuals at normoxic conditions (Groenewald et al., 2012), and the same was true here under a range of oxygen partial pressures.

According to Murray's law, physiological flow networks have been optimized, through evolution and natural selection, so that flow is achieved with the least possible biological work (i.e. uses the least amount of energy) (Murray, 1926; Sherman, 1981). Ferrara and Kirkton (Ferrara and Kirkton, 2013) found that tracheal dimensions within the metathoracic leg of the grasshopper *Schistocerca americana* follow Murray's law, which suggests that oxygen delivery occurs through convection. The processes of diffusion and passive convection require no work (energy expenditure) from the animal, whereas there is an energetic cost associated with active convection (body movements). It has been shown that diffusion can ensure sufficient O_2 uptake (without needing any active body movements) when metabolic demand is low (e.g. at low temperatures). Murray's law would predict that evolution and natural selection should favour a diffusion or passive convection-dominated system, as these two processes require no energy expenditure. One would therefore expect diffusion and/or passive convection to be the main modes of gas exchange present in insects. My results indicate that for *S. gregaria* gas exchange is most efficient (in terms of energy costs) at and approaching normoxic oxygen partial pressures (see Figure 3.6), because at these O_2 partial pressures diffusion-based gas exchange dominates in the DGC. At PO_2's

below 13 kPa or above 21 kPa gas exchange occurs mainly through active convection, leading to higher energetic costs of ventilation due to the presence of active body movements.

Chapter 4: A hierarchy of factors influence discontinuous gas exchange in the grasshopper *Paracinema tricolor* (Orthoptera: Acrididae)

This chapter is in press as "A hierarchy of factors influence discontinuous gas exchange in the grasshopper *Paracinema tricolor* (Orthoptera: Acrididae)" in *The Journal of Experimental Biology*, by Berlizé Groenewald, Steven L. Chown and John S. Terblanche (doi: 10.1242/jeb.102814).

4.1. Introduction

Insects display at least three different gas exchange patterns at rest. These patterns include continuous, cyclic and discontinuous gas exchange, typically described on the basis of spiracular behaviour (Marais et al., 2005). A discontinuous gas exchange cycle (DGC) consists of three phases including a closed (C)-, flutter (F)- and open (O)- spiracle phase. During the C-phase spiracles remain shut and no gas exchange occurs with the atmosphere (Levy and Schneiderman, 1966b). During this phase, pressure inside the tracheae falls as oxygen is used by metabolically active tissue and CO_2 accumulates and dissolves into the haemolymph. The F-phase includes the rapid opening and closing of the spiracles to regulate intratracheal O_2 levels with some limited CO_2 release (Levy and Schneiderman, 1966b; Lighton, 1994; Hetz and Bradley, 2005). Spiracles remain open during the O-phase, with gases being exchanged rapidly with the atmosphere by diffusion and convection (Lighton, 1988; Duncan et al., 2010; Harrison et al., 2013). During the C- and F-phase of the DGC, the CO_2 that accumulates in the insect dissolves into the haemolymph. This has consequences for the acid-base status of the insect's haemolymph (Wigglesworth, 1935; Harrison et al., 1995). Discontinuous gas exchange may therefore cause significant cyclic variation in haemolymph pH (Matthews and White, 2011a).

Discontinuous gas exchange is thought to have evolved primarily as a means to reduce respiratory water loss (hygric hypothesis) (Levy and Schneiderman, 1966b; Kestler, 1985; Hadley and Quinlan, 1993; Hadley, 1994a), because insects can control the opening and closing of their spiracles. By lengthening the C-phase and shortening the O-phase, insects can reduce the amount of respiratory water loss (Lighton, 1990; Hadley, 1994a; Lighton, 1996; Chown and Davis, 2003). However, several competing hypotheses have also been proposed to explain the origin and maintenance of the DGC: (1) the oxidative damage hypothesis – the DGC is an adaptation to minimize oxidative damage to tissue by regulating tracheal oxygen levels, while still ensuring adequate gas exchange, in a respiratory system evolved for high aerobic performance (Hetz and Bradley, 2005; Chown et al., 2006); (2) the chthonic hypothesis – the DGC facilitates gas exchange under hypoxic and/or hypercapnic conditions (Lighton, 1996; Lighton, 1998); (3) the chthonic-hygric hypothesis – the DGC is an adaptation to reduce respiratory water loss under hypoxic and/or hypercapnic conditions (Lighton and Berrigan, 1995); (4) the strolling arthropod hypothesis – the DGC is an adaptation to reduce the risk of parasitic infestation of the tracheae by increasing the

frequency of spiracle closure (Miller, 1964; Chown et al., 2006); and (5) the emergent property hypothesis – the DGC is a non-adaptive outcome of interactions between O_2 and CO_2 set-points (Chown and Holter, 2000). Despite much research investigating the evolutionary origin and current maintenance of the DGC, there is still no consensus regarding which of the proposed hypotheses is likely correct. New hypotheses have also been proposed, such as the neural hypothesis which states that the DGC is the consequence of the down-regulation of brain activity (Matthews and White, 2011b; Matthews and White, 2013). Much recent support has been found for both the hygric and oxidative damage hypotheses (e.g. Hetz and Bradley, 2005; White et al., 2007; Terblanche et al., 2008; Schimpf et al., 2009; Williams et al., 2010; Schimpf et al. 2011; but see Boardman et al., 2012), suggesting that both may provide mechanistic explanations for the evolution of the DGC. If this is indeed the case, the next logical step is to understand the prioritisation of control within the organism, so further elucidating the regulation of gas exchange, and the integration of inputs and homeostatic set-points in the system.

One potential way to investigate prioritisation in the gas exchange control cascade is by the explicit manipulation of potentially competing demands. Therefore, in this study I sought specifically to examine gas exchange responses of the DGC to both ambient oxygen and relative humidity (RH) variation, after acclimation to both conditions. It is relatively well established that insects can sense changes in ambient RH conditions primarily through hygrosensors located on sensilla of the antennae, which can respond to moist or dry air by elevating or altering signal transmission to the central nervous system, and also possibly through temperature-activated transient receptor potential (TRP) channels that are capable of discriminating between moist and dry conditions (see review by Chown et al., 2011). It is therefore reasonable to expect a physiological response to changes in rearing, or experimental, RH conditions of insects. By examining the effects that different factors and combinations thereof (e.g. haemolymph pH, ambient O_2 partial pressure, hydration state) have on an insect's gas exchange characteristics, those factors that an individual prioritizes, and the hierarchy of these priorities under different circumstances can potentially be identified. Such an approach is analogous to metabolic control analysis (e.g. Suarez and Darveau, 2005; Hofmeyr and Rohwer, 2011).

Here I aimed to investigate the prioritisation of factors which influence discontinuous gas exchange. I did so by assessing a suite of *a priori* predictions for whole-organism responses (see Table 4.1) against empirical data collected from the grasshopper *Paracinema tricolor* which readily shows discontinuous gas exchange cycles (DGCs) at rest. These predictions assume that tracheal and spiracle conductance is not a limiting factor and that morphology is not adjusted rapidly within the experimental time-frame. The predictions are: 1) if water saving is the key, short-term priority of the DGC, then the DGC should be absent under conditions of high RH, but present under conditions of low RH, as it can clearly contribute to respiratory water conservation. Under conditions of low RH, O-phase duration should also decrease, while C-phase duration should increase; 2) if the prevention of oxidative damage is the key priority of the DGC, the alteration of ambient RH conditions should have little or no effect on the presence or absence of the DGC. However, at high ambient partial pressure of O_2 (PO_2), the DGC should be present and cycle frequency should decrease (i.e. by increasing C-phase duration, while perhaps also decreasing O-phase duration) to lower the amount of oxidative damage to tissue; and 3) if haemolymph pH regulation is the key priority of the DGC, the DGC should be absent in dehydrated individuals because dehydration will lead to a decrease in haemolymph volume, which is likely problematic for the animal's acid-base status. Spiracles would therefore need to be kept open for longer so that the animal can release as much CO_2 as possible. Key to this idea is that respiratory acidosis is the main driver of pH change, though other changes due to haemolymph volume reduction may also be influential.

Specifically, in *P. tricolor*, the effects that different oxygen, water and haemolymph pH conditions have on gas exchange characteristics were investigated. Discontinuous gas exchange was assessed under different oxygen (5, 40 kPa O_2) and relative humidity (5, 90% RH) conditions, following acclimation to two different oxygen partial pressures (5, 40 kPa O_2), and following a hydration status acclimation (hydrated, dehydrated).

Table 4.1. Predictions for discontinuous gas exchange responses after acclimation to different conditions and exposure to varying environments.

Responses include presence/absence of the discontinuous gas exchange cycle (DGC), as well as modulation of the different phases. Responses are explained as predicted by the different hypotheses or abiotic factors influencing the DGC (O_2, H_2O and haemolymph pH).

	Treatment		Oxidative damage hypothesis		Hygric hypothesis		Haemolymph pH
Acclimation	**O_2**	**H_2O**	**DGC present?**	**Phase regulation**	**DGC present?**	**Phase regulation**	**DGC present?**
Dehydrated	Low		No		Yes[A]	Shorter O- and/or longer CF-phase	No[F]
	High		Yes	Shorter O- and/or longer CF-phase	Yes[A]	Shorter O- and/or longer CF-phase	No[F]
		Low	No		Yes[A]	Shorter O- and/or longer CF-phase	No[F]
		High	No		No		No[F]
Hydrated	Low		No[D]		No		
	High		Yes[D]	Shorter O- and/or longer CF-phase[D]	No		
		Low	No		Yes[E]	Shorter O- and/or longer CF-phase[B,C]	
		High	No		No[E]		
Hypoxia	Low		No		No		
	High		Yes	Shorter O- and/or longer CF-phase	No		
		Low	No		Yes	Shorter O- and/or longer CF-phase	
		High	No		No		

continued on next page…

Hyperoxia	Low		No		No		
	High		Yes	Shorter O and/or longer CF-phase	No		
		Low	No		Yes	Shorter O- and/or longer CF-phase	
		High	No		No		

DGC, discontinuous gas exchange cycle; O-phase, open phase; CF-phase, closed/flutter phase.
Hypoxia=5 kPa O_2; Hyperoxia=40 kPa O_2
Superscript letters indicate example references where the effect has been documented.
[A] Schimpf et al., 2011
[B] Lighton, 1990
[C] Chown and Davis, 2003
[D] Terblanche et al., 2008
[E] Sláma et al., 2007
[F] Hadley and Quinlan, 1993

4.2. Materials and methods

4.2.1. Study species and maintenance

Male and female individuals of *Paracinema tricolor* Thunberg 1815 (Orthoptera: Acrididae) were collected from a wetland in the JS Marais Park, Stellenbosch, South Africa (33°55'58"S 18°52'33"E). Both males and females are winged and flew readily when chased during collection. The population was maintained at a 14 h: 10 h light: dark photoperiod, a constant temperature of 25°C±1.5°C at 60-80% RH, and atmospheric O_2 and CO_2 conditions. Temperature and RH was verified with iButton hygrochron temperature/humidity electronic recorders (±0.5°C; 0.6% RH, Maxim/Dallas Semiconductors, Sunnyvale, CA, USA). Grasshoppers were provided with a diet of lettuce, spinach, oatmeal and fish food, as well as Restionaceae and Poaceae from their natural habitat. The rearing containers were sprayed with water daily to maintain a moist habitat, as the grasshoppers' natural habitat is wetlands (Picker et al., 2004). This association with mesic habitats could mean that they are sensitive to dehydration stress, and that they are thereby more likely to be influenced by hydration levels.

A total of 300 individuals were collected and divided into two major experimental blocks. The first experimental block investigated the effects of oxygen acclimation, and the second experimental block the effects of hydration status on the DGC (see Figure 4.1).

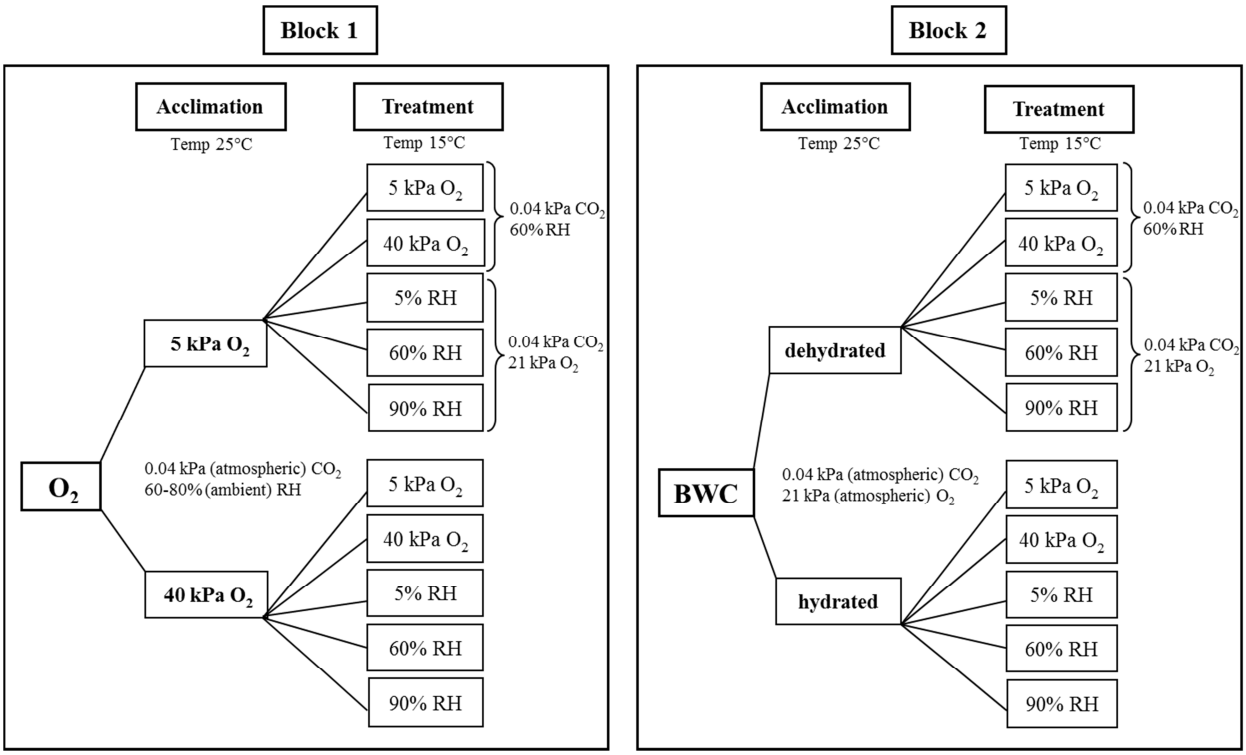

Figure 4.1. Schematic diagram of the experimental design for the oxygen and hydration status exposures (acclimations) and the range of treatments employed to assess modulation of the DGC.
BWC, body water content; Temp, temperature; RH, relative humidity.

4.2.2. Acclimation to variation in ambient oxygen

The acclimation period commenced immediately after collection of sufficient individuals. Individuals (males and females) were randomly divided into two groups: hypoxia (5 kPa O_2) and hyperoxia (40 kPa O_2,). The acclimation period lasted for more than seven days and took place under the same temperature, RH and photoperiod conditions at which the initial collected population was kept. For the two different treatment gases (5 or 40 kPa O_2, balance Nitrogen), a pressurised gas cylinder (Air Products South Africa Pty., Cape Town) was used to feed the gas into the acclimation containers at a constant flow rate of 150 ml min^{-1}, which was verified with a hand-held electronic flow meter (Model ADM1000, Agilent technologies, Wilmington, DE, USA). An oxygen analyser (Pacific CA Systems, Washington, USA) was used to confirm that the oxygen partial pressures were at the desired levels within the acclimation containers. Temperature and humidity conditions within the containers were recorded with temperature/humidity iButtons.

4.2.3. Acclimation to variation in moisture availability

Individuals were taken from the collected population and transferred to separate 300 ml plastic jars containing a layer of silica gel to lower the RH inside the jar. Either oats (for the 'dehydrated' status) or lettuce (for the 'hydrated' status) was provided as food and all individuals were kept at 25°C. Individuals were then weighed regularly to determine the amount of body mass (BM) lost. Grasshoppers were classified as 'dehydrated' if more than 10% of their BM was lost (calculated as (start mass – end mass) / start mass x 100). Grasshoppers in both hydration groups experienced some mass loss during the treatment. Individuals in the hydrated group displayed a mean loss of 5.2±1.4% of their body mass, while dehydrated individuals showed a mean loss of 13.2±2.4% of their body mass (sample size 33 and 40, respectively).

4.2.4. Respirometry treatment conditions

Measurements of CO_2 release rate ($\dot{V}CO_2$) were undertaken at 15°C on two separate grasshoppers in two separate respirometry systems simultaneously. Individuals from each of the acclimations were recorded under each of the following treatments: 1) 5 kPa O_2; 2) 40 kPa O_2; 3) 5% RH; and 4) 90% RH. The treatment for the control group consisted of 21 kPa O_2 and 60% RH. Oxygen treatments were conducted at 0 kPa CO_2 and 60% RH, and relative humidity treatments were conducted at 0 kPa CO_2 and 21 kPa O_2 (see Figure 4.1). An average sample size of seven individuals from each acclimation was recorded per experimental treatment.

Flow-through CO_2 based respirometry was undertaken to record $\dot{V}CO_2$ (see Lighton, 2008). For the two different oxygen treatments (5 and 40 kPa O_2, balance nitrogen), a pressurised gas cylinder (Air Products South Africa Pty., Cape Town) fed one of the gas mixtures into soda lime (MERCK, Gauteng, RSA) and 50: 50 silica gel/Drierite (silica gel, MERCK, Gauteng, RSA; Drierite, Sigma-Aldrich, Ohio, USA) scrubber columns. For the treatment at normoxia (21 kPa O_2) an aquarium pump was used to feed atmospheric air through the scrubber columns. Air was regulated at a constant flow rate of 200 ml min^{-1} by a flow control valve (Sierra Side-Trak, Sierra Instruments Inc., Monterey, CA, USA) connected to a mass flow controller (MFC-2, Sable Systems, Las Vegas, NV, USA). Thereafter, air flowed

through the zero channel of an infra-red CO_2-H_2O analyser (Li-7000, LiCor, Lincoln, NE, USA).

During the oxygen treatment trials air was humidified to prevent desiccation stress. A custom made air bubbler, consisting of a 250 ml Schott jar two-thirds filled with doubly-distilled water, plus 1 ml of a 1 mol l^{-1} aqueous solution of sodium hydroxide, to prevent CO_2 in the water from diffusing into the air stream (following Stevens et al., 2010), was used to humidify air leaving the gas analyser. The air bubbler was immersed in a water bath (Grant GD-120, Cambridge, England, UK), which controlled the selected temperature at 7°C, to maintain a RH of 60% within the airstream flowing to the cuvette. During the RH treatment trials, to obtain a RH of 90%, temperature of the water bath containing the air bubbler was set to 13°C, and to obtain a RH of less than 5%, air was scrubbed of water by silica gel and Drierite. The humidified air then passed through a plastic coil of Bev-A-Line tubing before passing over the test animal in the cuvette, both located inside a second water bath (Grant GP200-R4, Cambridge, England, UK) which maintained the temperature at 15°C. Air leaving the cuvette passed through a small custom made scrubber column containing re-activated Drierite to remove water vapour from the airstream before it entered the gas analyser (following White et al., 2006). This was done to prevent condensation within the gas analyser cell which may damage the analyser. The scrubbed air then entered the gas analyser through another channel, which recorded the difference in CO_2 concentration of the air before and after it flowed through the respirometry cuvette, at 1 s intervals. The output of the analysers ($\dot{V}CO_2$) was computed/stored via Li-7000 software on a standard desktop computer. Activity was recorded for individual grasshoppers using infrared activity detectors (AD2, Sable Systems, Las Vegas, Nevada, USA). Activity traces were only used to ensure that the extracted DGCs represented inactive periods. Activity data were not used for any further analyses.

Each individual was weighed to 0.1 mg prior to and after each trial by using a digital microbalance (Model MS104S, Mettler Toledo, Greifensee, Switzerland). In each respirometry system, a single grasshopper was placed in a darkened cuvette and given approximately 20 minutes to settle in the cuvette (at 15°C) before recording of CO_2 release commenced. Baselines (between 15 and 30 minutes) were recorded at the beginning and end

of each trial by using an identical setup, but without the test animal. The rate of CO_2 release was measured and recorded for individual grasshoppers for a period of 6-12 hours per individual. Owing to sexual size dimorphism, female grasshoppers were measured in a 20 ml cuvette and males in a 10 ml cuvette. Individuals were fasted for at least 8 hours before recording of CO_2 release commenced.

4.2.5. Haemolymph pH measurement

Haemolymph pH of individual grasshoppers was measured for both a control (hydrated, *n*=12) and treatment (dehydrated, *n*=5) group. The small sample size of the treatment group when compared with the control group is due to the fact that haemolymph volume decreases in dehydrated individuals. This made it difficult to obtain a large enough volume of haemolymph to measure from individuals in the treatment group. To obtain different levels of dehydration within the individuals, individuals were treated as previously described (see Figure 4.1 - Block 2). Once the desired level of dehydration was reached, haemolymph was extracted from the individual by removal of the hind legs. Haemolymph was extracted from the wound with a 100 µl micropipette (Eppendorf Research, MERCK Millipore) and transferred to a 200 µl Eppendorf tube. The average volume of haemolymph extracted per individual was 5-15 µl. After extraction, measurements were made as quickly as possible to minimize the amount of time that the haemolymph came into contact with atmospheric air. Needle type pH microsensors (140 µm diameter), connected to a PreSens pH-1 micro fiber optic pH transmitter (PreSens GmbH, Regensburg, Germany), were used to measure haemolymph pH. The pH sensors were calibrated at 15°C using seven sodium phosphate buffers that ranged from pH 5 to 8 in 0.5 pH increments. Because the pH sensors are sensitive to the ionic strength of a sample, the sodium phosphate buffers were made up with an ionic strength the same as locust Ringer's solution (0.172, from Pearson and Robertson, 1981). The sodium phosphate buffers were checked with a pH meter (inoLab, pH 720, WTW GmbH, Weilheim, Germany) calibrated with standard pH 4 and 7 buffer solutions. To measure haemolymph pH, a hole was pierced through the Eppendorf lid containing the haemolymph, so that the pH probe could be inserted while minimizing contact of the haemolymph with atmospheric air. Haemolymph pH was measured every 1 s for a period of three minutes to allow pH readings to stabilize. Haemolymph pH readings were recorded with pH1-view software via a standard desktop computer.

4.2.6. Data analyses

Respirometry data ($\dot{V}CO_2$) were converted to ml CO_2 h^{-1} and data were drift corrected by using ExpeData data acquisition and analysis software (Version 1.1.25, Sable Systems, Las Vegas, NV, USA). For treatments in which individuals maintained DGCs, data from 2-5 consecutive DGCs per individual were extracted in ExpeData. A DGC was measured from the onset of the closed/flutter (CF)-phase until the end of the O-phase. For each DGC the mean duration and mean $\dot{V}CO_2$ of a total DGC (O+CF-phases), O-phases and CF-phases, as well as the O-phase emission volumes were extracted. Closed and F-phases were combined due to the difficulty of differentiating the F-phase in all individuals, and because the F-phase may commence before CO_2 release is detected (see Figure 4.2 for an example data trace) (Hadley and Quinlan, 1993; Wobschall and Hetz, 2004; Groenewald et al., 2012). Water loss rates (WLR) were estimated gravimetrically by subtracting the mass of an individual at the end of an experiment from the mass of the individual at the start of the experiment, and dividing by the duration of the trial.

Individual grasshoppers were assessed for the presence or absence of the DGC based on their ($\dot{V}CO_2$). Contingency tables were then generated for the number of individuals that showed DGCs under control and treatment conditions. Mean duration and mean $\dot{V}CO_2$ for the different phases (CF-phases and O-phases) were compared between different treatments and acclimations. These data (presence/absence of the DGC and different phase responses) were then used to test the *a priori* predictions regarding water, oxygen and pH regulation (Table 4.1).

Mass was a significant covariate for all parameters ($p<0.05$ in all cases), except for mean O-phase duration and WLR. Therefore, to adjust for the effects of size the residuals of the dependent variable-body mass relationship were used when drawing correlations. Generalized linear models (GLZs, normal distribution, identity link function) were used for data analysis as several assumptions were not met, and GLZs are more robust to the violation of these assumptions. Generalized linear models were used to test for effects of acclimation and treatment on mean DGC phase durations and $\dot{V}CO_2$, as well as on O-phase volume and WLR. An interaction between acclimation and treatment was always tested for and any significant covariates were included in the analyses. For variables in which treatment or

acclimation was found to have a significant effect, graphs including 95% confidence intervals were drawn to distinguish which of the treatments or acclimations differ significantly from the others. In addition, estimates of effect sizes were calculated for all the recorded variables for each of the different acclimation and treatment conditions (Table 4.2). Effect sizes were calculated as the mean deviation from the grand mean. Weighted least squares were used to test for correlations between the different variables, as this analysis is more robust to outliers and data that are not normally distributed. All statistical analyses were carried out in STATISTICA v.11 (StatSoft, Tulsa, OK, USA).

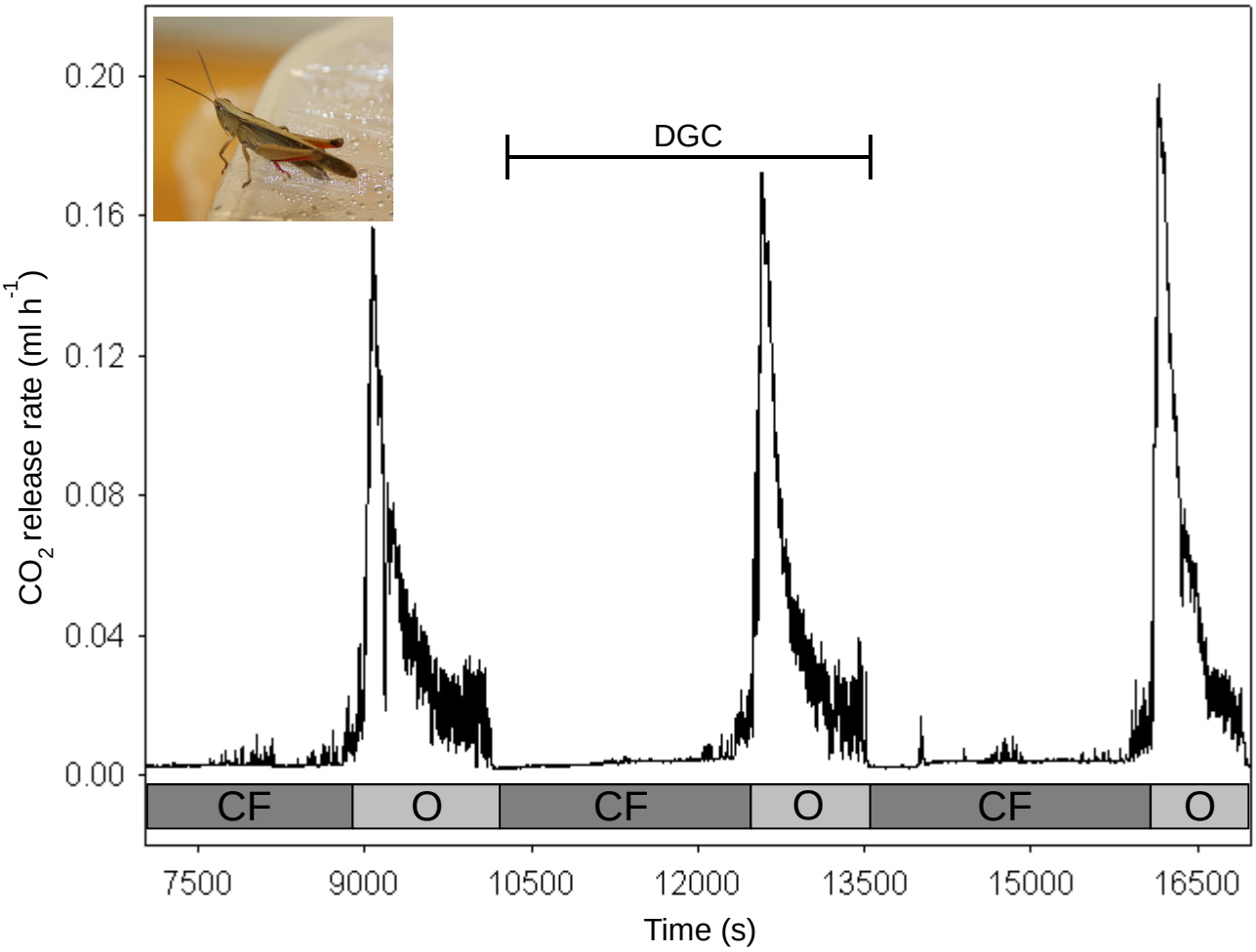

Figure 4.2. Typical CO_2 emission trace by a 0.2442 g male *Paracinema tricolor* (inset) at 15°C.
This individual was acclimated to 40 kPa O_2 and recorded under treatment conditions of 21 kPa O_2 and 60% relative humidity. Flow rate was 200 ml min^{-1}. The different phases of the discontinuous gas exchange cycle that were used for data analysis are indicated by the different bars. During the open phase (O, light grey bar) a burst of CO_2 release is observed, while during the closed/flutter phase (CF, dark grey bar) practically no CO_2 release occurs. A discontinuous gas exchange cycle (DGC) consists of a CF- and an O-phase.

Table 4.2. Mean effect sizes and their signs for all recorded variables for each of the different acclimations (5 kPa O_2, 40 kPa O_2, hydrated, dehydrated) at each of the different treatments (5% RH, 90% RH, 5 kPa O_2, 40 kPa O_2, control), calculated as the mean deviation from the grand mean.
Negative values are highlighted in bold.

		% Deviation from the grand mean							
		Mean duration			Mean $\dot{V}CO_2$			CO_2 release volume	
Acclimation	**Treatment**	**DGC**	**CF-phase**	**O-phase**	**DGC**	**CF-phase**	**O-phase**	**O-phase**	**WLR**
5 kPa O_2	5% RH	17.3	24.6	**-4.5**	**-15**	**-6.9**	**-8.2**	**-2.3**	66.4
	90% RH	**-11.1**	**-4.9**	**-11.3**	**-6**	**-18.9**	**-6.4**	**-15.9**	**-74.2**
	Control	4.2	21.6	**-18.6**	**-13.6**	**-14**	11.5	**-20.2**	**-65.7**
	5 kPa O_2	27.8	13.1	38.4	**-1.6**	12.4	**-22.8**	13.8	**-28.9**
	40 kPa O_2	**-31.6**	**-19.5**	**-46.7**	**-5.4**	1.7	20	**-35.8**	**-63.4**
40 kPa O_2	5% RH	**-24**	**-12.3**	**-13.3**	3.6	**-16**	29.3	**-8.1**	123.8
	90% RH	**-33.1**	**-31.3**	**-12.2**	**-2.2**	**-20.1**	0.8	**-35.6**	**-117.4**
	Control	59.3	70	**-22.1**	**-25.6**	**-27.1**	**-6**	22.5	**-80.2**
	5 kPa O_2	0.1	**-13.2**	39.5	0.7	34	**-22.6**	2.9	**-28.0**
	40 kPa O_2	4.4	20.6	**-40.7**	**-28.8**	**-21.2**	**-1.5**	**-26.9**	**-51.5**
Hydrated	5% RH	**-7.6**	**-9.1**	**-4.3**	5.2	12.3	**-8.2**	0.4	75.5
	90% RH	**-13.9**	**-16.4**	**-6.7**	14.2	**-0.7**	6.6	3.7	191.5
	Control	15	18.9	**-0.4**	**-13.3**	**-7.6**	**-3.3**	16.7	**-34.9**
	5 kPa O_2	**-10.5**	**-36.6**	43.9	22.3	55.9	**-36.2**	8.1	**-5.4**
	40 kPa O_2	4	6.2	1	22.8	4.3	12.6	34.2	17.0
Dehydrated	5% RH	1.8	0.1	**-0.2**	7	5.3	**-0.3**	12.5	46.0
	90% RH	**-4.9**	**-13.2**	15.4	22.2	11.7	11.5	22.7	**-72.8**
	Control	7.1	**-4.5**	34.5	**-1.3**	**-20.1**	**-8.2**	**-24.1**	60.6
	5 kPa O_2	14.4	**-47.2**	146.7	35.8	18	**-30.1**	96.6	**-36.5**
	40 kPa O_2	**-16.4**	**-17.8**	**-11.7**	23.6	32.2	38.1	9.8	41.4

DGC, discontinuous gas exchange cycle; CF-phase, closed/flutter phase; O-phase, open phase; $\dot{V}CO_2$, rate of CO_2 release; RH, relative humidity; WLR, water loss rate.

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*Mass was a significant covariate in all cases, except for mean O-phase duration and WLR.
Where mass was a significant covariate the residual data were used in the analysis to compute effect sizes.

4.3. Results

Discontinuous gas exchange was present under all experimental treatments in *P. tricolor* from both the hyperoxia and hydrated status acclimation conditions. For the hypoxia acclimation only one individual did not show DGCs, while for the dehydrated acclimation only 67% of individuals maintained DGCs (24/36 individuals), differing significantly from all other acclimations (Table 4.3). Within the dehydrated acclimation group, significantly fewer individuals in the 5% RH and 5 kPa O_2 treatment group maintained DGCs when compared to the other treatment groups (Table 4.3).

4.3.1 Modulation of discontinuous gas exchange

Both acclimation and treatment had significant effects on O-phase duration (Table 4.4). Assessed across all acclimation groups the treatment at [5 kPa O_2, 60% RH] resulted in a significantly longer O-phase duration when compared to the treatments of [21 kPa O_2, 5% RH], [21 kPa O_2, 90% RH], and [40 kPa O_2, 60% RH] ($p<0.05$ in all cases). Acclimation significantly affected mean DGC $\dot{V}CO_2$ (Table 4.4), with the acclimation at 5 kPa O_2 (taken across all treatment groups) resulting in a significantly lower $\dot{V}CO_2$ than the hydrated acclimation ($p=0.004$). For the mean $\dot{V}CO_2$ during a DGC, at the 5 kPa O_2 acclimation, all treatment groups had negative deviations from the overall mean (Table 4.2).

For the experimental treatment of 5 kPa O_2, the duration of the O-phase was significantly longer for the dehydrated acclimation than for any of the other acclimation conditions (Figure 4.3). Across all individuals from all treatment and acclimation groups, both the mean duration of the O-phase and the mean duration of the CF-phase increased with the mean duration of a DGC (Figure 4.4; $r=0.233$, $n=138$, $p=0.006$ and $r=0.227$, $n=138$, $p=0.008$, respectively). The dehydrated acclimation resulted in a significant decrease in haemolymph pH from 7.0±0.3 to 6.6±0.1 (mean±standard deviation; Mann-Whitney U: U=7, Z=2.372; $p=0.018$).

The recommended (post-hoc) sample sizes for three different extreme cases in the results were calculated using STATISTICA software power calculator, assuming $\alpha=0.05$ and $\beta=0.8$ (Crawley, 2007). This analysis suggested a sample size of approximately 34 individuals per

treatment, meaning that for my experimental design a total sample size of 680 individuals would be required.

Table 4.3. Contingency table for individuals that abandoned discontinuous gas exchange under the various experimental treatments (5% RH, 90% RH, 5 kPa O_2, 40 kPa O_2, control) within each acclimation (5 kPa O_2, 40 kPa O_2, hydrated, dehydrated).

		Acclimation													
		Hypoxia			Hyperoxia			Hydrated			Dehydrated			Total	
Treatment	**d.f.**	χ^2	***p*-value**	**DGC present**	χ^2	***p*-value**	**DGC present**	χ^2	***p*-value**	**DGC present**	χ^2	***p*-value**	**DGC present**	χ^2	***p*-value**
5% RH	4	0.861	0.93	10 (10)	0.775	0.942	9 (9)	0.689	0.953	8 (8)	**19.152**	**<0.005**	3 (7)	0.393	0.983
90% RH	4	0.603	0.963	7 (7)	0.775	0.942	9 (9)	0.775	0.942	9 (9)	0.603	0.963	7 (7)	2.755	0.6
40 kPa O_2	4	0.603	0.963	7 (7)	0.689	0.953	8 (8)	0.43	0.98	5 (5)	0.141	0.998	7 (8)	0.825	0.935
5 kPa O_2	4	0.065	0.999	8 (9)	0.603	0.963	7 (7)	0.517	0.972	6 (6)	**19.152**	**<0.005**	3 (7)	2.51	0.643
Control	4	0.603	0.963	7 (7)	0.861	0.93	10 (10)	0.344	0.987	4 (4)	**9.537**	**0.049**	4 (7)	0.144	0.998
Total	3	1.734	0.629		3.702	0.295		2.755	0.431		**25.561**	**<0.001**		**57.778**	**<0.001**

DGC, discontinuous gas exchange cycle; RH, relative humidity; d.f., degrees of freedom.
Hypoxia=5 kPa O_2; Hyperoxia=40 kPa O_2.
Numbers in brackets indicate the total number of individuals recorded for the specific treatment.
Bold indicates significant differences.
Chi-square (χ^2) values were calculated with Pearson's Chi squared test (see Crawley (2007) pp. 303-304) for individuals that abandoned the DGC.
Total values of rows indicate χ^2 values for the different treatment groups, while total values of columns indicate χ^2 values for the different acclimation groups.

Table 4.4. Summary of statistics for generalized linear models testing for effects of acclimation and experimental treatment, as well as interactions between acclimation and experimental treatment, on the mean discontinuous gas exchange cycle and phase durations, rate of CO_2 release, as well as O-phase emission volume and water loss rate.

	Acclimation			Treatment			Interaction		
	Wald χ^2	**d.f.**	***p*-value**	**Wald χ^2**	**d.f.**	***p*-value**	**Wald χ^2**	**d.f.**	***p*-value**
Mean DGC duration	0.501	3	0.919	3.809	4	0.432	6.925	12	0.863
Mean O-phase duration	**21.631**	**3**	**<0.001**	**57.023**	**4**	**<0.001**	14.183	12	0.289
Mean CF-phase duration	5.020	3	0.170	7.220	4	0.125	6.478	12	0.890
Mean DGC $\dot{V}CO_2$	**7.972**	**3**	**0.047**	3.180	4	0.528	9.234	12	0.683
Mean O-phase $\dot{V}CO_2$	0.045	3	0.997	6.340	4	0.175	13.458	12	0.337
Mean CF-phase $\dot{V}CO_2$	4.224	3	0.238	7.687	4	0.104	6.540	12	0.886
Mean O-phase volume	7.318	3	0.062	5.457	4	0.244	13.758	12	0.316
Water loss rate	5.633	3	0.131	7.437	4	0.115	17.029	12	0.149

CF-phase, closed/flutter phase; O-phase, open phase; DGC, discontinuous gas exchange cycle; d.f., degrees of freedom; $\dot{V}CO_2$, rate of CO_2 release.
Bold indicates significant effects ($p<0.05$).
*Mass was a significant covariate in all cases, except for mean O-phase duration and water loss rate.

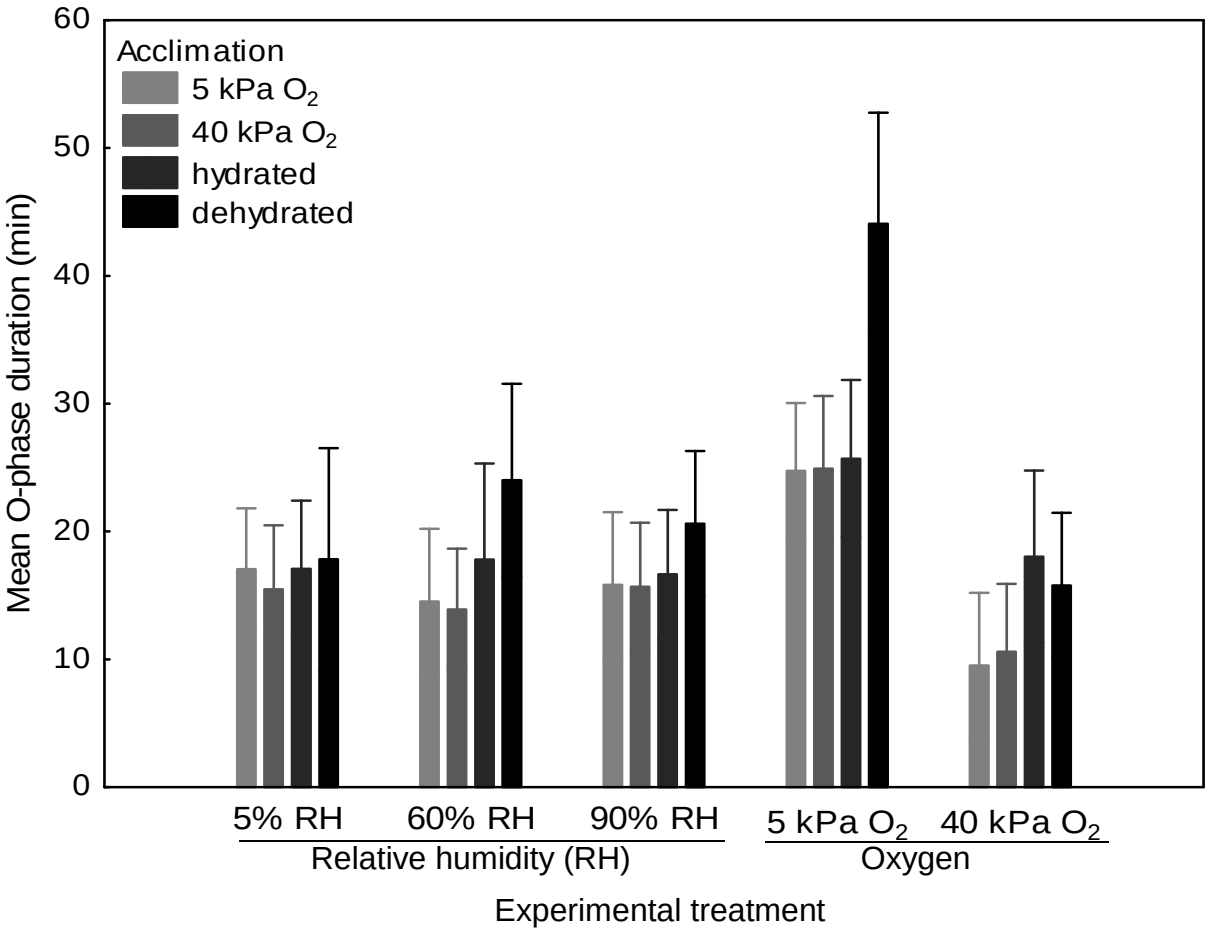

Figure 4.3. Summary results of the effects of experimental treatment and acclimation (means+95% confidence intervals) on the mean duration of the open (O) phase.
Both experimental treatment and acclimation had a significant effect on O-phase duration (Wald χ^2=57.023, d.f.=4, p<0.001; Wald χ^2=21.631, d.f.=3, p<0.001, respectively). Mass did not have a significant relationship with the duration of the O-phase (p=0.868) and was therefore not included as a covariate.

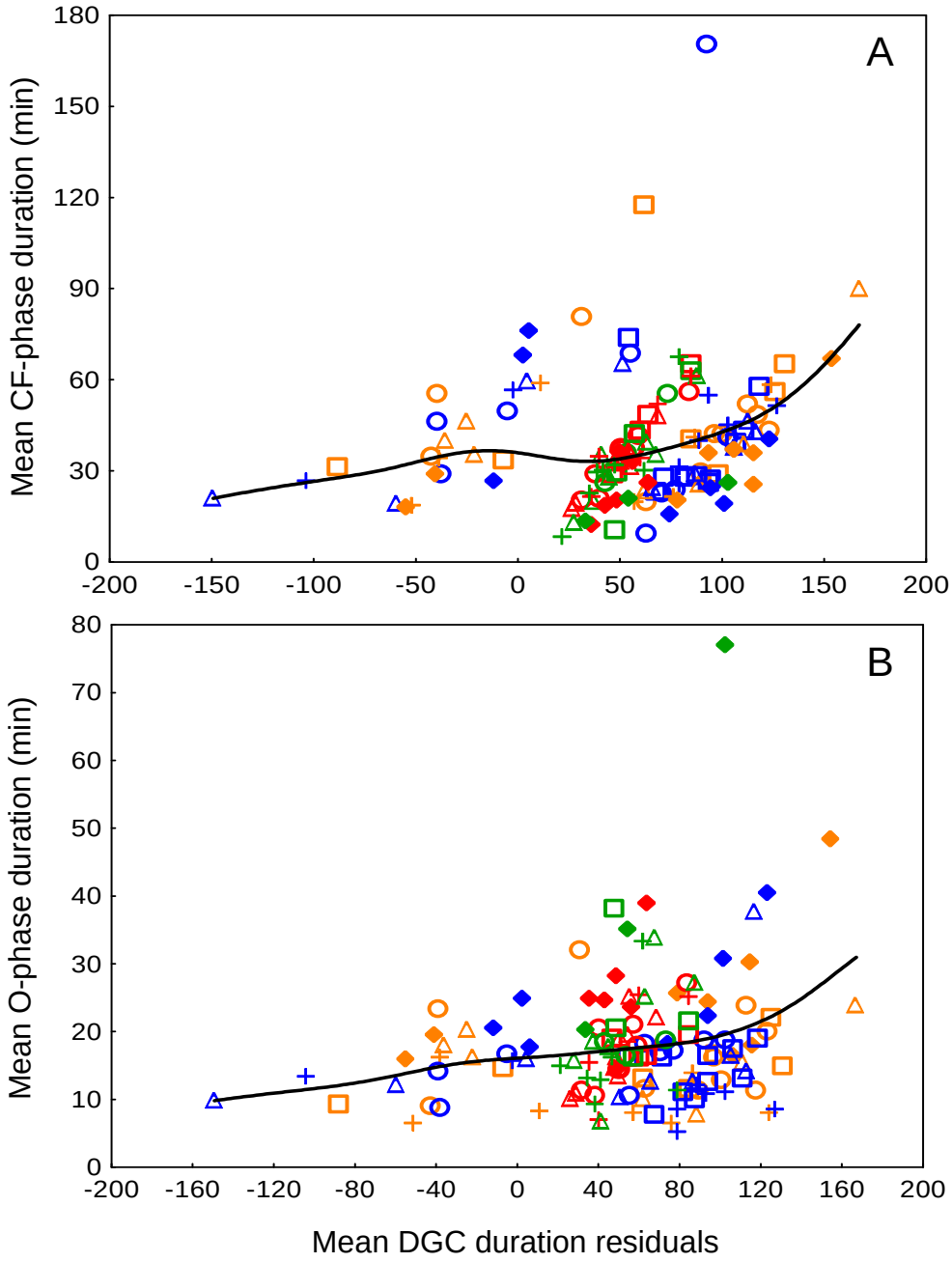

Figure 4.4. Relationship between (A) the mean duration of a closed/flutter (CF) phase with the residuals of the mean discontinuous gas exchange cycle (DGC) duration (i.e. DGC-body mass relationship) (*y*=1988.786 + 0.091*x*; *r*=0.227; *p*=0.008); and (B) the mean duration of an open (O) phase with the residuals of the mean duration of a DGC (*y*=933.820 + 0.041*x*; *r*=0.2325; *p*=0.006).

All individuals were included in the analysis (*n*=138) and each point on the graphs represents the mean value of 2-5 DGCs per individual. Residual data were used for mean DGC duration, as mass was a significant covariate for this variable (Wald χ^2=5.661, d.f.=1, *p*=0.017). Fitted lines are distance weighted least squares. The different acclimations are represented by different colours (blue=hyperoxia; orange=hypoxia; green=dehydrated; red=hydrated), while the different experimental treatment groups are represented by different symbols ([○ 21 kPa O_2, 5% RH]; [□ 21 kPa O_2, 60% RH]; [Δ 21 kPa O_2, 90% RH]; [♦ 5 kPa O_2, 60% RH]; [+ 40 kPa O_2, 60% RH]). For example, an orange triangle indicates that an individual received an acclimation treatment of 5 kPa O_2, and its CO_2 release was recorded at the experimental treatment of 21 kPa O_2 and 90% RH.

4.4. Discussion

The insect respiratory system has evolved to facilitate rapid oxygen delivery during periods of activity, but during periods when the insect's metabolic rate is low, these high supplies of O_2 could be harmful to tissues (Hetz and Bradley, 2005). The oxidative damage hypothesis is based on this assumption and is supported in moth pupae, which regulate their internal PO_2 at low levels during the F-phase, even when exposed to hyperoxic atmospheres (Hetz and Bradley, 2005; see also Terblanche et al., 2008; Boardman et al., 2012). However, for *Locusta migratoria* internal PO_2 is dependent on ambient O_2 partial pressures when exposed to hyperoxic conditions (Matthews et al., 2012). According to my *a priori* predictions, there should be a decrease in the O-phase and/or an increase in CF-phase duration under hyperoxic conditions to lend support to the oxidative damage hypothesis (Table 4.1). However, no significant decrease in O-phase duration (Figure 4.3) or increase in CF-phase duration (Table 4.5) was found in *Paracinema tricolor*. Therefore, the predictions of the oxidative damage hypothesis were not supported here.

Table 4.5. Means and standard errors for the recorded variables for each of the different acclimations (5 kPa O_2; 40 kPa O_2; hydrated; dehydrated) at each of the different treatments (5% relative humidity (RH); 90% RH; 5 kPa O_2; 40 kPa O_2; control).
Mass was a significant covariate in all cases, except for mean O-phase duration and water loss rate. Sample sizes (*n*) and mean mass for each treatment group within an acclimation are also indicated.

				Mean duration (min)±SE			Mean $\dot{V}CO_2$ (ml h^{-1})±SE			CO_2 release volume (ml)± SE	
Acclimation	**Treatment**	***n***	**Mean mass (g)**	**DGC**	**CF-phase**	**O-phase**	**DGC**	**CF-phase**	**O-phase**	**O-phase**	**WLR±SE (mg h^{-1})**
5 kPa O_2	5% RH	10	0.398	65.5±17.0	47.6±12.6	17.1±2.4	0.0346±0.0032	0.0114±0.0019	0.1031±0.0141	0.0291±0.0067	1.549±0.506
	90% RH	7	0.440	49.7±20.4	36.3±15.0	15.8±2.9	0.0382±0.0038	0.0010±0.0022	0.1051±0.0168	0.0250±0.0080	0.240±0.604
	Control	7	0.442	58.2±20.4	46.5±15.0	14.5±2.9	0.0351±0.0038	0.0106±0.0022	0.1252±0.0168	0.0238±0.0080	0.320±0.604
	5 kPa O_2	8	0.368	71.3±19.1	43.2±14.1	24.7±2.7	0.0400±0.0036	0.0138±0.0021	0.0867±0.0157	0.0339±0.0075	0.662±0.565
	40 kPa O_2	7	0.436	38.2±20.4	30.7±15.0	9.5±2.9	0.0385±0.0038	0.0125±0.0022	0.1347±0.0168	0.0191±0.0080	0.340±0.604
40 kPa O_2	5% RH	9	0.492	42.4±18.0	33.5±13.3	15.5±2.5	0.0421±0.0034	0.0103±0.0020	0.1452±0.0148	0.0274±0.0070	2.083±0.533
	90% RH	9	0.466	37.4±18.0	26.2±13.3	15.7±2.5	0.0398±0.0034	0.0098±0.0020	0.1132±0.0148	0.0191±0.0070	-0.162±0.533
	Control	10	0.286	89.0±17.0	65.0±12.6	13.9±2.4	0.0303±0.0032	0.0090±0.0019	0.1056±0.0141	0.0365±0.0067	0.184±0.506
	5 kPa O_2	7	0.436	55.9±20.4	33.2±15.0	24.9±2.9	0.0409±0.0038	0.0164±0.0022	0.0869±0.0168	0.0307±0.0080	0.671±0.604
	40 kPa O_2	8	0.390	58.3±19.1	46.1±14.1	10.6±2.7	0.0289±0.0036	0.0097±0.0021	0.1106±0.0157	0.0218±0.0075	0.451±0.604
Hydrated	5% RH	8	0.487	51.6±19.1	34.7±14.1	17.1±2.7	0.0428±0.0036	0.0138±0.0021	0.1030±0.0157	0.0299±0.0075	1.634±0.565
	90% RH	9	0.439	48.1±18.0	32.0±13.3	16.7±2.5	0.0465±0.0034	0.0122±0.0020	0.1197±0.0148	0.0309±0.0070	2.714±0.533
	Control	4	0.672	64.2±27.0	45.4±19.9	17.8±3.8	0.0352±0.0050	0.0114±0.0030	0.1086±0.0223	0.0348±0.0106	0.606±0.799
	5 kPa O_2	6	0.530	50.0±22.0	24.2±16.2	25.7±3.1	0.0497±0.0041	0.0191±0.0024	0.0716±0.0182	0.0322±0.0086	0.881±0.653
	40 kPa O_2	5	0.448	58.1±24.1	40.6±17.8	18.0±3.4	0.0499±0.0045	0.0128±0.0027	0.1265±0.0199	0.0400±0.0094	1.089±0.715

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	5% RH	3	0.621	56.8±31.1	38.3±23.0	17.8±4.4	0.0435±0.0058	0.0129±0.0034	0.1119±0.0257	0.0335±0.0122	1.360±0.923
	90% RH	7	0.416	53.1±20.4	33.2±15.0	20.6±2.9	0.0497±0.0038	0.0137±0.0022	0.1253±0.0168	0.0366±0.0080	0.253±0.604
Dehydrated	Control	4	0.416	59.8±27.0	36.5±19.9	24.0±3.8	0.0402±0.0050	0.0098±0.0030	0.1031±0.0223	0.0226±0.0106	1.495±0.799
	5 kPa O_2	3	0.465	63.9±31.1	20.2±23.0	44.1±4.4	0.0553±0.0058	0.0145±0.0034	0.0785±0.0257	0.0586±0.0122	0.591±0.923
	40 kPa O_2	7	0.441	46.7±20.4	31.4±15.0	15.8±2.9	0.0503±0.0038	0.0162±0.0022	0.1550±0.0168	0.0327±0.0080	1.316±0.604

DGC, discontinuous gas exchange cycle; CF-phase, closed/flutter phase; O-phase, open phase; $\dot{V}CO_2$, rate of CO_2 release; RH, relative humidity; WLR, water loss rate.
*Mass was a significant covariate in all cases, except for mean O-phase duration and water loss rate.
Where mass was a significant covariate the residual data were used to calculate mean values.

After oxygen regulation, the next major abiotic factor which could influence the presence/absence and modulation of the DGC is a need to conserve respiratory water. This is typically studied under the rubric of the hygric hypothesis, or some derivation thereof (reviewed in e.g. Chown et al., 2011). During a DGC, O_2 consumption and CO_2 production within the animal is a continuous process, while the external exchange of gases between the animal and the atmosphere is discontinuous. While the spiracles are kept closed (CF-phase) there is no exchange of respiratory gases (O_2, CO_2, H_2O) with the atmosphere, thereby resulting in a decrease in respiratory water loss (RWL). Insects may therefore face a trade-off between the need to support aerobic metabolism and the need to conserve water (Woods and Smith, 2010). For the hygric hypothesis to be supported, a decrease in O-phase duration and/or an increase in CF-phase duration need to be present under low RH conditions, and/or when an individual is dehydrated, the latter of which is assumed could gain the most benefit from further reductions in water loss (Table 4.1). In *P. tricolor* there was no significant decrease in O-phase or increase in CF-phase duration under low RH conditions or in dehydrated individuals (Figure 4.3; Table 4.5). If the DGC functions simply to reduce RWL, then a positive relationship should exist between the frequency of DGCs and ambient RH, because in conditions of low RH the C-phase duration should increase to reduce RWL (see e.g. White et al., 2007). However, no significant difference between DGC frequency for the different RH treatments (5, 60 and 90% RH) or acclimation conditions (5, 40 kPa O_2, dehydrated and hydrated) were found. While this prediction makes use of comparative responses to predict mechanistic responses by the organism to a certain environmental variable, this approach may be confounded by short-term responses (White et al., 2007). The acute responses expressed by the organism to a specific environmental condition may be opposite to the evolutionary response (Arendt and Wilson, 1999; Marcil et al., 2006). However, the approach adopted here and the associated inference of mechanistic responses remains an essential step at the experimental level for understanding how organisms function in a given environment.

With no support found for the predictions of either the hygric or oxidative damage hypotheses, I reasoned that neither protecting tissues against oxidative damage nor minimizing respiratory water loss is the main factor driving the DGC in this species. The last factor hypothesized for influencing the presence/absence or modulation of the DGC is haemolymph pH regulation (Table 4.1). Here, I found that dehydrated individuals had a

longer O-phase (although not always significantly so) than individuals from any of the other acclimations at all treatments except the 40 kPa O_2 treatment. For dehydrated individuals, the only treatment that had a significant effect on O-phase duration was 5 kPa O_2, which resulted in a significantly longer O-phase than any of the other acclimations or treatments. For the dehydrated acclimation, the number of individuals that abandoned the DGC was significantly higher than for the other acclimation groups (Table 4.3), supporting the prediction that within dehydrated individuals the DGC will be abandoned to ensure adequate haemolymph pH regulation (Table 4.1). Hadley and Quinlan (Hadley and Quinlan, 1993) also found that the occurrence of the DGC decreases if the Eastern lubber grasshopper (*Romalea guttata*), a relatively mesic species, is dehydrated.

Dehydrating an insect should affect the acid-base status of the insect's haemolymph and lead to a decrease in haemolymph volume (Wharton, 1985). Dehydration, likely primarily through a reduction in available free water, or possibly also through a reduction in capacity to buffer free H^+ ions and more rapid build-up of other ions to toxic levels, could therefore also affect haemolymph pH and its associated buffer capacity and could influence the occurrence and/or modulation of the DGC. Indeed, ion homeostasis is a critical aspect of cell and tissue survival post-dehydration or thermal stress in insects (Hadley, 1994a; Nation, 2002; Chown and Terblanche, 2007; Boardman et al., 2011). The abolition of the DGC or the increase in O-phase duration that I observed could be due to the animal attempting to regulate its haemolymph pH. If the O-phase duration is increased, spiracles are kept open for longer, leading to longer periods in which gases can be exchanged between the tracheal system and the atmosphere. Apart from elevated RWL, keeping the spiracles open for longer can also result in respiratory alkalosis via an excess dumping of CO_2. I therefore propose that the increased O-phase observed under dehydrated conditions is likely a consequence of respiratory acidosis and the need to excrete high levels of CO_2 that have dissolved into the reduced volume of haemolymph.

A similar argument has been made by Woods and Smith (Woods and Smith, 2010) who found that insects have higher transpiration ratios than predicted by their universal model for water costs of gas exchange. They argue that the reason for this is because most of the insects included in their model use cyclic patterns or the DGC. These insects therefore need to

increase their O-phase duration above that which would be necessary solely for sufficient O_2 uptake, as during the C-phase, CO_2 dissolves into the body fluids and needs to be expelled during the O-phase to maintain acid-base homeostasis (Woods and Smith, 2010). Consequently, more respiratory water is lost to the atmosphere owing to the spiracles being kept open for longer periods to achieve pH regulation. Harrison (Harrison, 1989) found that in the American locust the acid-base status of the insect's haemolymph is regulated by ventilation, with a lower haemolymph pH (as would be present in dehydrated individuals, see below) leading to increased ventilation frequencies. Snyder et al. (Snyder et al., 1980) also suggested that pH controls ventilation frequency in the cockroach (*Nauphoeta cinerea*) (and see Matthews and White, 2011a). An increase in ventilation frequency will lead to a decrease in the duration of the different phases of the DGC, and could ultimately lead to the abolition of the DGC altogether. The finding that dehydrated individuals of *P. tricolor* abandon the DGC therefore fails to reject the predictions of haemolymph pH regulation acting as a factor controlling the DGC (Table 4.1). Nonetheless, other factors associated with haemolymph volume reduction may be involved. In addition, a post-hoc assessment of sample size that might affect my conclusions suggested a sample size of approximately 34 individuals per treatment (see results). Bearing this caveat in mind for the weaker effects, the major significant effects detected in this study nevertheless provide substantial insight into insect respiratory and control of the DGC.

With haemolymph pH regulation being the only factor for which the *a priori* predictions were not rejected, the next step is to examine whether dehydrated individuals do indeed have lower haemolymph pH values when compared with hydrated individuals. There was a significant reduction in haemolymph pH in dehydrated versus control individuals (6.6±0.1 and 7.0±0.3, respectively; p=0.018). These values fall well within the range of haemolymph pH values found for other grasshopper and locust species (*Melanoplus bivittatus* pH=7.12 (Harrison, 1988); *Taeniopoda eques* pH=6.98 (Harrison and Kennedy, 1994); *Schistocerca gregaria* pH=7.31 (Harrison et al., 1990)). These results confirm my predictions and suggest that in *P. tricolor* the absence/presence of the DGC, as well as the modulation of different parameters of the DGC when present, are being used to regulate haemolymph pH in dehydrated individuals. The results also indicate that this is a higher priority than the potential importance of reducing respiratory water loss or protecting tissues against oxidative damage. I therefore propose a scheme (outlined in Figure 4.5) for a hierarchy of abiotic stressors

influencing the DGC and its ability to reduce respiratory water loss or oxidative damage. The following stressors may be prioritised by *P. tricolor* in decreasing order of importance: 1) maintain oxygen supply to match cellular demand; 2) excrete CO_2 and avoid excess pH variation; and finally, 3) protect against oxidative damage and save water. The one overarching factor that influences all of the stressors and their relative importance is the morphology of the organism, which will affect for example, the organism's spiracular/tracheal conductance, as well as modes of gas exchange (diffusion *vs* convection, e.g. Wobschall and Hetz, 2004; Duncan et al., 2010; Groenewald et al., 2012). Once the two main priorities of taking up enough oxygen for aerobic metabolism and releasing CO_2 that accumulated due to cellular respiration have been satisfied, only then can the DGC be employed to attempt to regulate any of the subsequent stressors (e.g. energetic cost minimization, oxidative damage, water conservation). For example, I found that for dehydrated individuals, at the treatments where O_2 levels remained at 21 kPa O_2 and only RH levels were altered, an increase in O-phase duration was observed, although not significantly. Because oxygen levels remain at normoxia, priority 1 (maintaining adequate O_2 supply; Figure 4.5) can easily be satisfied and the animal can therefore move on to priority 2 (avoid excess pH variation; Figure 4.5). The increase in O-phase duration could thus be due to the animal trying to regulate its haemolymph pH, as its oxygen demand (the top priority) has already been satisfied. However, dehydrated individuals at the treatment of 5 kPa O_2 need to regulate their spiracular activity to both maintain an adequate O_2 supply to tissues, as well as regulate haemolymph pH levels (priority 1 and 2; see Figure 4.5). Interactions occur between these two stressors (Figure 4.5), and the effects of hypoxia and decreased haemolymph pH could therefore be synergistic, as these two factors together lead to a greater increase in O-phase duration than either did alone. Such a line of reasoning is supported by findings of Matthews and White (Matthews and White, 2011a) who showed that in the cockroach *Nauphoeta cinerea* conditions of hypoxia and hypercapnia are synergistic and lead to an increase in ventilation frequency.

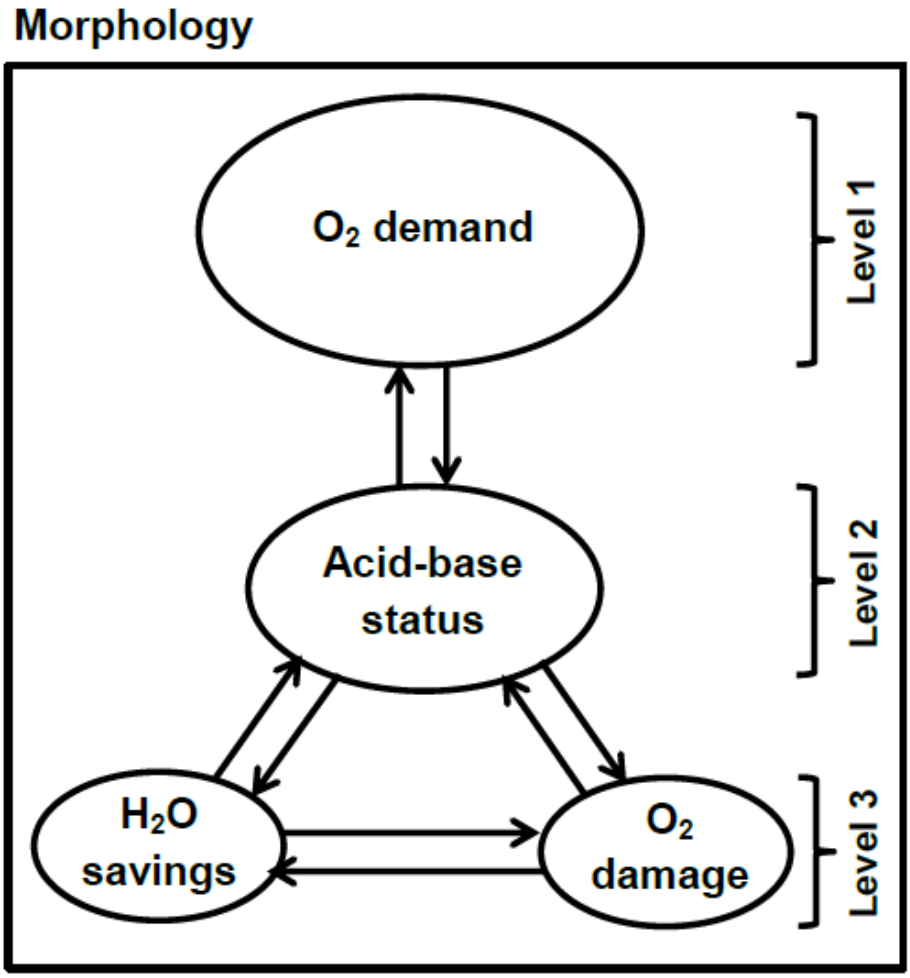

Figure 4.5. Schematic hierarchy of abiotic stressors influencing discontinuous gas exchange responses (see Discussion for detailed explanation).
Different levels indicate stressors prioritized by the insect in decreasing order of importance, with level 1 indicating the stressor that is of highest priority to the animal; level 3 indicates stressors that can only be regulated once priorities from level 1 and 2 have been satisfied. Arrows represent the interactions between the various stressors.

Chapter 5: General discussion

Schwenk et al. (Schwenk et al., 2009) identified five grand challenges which can act as a guideline for future research in organismal biology. The development of new techniques, methods and technologies makes it possible to now investigate long-standing questions that until now have been left unanswered. An important aspect which is highlighted by Schwenk et al. (Schwenk et al., 2009) is the need for collaboration and integration across different fields. The five grand challenges which they identified are:

1) To understand the organism's role in the environment and its responses to changes in the environment.
2) To investigate ecosystem dynamics and functioning, as well as biodiversity.
3) To integrate different research disciplines e.g. engineering, mathematical and physiological principles.
4) To understand organismal development and evolution.
5) To understand organismal behaviour and adaptation "understanding how organisms walk the tightrope between stability and change".

The second chapter of my dissertation addressed the third challenge identified by Schwenk et al. (Schwenk et al., 2009) by integrating the recording of different variables to better the understanding of the mechanisms behind insect gas exchange. In my third chapter I investigated the effect which different conditions have on insect gas exchange dynamics by integrating the recording of different gas exchange variables in different environments (different oxygen partial pressures), thereby addressing the first and third challenges as identified by Schwenk et al. (Schwenk et al., 2009). My fourth chapter investigated the effect which different environmental variables (oxygen, relative humidity) have on insect gas exchange, as well as on the haemolymph pH status of the insect, thereby addressing the first, third and fifth challenges identified by Schwenk et al. (Schwenk et al., 2009). Briefly, the main aims for my dissertation were to investigate the mechanistic basis of gas exchange patterns, to examine the different modes of gas exchange (diffusion *vs* convection), to study the effect of ventilation on haemolymph acid-base status of insects, to test the oxidative damage and hygric hypotheses and to examine prioritisation of factors by the insect within the gas exchange control cascade. To accomplish these aims I used an integrated approach by including multiple physiological as well as biomechanical parameters and measurements (e.g. intratracheal pressure, activity (body movements), CO_2 release, haemolymph pH, water balance).

A major finding in my second chapter is that for the desert locust, *Schistocerca gregaria*, there is no distinct intratracheal pressure pattern that coincides with the discontinuous gas exchange cycle (DGC). This finding differs from studies conducted on moth pupae, which show a distinct intratracheal pressure cycle that coincides with the discontinuous gas exchange pattern (Hetz and Bradley, 2005; Terblanche et al., 2008). I found that the intratracheal pressure of *S. gregaria* is very variable even between individuals (see Figure 2.2 and Table 2.2) and is therefore not a good indicator to distinguish between the different phases of the DGC. I also found that these locusts exhibit huge positive pressure peaks during their interburst phase (see Figure 2.2). This finding is surprising as one would expect intratracheal pressure values to decrease during the interburst phase as oxygen is used by metabolically active tissues and carbon dioxide dissolves into the haemolymph. I hypothesised that these high pressure pulses could be used to mix gases within the closed tracheal system. This mixing would lead to a more efficient distribution of O_2 within the tracheal system, and by doing so could allow the insect to increase its interburst phase. This hypothesis has been investigated by Huang et al. (Huang et al., 2014) for *S. gregaria*. They found that ventilatory movements (body movements) are used to mix gases in the tracheal system, thereby ensuring efficient oxygen transport to tissues during the interburst phase (Huang et al., 2014).

In Chapter 3, I found that for *S. gregaria* there is a switch from active convection to passive diffusion at a certain oxygen partial pressure (Figure 3.5). The method that I used to determine less *vs* more body movements (i.e. the coefficient of variation (CV) of activity) is reliable and robust, as one can clearly see by visible inspection of the data traces (see Appendix 1, FiguresA1-A5). Individuals that display less body movements have lower CV values, while individuals that display more body movements have higher CV values. At oxygen partial pressures below 13 kPa O_2 or above 21 kPa O_2 gases are exchanged mainly through active convection, while at or nearing normoxic levels, diffusion-based gas exchange dominates (Figure 3.5). This suggests that gas exchange is more energy efficient at and approaching normoxic oxygen levels (see Figure 3.6). A potentially important trend can be observed in figure 3.7 regarding the volume of the open (O)-phase and the associated rate of CO_2 release ($\dot{V}CO_2$) under different oxygen partial pressures (see Figure 5.1). This trend shows that, relative to normoxia, there is a shift towards lower O-phase volumes and slightly higher $\dot{V}CO_2$ (i.e. a left and vertical upwards shift in the curve) at the oxygen treatment of 35

kPa, while at the oxygen treatment of 8 kPa, there is a shift towards a lower $\dot{V}CO_2$, relative to the normoxic treatment (i.e. a vertical downwards shift in the curve). This perhaps indicates that it is not the build-up of CO_2 during the flutter (F)-phase that triggers the O-phase, as the volume of CO_2 released during the O-phase decreases for the 35 kPa O_2 treatment, relative to the normoxic treatment. This decrease in the volume of CO_2 released during the O-phase indicates that less CO_2 build-up occurred during the F-phase, therefore less CO_2 needed to be expelled during the O-phase. This brings into question whether the start of the O-phase is triggered due to a build-up of CO_2 during the F-phase, as is predicted by the widely accepted model regarding the triggering of the opening and closing of the spiracles for the different phases of the DGC. This model states that the O-phase is triggered by the build-up of CO_2 during the F-phase to a certain threshold (Förster and Hetz, 2010). However, my results indicate that this is not the case for *S. gregaria* and that the model for spiracle triggering needs to be revisited, at least for this model DGC system. Another finding that is indicated by the schematic (Figure 5.1) is that elevating ambient O_2 conditions does not lead to the inverse effect of decreasing ambient O_2 conditions. If increasing O_2 was simply the inverse of decreasing O_2, then the same, but reversed, trend should have been visible in both the low and high O_2 treatments, i.e. a shift left for high O_2 conditions and a shift right for low O_2 conditions, or, a downward shift for low O_2 conditions and an upward shift for high O_2 conditions, relative to normoxia. This finding indicates that different mechanisms are used by the insect for respiratory gas exchange in low *vs* high O_2 conditions. One possible interpretation of this result is that the limit for gas exchange in low O_2 partial pressures is set by diffusion, while the upper limit for gas exchange in high O_2 partial pressures is set by a different factor, such as maximal tracheal conductance. Several studies have found asymmetrical responses to oxygen e.g. Klok et al. (Klok et al., 2009) found that rearing *Drosophila melanogaster* in hypoxic conditions led to the flies having smaller body sizes, while rearing the flies in hyperoxia did not lead to flies having larger body sizes. Furthermore, Stevens et al. (Stevens et al., 2010) found that for the isopod *Porcellio scaber* and the beetle *Tenebrio molitor* conditions of hypoxia reduce the critical thermal maximum (CT_{max}), while hyperoxic conditions do not necessarily increase the CT_{max}.

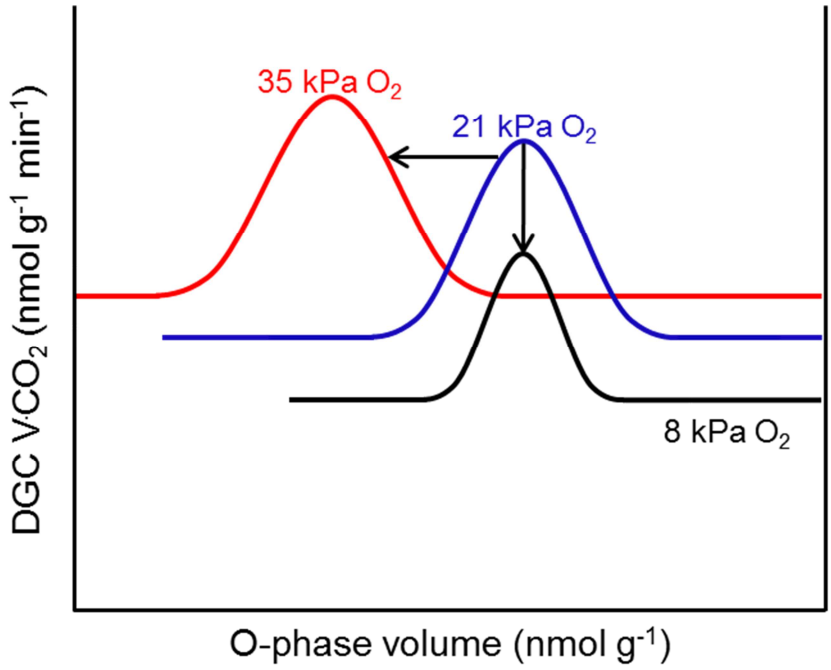

Figure 5.1. Schematic indicating trends regarding the volume of the open (O)-phase and the associated rate of CO_2 release ($\dot{V}CO_2$) during the discontinuous gas exchange cycle (DGC) under different oxygen partial pressures (8, 21 and 35 kPa O_2).

Lastly, in chapter 4, I investigated the prioritisation of abiotic factors which influence the DGC by examining oxygen, water and haemolymph pH regulation in the grasshopper *Paracinema tricolor*. I found that the DGC is significantly less common in dehydrated grasshoppers (Table 4.3), that dehydration leads to a significant decrease in haemolymph pH, and also significantly increases the duration of the O-phase (Figure 4.3). By using the outcomes of this study, I generated a hierarchy of abiotic factors which influence the DGC in *P. tricolor* (Figure 4.5). In this hierarchy the following stressors are prioritised in decreasing order of importance: 1) maintain adequate oxygen supply to tissues; 2) excrete built up CO_2; 3) modulate acid-base status; 4) protect tissues against oxidative damage; and 5) reduce respiratory water loss (Figure 4.5). This hierarchy suggests that the maintenance of the DGC is not due to a single factor, but that multiple factors can play a role in the exhibition of the DGC.

This dissertation examined gas exchange dynamics and characteristics using Orthoptera as a model system. This study showed that gas exchange dynamics of Orthoptera differ substantially from those of the Lepidoptera. This leads to the question whether gas exchange dynamics for other less examined insect orders (e.g. Hymenoptera) adhere to the model for

Orthoptera or Lepidoptera, or if their gas exchange dynamics differ completely from these two insect orders. Gas exchange dynamics and the mechanistic basis of the DGC have mostly been investigated in larger insects, due to the difficulty of undertaking surgical procedures on insects with very small body sizes. This has led to an imbalance in the insect gas exchange literature, with most studies focusing on insects with larger body sizes. Examining insect gas exchange dynamics across a range of different body sizes, as well as for different insect orders, will help to determine the scaling relationship between insect metabolic rate and body size, at both the intra- and interspecific levels. This knowledge will help to better the understanding of energy use of insects of different sizes (Chown and Nicolson, 2004).

The next logical step would therefore be to examine gas exchange dynamics, and more specifically the mechanistic basis of the DGC, for insects with a range of different body sizes, as well as for species from the under-represented insect orders. The simultaneous measurement of intratracheal partial pressure, tracheal partial pressure of O_2 (PO_2), $\dot{V}CO_2$, water loss rates, haemolymph pH, and body movements (activity) in conjunction with synchrotron X-ray imaging (see Westneat et al., 2003) will provide valuable insights into ventilatory mechanisms, the mechanics behind the DGC, as well as testing the different hypotheses explaining the DGC. Also, the direct measurement of insect haemolymph volumes would provide valuable information regarding haemolymph acid-base regulation in insects. The measurement of these variables at a range of different oxygen concentrations will help to elucidate whether acidification of the haemolymph occurs during exposure to certain oxygen concentrations. The most extensively investigated insect orders regarding gas exchange dynamics are the Orthoptera, Lepidoptera (pupae) and Blattodea. Of these orders the Blattodea has mostly lent support to the hygric hypothesis and the Lepidoptera to the oxidative damage hypothesis, while results from Orthoptera are variable. Results from this experiment will help to elucidate whether each insect order adheres to a different model regarding gas exchange dynamics, and then potentially also why different insect orders use different gas exchange mechanisms. These results will also help to determine whether the DGC arose and is maintained for different reasons within different insect orders.

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Appendix 1 – Example traces of CO_2 release and activity for *Schistocerca gregaria*

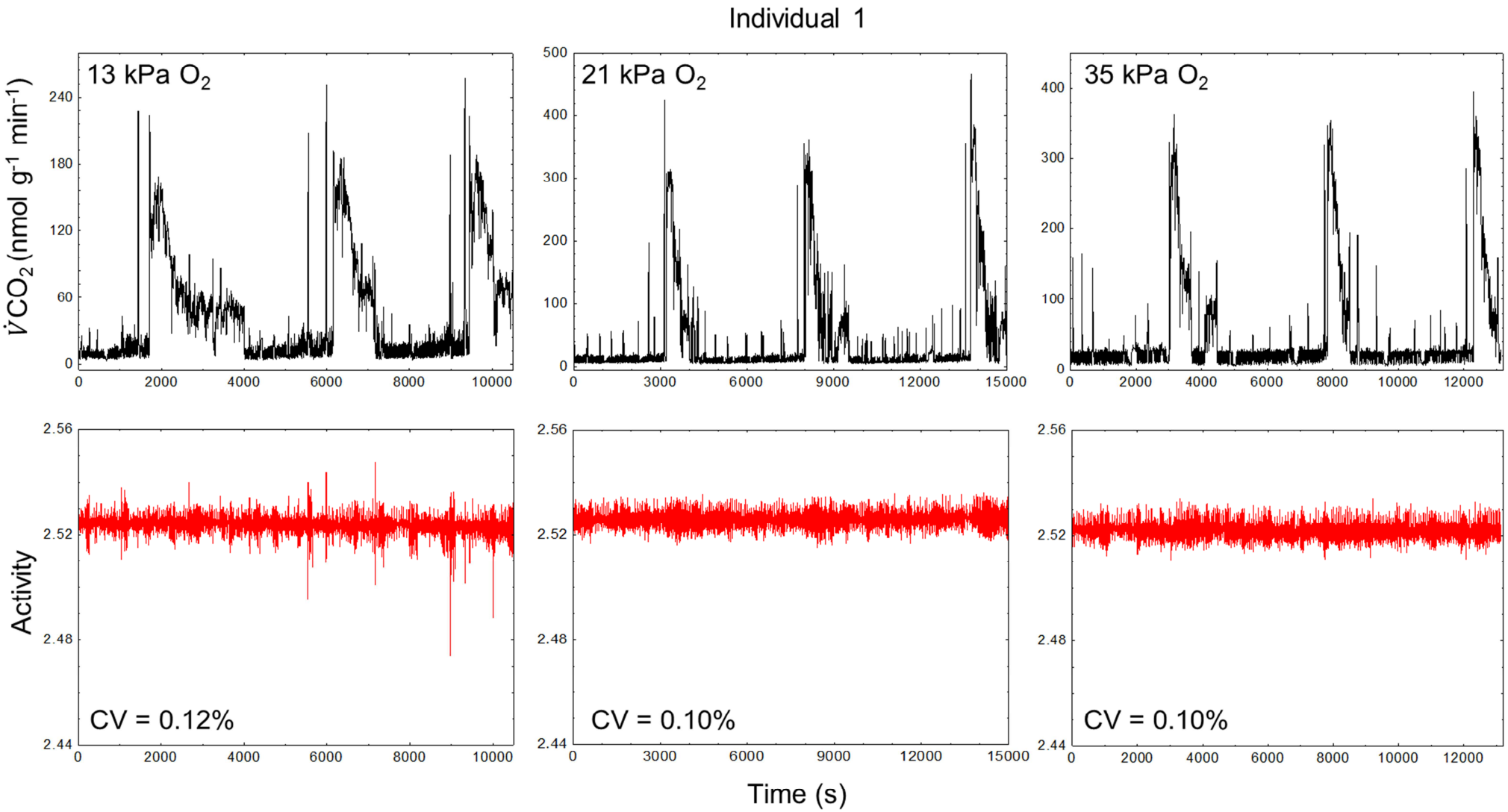

Figure A1. Recordings of CO_2 release rate ($\dot{V}CO_2$, nmol g^{-1} min^{-1}) and body movements (i.e. activity) for a single *Schistocerca gregaria* individual (mass 1.8162 g) at different oxygen treatments (13, 21 and 35 kPa O_2). The coefficient of variation (CV) of activity is indicated on the activity graphs as a percentage.

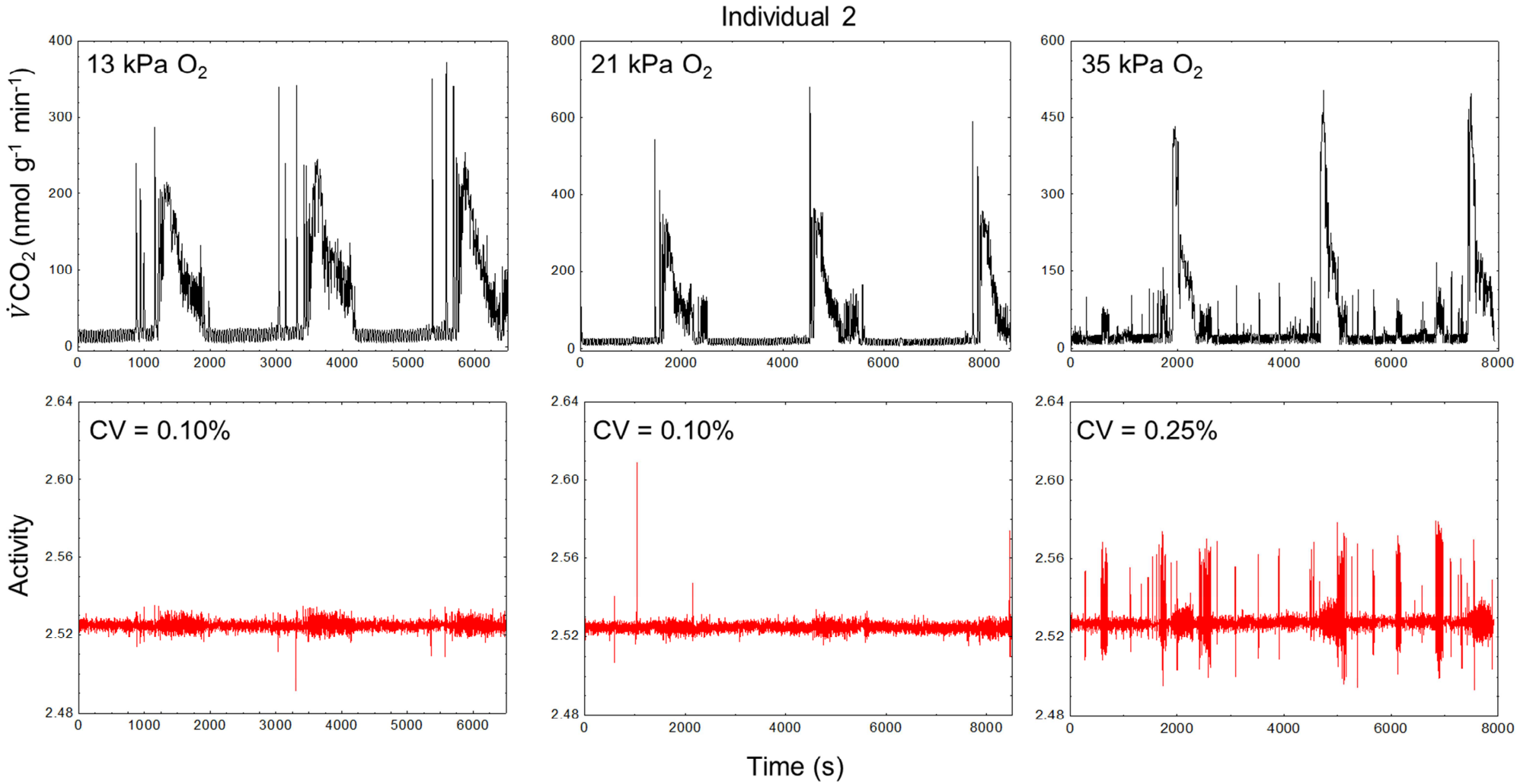

Figure A2. Recordings of CO_2 release rate ($\dot{V}CO_2$, nmol g^{-1} min^{-1}) and body movements (i.e. activity) for a single *Schistocerca gregaria* individual (mass 1.5045 g) at different oxygen treatments (13, 21 and 35 kPa O_2). The coefficient of variation (CV) of activity is indicated on the activity graphs as a percentage.

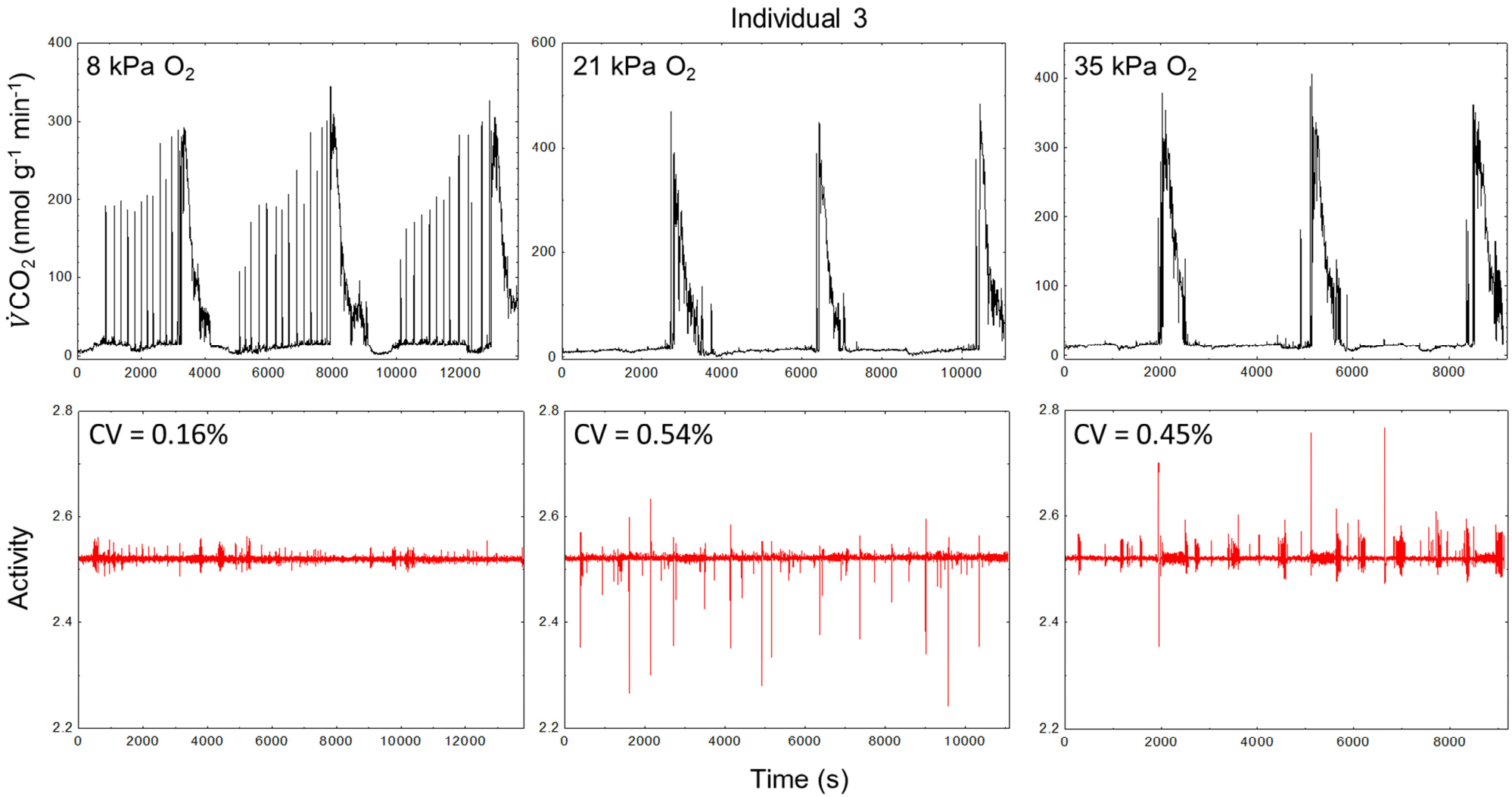

Figure A3. Recordings of CO_2 release rate ($\dot{V}CO_2$, nmol g^{-1} min^{-1}) and body movements (i.e. activity) for a single *Schistocerca gregaria* individual (mass 1.7138 g) at different oxygen treatments (8, 21 and 35 kPa O_2). The coefficient of variation (CV) of activity is indicated on the activity graphs as a percentage.

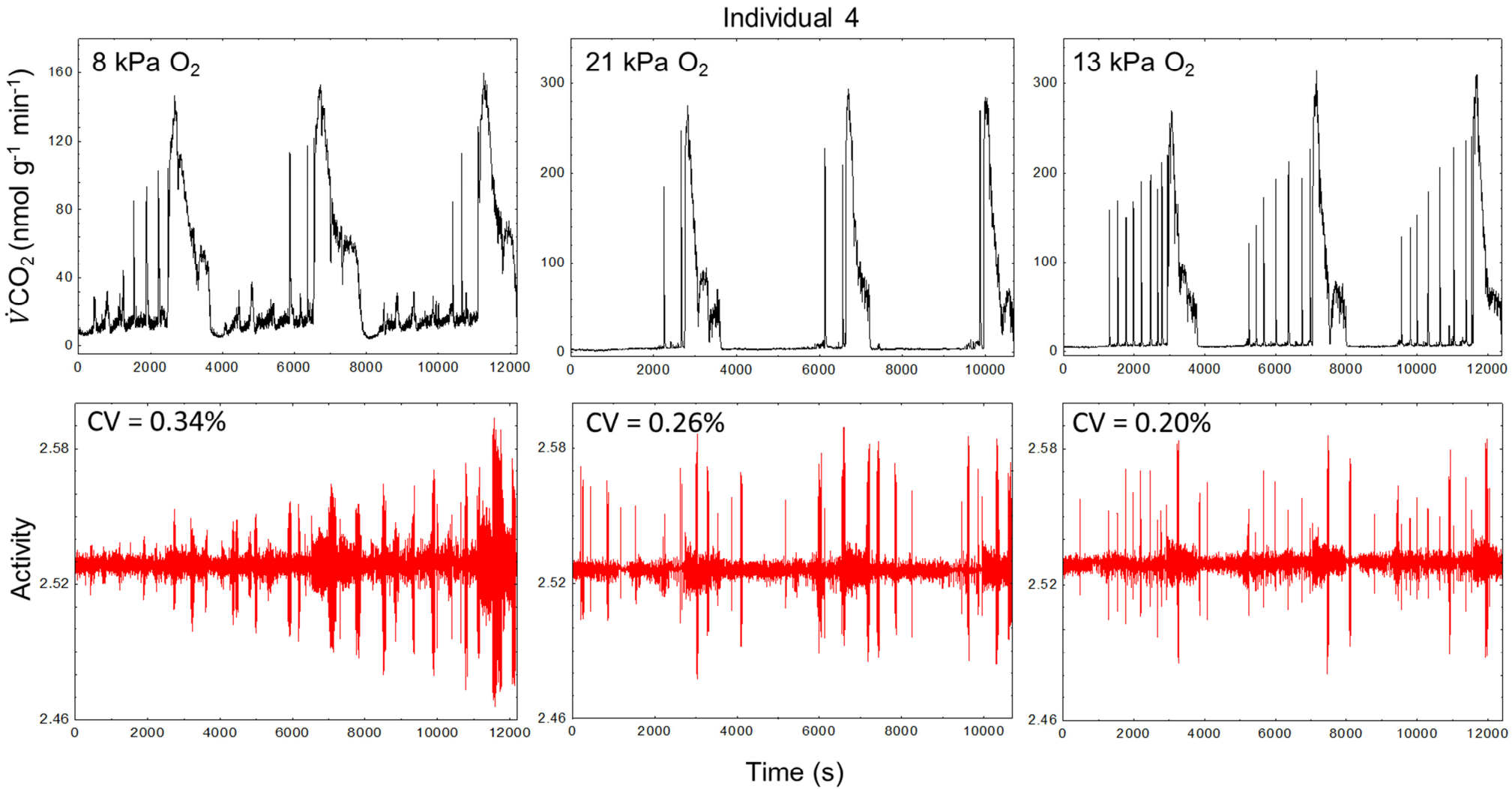

Figure A4. Recordings of CO_2 release rate ($\dot{V}CO_2$, nmol g^{-1} min^{-1}) and body movements (i.e. activity) for a single *Schistocerca gregaria* individual (mass 2.2869 g) at different oxygen treatments (8, 21 and 13 kPa O_2). The coefficient of variation (CV) of activity is indicated on the activity graphs as a percentage.

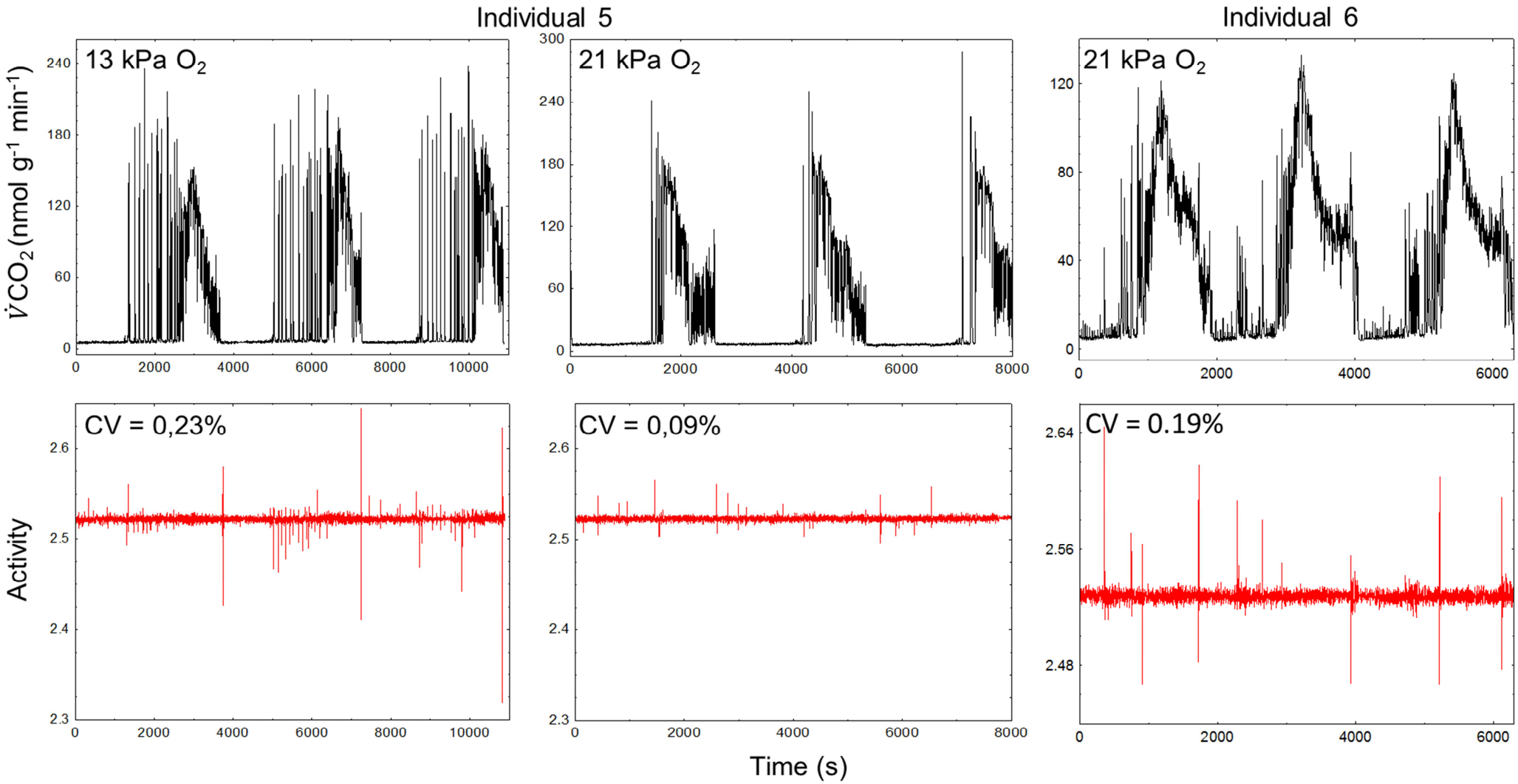

Figure A5. Recordings of CO_2 release rate ($\dot{V}CO_2$, nmol g^{-1} min^{-1}) and body movements (i.e. activity) for two different *Schistocerca gregaria* individual (Individual 5 - mass 1.4529 g; Individual 6 – mass 1.7705g) at different oxygen treatments (13 and 21 kPa O_2). The coefficient of variation (CV) of activity is indicated on the activity graphs as a percentage.

Appendix 2 – Supplementary papers published during the course of doctoral studies in collaboration with other projects

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RESEARCH ARTICLE

Gas exchange patterns and water loss rates in the Table Mountain cockroach, *Aptera fusca* (Blattodea: Blaberidae)

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SUMMARY

The importance of metabolic rate and/or spiracle modulation for saving respiratory water is contentious. One major explanation for gas exchange pattern variation in terrestrial insects is to effect a respiratory water loss (RWL) saving. To test this, we measured the rates of CO_2 and H_2O release ($\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$, respectively) in a previously unstudied, mesic cockroach, *Aptera fusca*, and compared gas exchange and water loss parameters among the major gas exchange patterns (continuous, cyclic, discontinuous gas exchange) at a range of temperatures. Mean $\dot{V}_{CO_2}$, $\dot{V}_{H_2O}$ and $\dot{V}_{H_2O}$ per unit $\dot{V}_{CO_2}$ did not differ among the gas exchange patterns at all temperatures (P>0.09). There was no significant association between temperature and gas exchange pattern type (P=0.63). Percentage of RWL (relative to total water loss) was typically low (9.79±1.84%) and did not differ significantly among gas exchange patterns at 15°C (P=0.26). The method of estimation had a large impact on the percentage of RWL, and of the three techniques investigated (traditional, regression and hyperoxic switch), the traditional method generally performed best. In many respects, *A. fusca* has typical gas exchange for what might be expected from other insects studied to date (e.g. $\dot{V}_{CO_2}$, $\dot{V}_{H_2O}$, RWL and cuticular water loss). However, we found for *A. fusca* that $\dot{V}_{H_2O}$ expressed as a function of metabolic rate was significantly higher than the expected consensus relationship for insects, suggesting it is under considerable pressure to save water. Despite this, we found no consistent evidence supporting the conclusion that transitions in pattern type yield reductions in RWL in this mesic cockroach.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/216/20/3844/DC1

Key words: water conservation, hyperoxic switch method, regression method, metabolic rates, metabolic efficiency.

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INTRODUCTION

Given their small body size and relatively large surface area-to-volume relationships, water balance in terrestrial environments can pose significant challenges for many insects. Most water is lost by passive diffusion to the outside air through the cuticle (i.e. cuticular water loss, CWL) or through the spiracles (respiratory water loss, RWL) while exchanging gas with their environment (reviewed in Hadley, 1994; Benoit and Denlinger, 2010; Chown et al., 2011). However, the contribution of RWL to total water loss may be influenced by gas exchange pattern (e.g. Schimpf et al., 2009; Williams et al., 2010) or metabolic rate modulation (e.g. Terblanche et al., 2010), particularly under xeric conditions (e.g. Gefen, 2011), and may therefore be linked to evolutionary fitness (e.g. Schimpf et al., 2012). Manipulation of environmental conditions (such as temperature, moisture or oxygen availability) or the insect's state (rest *versus* active, dehydrated *versus* hydrated) can have a dramatic influence on the pattern and overall flux rates exhibited (see Lighton and Turner, 2008; Schimpf et al., 2009; Terblanche et al., 2008; Terblanche et al., 2010; Williams et al., 2010; Matthews and White, 2011a; Matthews and White, 2011b).

Insects produce at least three distinct gas exchange patterns at rest: continuous gas exchange (CGE), cyclic gas exchange or burst–interburst, and discontinuous gas exchange (DGE) (e.g. Marais and Chown, 2003; Gibbs and Johnson, 2004; Marais et al., 2005; Contreras and Bradley, 2011). A DGE cycle consists of three phases that can be identified on the basis of spiracular behaviour (Schneiderman and Williams, 1955; Schneiderman, 1960) and their associated CO_2 emission patterns: the open (O) spiracle phase, when gas exchange takes place freely through diffusion [although sometimes aided by active convection (e.g. Loveridge, 1968; Miller, 1973; Groenewald et al., 2012)]; the closed (C) spiracle phase, when there is no exchange of gas between the insect's tracheae and the outside environment; and the flutter (F) phase, when spiracles open and close rapidly and some exchange of gases occurs (reviewed in Lighton, 1996; Chown et al., 2006a; Bradley, 2007; Hetz, 2007).

There are several adaptive and non-adaptive theories that have been proposed to explain the origin and maintenance of DGE in tracheated arthropods (see Chown et al., 2006a; Matthews and White, 2011a). One of the most prominent, and perhaps well supported, is the hygric hypothesis, which states that DGE evolved to reduce RWL by extending the C phase or reducing the O phase (Kestler, 1985; Lighton, 1994; Chown, 2002; Duncan et al., 2002; Chown and Davis, 2003; Chown et al., 2006b; White et al., 2007; Schimpf et al., 2009; Terblanche et al., 2010; Williams et al., 2010; Chown, 2011). However, this notion is not without controversy (see discussions in Chown, 2011; Contreras and Bradley, 2011; Matthews and White, 2011a), at least partly owing to the mechanisms by which RWL and CWL are regulated, their relative contribution to total water loss (TWL) (Chown,

2002; Chown and Davis, 2003; Terblanche et al., 2010) and the fact that many insects abandon DGE under conditions when it is thought to be most useful for water saving [e.g. higher temperatures, dehydration (Quinlan and Hadley, 1993)]. Alternatively, DGE or cyclic patterns are present in some mesic species, such as water striders, which are expected not to be under desiccation stress (Contreras and Bradley, 2011).

Some studies have argued that RWL is a negligible component of TWL (Edney, 1977; Hadley, 1994; but see Chown and Davis, 2003). In the German cockroach, for example, ~95% of water loss occurs through cuticular transpiration, while respiratory transpiration accounts for 3.4–4.4% of TWL (Dingha et al., 2005). However, RWL can be of importance for insects that have evolved under dry conditions, as the amount saved may be the difference between life or death for a xeric species, and indeed, may therefore be the subject of natural selection for pattern variation (Chown and Davis, 2003; Duncan et al., 2002; Gibbs et al., 2003; Benoit and Denlinger, 2007; Schimpf et al., 2012). Furthermore, the regulation of metabolic rate (MR) and gas exchange patterns have considerable impacts on the way in which RWL can be adjusted (Terblanche et al., 2010; Woods and Smith, 2010; Weldon et al., 2013), which in turn may be influenced by life-style factors (e.g. aptery, diapause).

Here, we investigate gas exchange and water loss rates of the Table Mountain cockroach, *Aptera fusca* (Thunberg 1784), a poorly studied mesic insect species. We hypothesize that if DGE is a water saving strategy, *A. fusca* will favour DGE above other gas exchange patterns under desiccating conditions (e.g. high temperatures, low humidity), and that $\dot{V}_{H2O}$ per unit $\dot{V}_{CO2}$ (where $\dot{V}_{H2O}$ and $\dot{V}_{CO2}$ are the rates of H_2O and CO_2 release, respectively) will be lower during DGE than CGE (see Williams et al., 2010). We also expect that the percentage of RWL relative to TWL will be lower during DGE than other gas exchange patterns. Because measurement of RWL for gas exchange patterns other than DGE (i.e. CGE and cyclic gas exchange) is not straightforward, we compare three techniques for partitioning CWL and RWL, and test their repeatability (following Gray and Chown, 2008). Finally, to better comprehend how the study species fits into a broader (global) perspective, we compare our results with those for other insect species.

MATERIALS AND METHODS

Animals

Adult *Aptera fusca* (Blattodea: Blaberidae) females were collected from two localities in the Western Cape, South Africa. Ten individuals were collected from Jonaskop, Villiersdorp (33°58′00″S, 19°30′00″E), and eight individuals were collected from Landdroskop, Hottentots Holland Nature Reserve (34°0′6.48″S, 19°1′18.12″E), in late autumn/early winter. Animal field collection was undertaken under Cape Nature permit number 0056-AAA007-00006. Animals were maintained at a constant temperature of 18±4°C, a relative humidity (RH) of 50–90% and a 10 h:14 h light:dark photoperiod. Temperature and RH conditions were verified with iButton hygrochron temperature/humidity loggers (Maxim/Dallas Semiconductors, Sunnyvale, CA, USA).

Cockroaches from the two different localities were placed in separate glass terrariums, thereby creating two separate colonies. Individuals for each experimental trial were selected at random from the different colonies, as preliminary trials suggested no body mass or resting $\dot{V}_{CO2}$ differences between individuals from the different colonies. The terrariums contained sterilized potting soil, empty egg cartons (to create refugia), as well as restios (Restionaceae) and wood/bark from their natural environment. Animals were fed a diet of mixed nuts (Montagu dried fruit: Mixed nuts raw; Montagu, Western Cape, South Africa) and seeds (Montagu dried fruit: Seed & almond mix), oats, fish food (Tetra goldfish flakes; Melle, Osnabrück, Germany), fresh lettuce and apple slices *ad libitum*. Water was provided as soaked cotton wool and the containers were lightly sprayed with distilled water daily to maintain high humidity levels (~50–90%). Animals were fasted, but allowed access to water, for at least 12 h before respirometry commenced.

Respirometry

Each individual was weighed to 0.1 mg before and after each trial using an electronic microbalance (Model MS104S, Mettler Toledo, Greifensee, Switzerland). $\dot{V}_{H2O}$ and $\dot{V}_{CO2}$ were recorded in a darkened 40 ml cuvette. A short plastic rod was placed in each cuvette for the animal to grasp (simulating their host plants) to reduce activity of the animal during respirometry trials. Individuals were given a period of at least 5 min to settle in the cuvette before recording commenced. When handling the animals, care was taken to minimize contact with the cuticle to avoid accidental abrasion, which could elevate CWL rates (see Johnson et al., 2011). Pilot trials with visual observation or recordings with a custom-built electronic activity detector clearly showed that activity abolished cyclic or DGE patterns, and resulted in the exhibition of CGE.

Flow-through respirometry was undertaken to record $\dot{V}_{CO2}$ and $\dot{V}_{H2O}$. An infrared CO_2/H_2O analyzer (Li-7000, Li-Cor, Lincoln, NE, USA) was set up as follows. For the gas switches (100% O_2 and 100% N_2), a pressurized gas cylinder (Air Products South Africa, Cape Town, South Africa) fed one of the gases into the system. Where normoxic conditions were required, an aquarium pump (AC-9610, Hailea Group, Guangdong, China) was used to feed atmospheric air into the system. The air stream was then fed through a scrubber column containing soda lime (Merck, Gauteng, RSA) and another scrubber containing silica gel/Drierite (ratio 1:1) (Merck, Gauteng, RSA/Sigma-Aldrich, St Louis, MO, USA) to remove CO_2 and water vapour from the air stream. Scrubbed air was then fed through a flow control valve (Model 840, Side-Trak, Sierra Instruments, Monterey, CA, USA) and regulated at a constant flow rate of 200 ml min^{-1} by a mass flow control unit (Sable Systems, MFC-2, Las Vegas, NV, USA). Thereafter, air flowed through the zero channel of the CO_2/H_2O analyzer and through the cuvette containing the cockroach, which was placed into a programmable water bath (Huber CC-410-WL, Peter Huber Kältemaschinenbau, Offenburg, Germany) to regulate the temperature of the cockroach. Air leaving the cuvette then entered the gas analyzer through another channel, which thus recorded the difference in CO_2 and H_2O concentration of the air before and after it flowed through the respirometry cuvette, at 1 s intervals. The output of the analyzer ($\dot{V}_{CO2}$ and $\dot{V}_{H2O}$) was recorded *via* Li-7000 software on a standard desktop computer. Only periods where no activity was visible, based on recordings of movement from the electronic activity detector, were used in analyses. The time constant of the cuvette was determined to be 12 s (40/200×60 s), meaning that it takes 60 s (12×5 s) for 99% of the CO_2 that is released by the animal to be removed from the cuvette and detected by the analyzer (Gray and Bradley, 2006). The shortest C-phase duration that we recorded was 564 s; therefore, we were confident that the different phases of the DGE cycles could be accurately detected.

Gas exchange pattern

Trials were conducted under normoxic conditions, at 0% RH and 15°C. Each individual (N=13) was run in three separate trials (allowing a rest period of 2–5 days between trials) to allow analyses of repeatability. Time of day was randomized among individuals' trials to avoid potential diurnal effects on gas

exchange. Gas exchange patterns were identified based on $\dot{V}_{CO_2}$. Cyclic gas exchange was identified according to methods described in Marais et al. (Marais et al., 2005), namely that if a line is drawn through the middle of the data trace, <30% of the data points should be above the line. If >30% of the data points are above the line, then the pattern would be identified as CGE. Discontinuous gas exchange was identified on the basis of a true C phase being present. The F phase of the DGE cycle could not be identified for all individuals when looking at the $\dot{V}_{CO_2}$ trace. For this reason, and because the F phase may begin before CO_2 release is detected, C and F phases were combined to form the closed/flutter (CF) phase and analyzed as such (e.g. Wobschall and Hetz, 2004; Groenewald et al., 2012).

Hyperoxic switch

Trials were conducted at 0% RH and 15°C. Each trial began with a period of 3–4 h at normoxic conditions. Air was then switched to 100% O_2 (hyperoxic switch) for 30 min, followed by a switch to 100% N_2 (anoxic switch) for another 30 min. Thereafter, the gas was switched to normoxic air. A period of 30 min was gauged as adequate time to ensure maximum spiracle opening and closing to evaluate the highest and lowest steady-state water loss rates, respectively. Time of day was randomized among individuals' trials to avoid potential diurnal effects on gas exchange.

Temperature switches

To investigate the effects of temperature on gas exchange pattern and water loss rate (WLR), individuals (N=10) were subjected to increasing temperatures using a programmable water bath. The same respirometry setup was used as in the hyperoxic switch experiment. Trials were conducted under normoxic conditions at 0% RH, and individuals were exposed to temperatures in the following order: 15, 20 and 25°C for 3 h at each temperature, and 30°C for 2 h. The heating rate between each of the temperatures was 1°C min^{-1}. The temperature range of 15–30°C represents the mean annual to mean maximum annual temperatures that *A. fusca* experience in their natural environment [see table 3 in Clusella-Trullas et al. (Clusella-Trullas et al., 2009)].

Data extraction

Data were extracted using ExpeData data acquisition and analysis software (v. 1.1.25, Sable Systems) and statistical analyses were performed using STATISTICA v. 10 (StatSoft, Tulsa, OK, USA). Data were inspected for outliers, defined as individuals having a mass-specific $\dot{V}_{CO_2}$ that is more than two standard deviations higher or lower than the mean for all animals. No overall outliers were found, although for some tests where subsets of data were used, significant outliers were excluded, and these are stated below. Individuals were assessed for different gas exchange patterns based on their $\dot{V}_{CO_2}$. Where individuals displayed cyclic gas exchange or DGE, data from one to five consecutive cycles per individual were extracted. A DGE cycle was measured from the onset of the C phase until the end of the O phase. Examples of the different gas exchange patterns that were detected are shown in Fig. 1A–C.

Water loss partitioning methods

Three different methods were used to partition CWL from RWL.

Traditional method

In the traditional method, RWL is calculated as the difference between TWL (O phase) and CWL (CF phase) (e.g. Lighton, 1992; Hadley and Quinlan, 1993).

Hyperoxic switch method

In the hyperoxic switch method, RWL is calculated by subtracting CWL [hyperoxic C (hypC) phase] from TWL (anoxic burst). For data extraction from the hypC phase, a period of 2 min was given after the gas switch occurred to ensure that the treatment gas had reached the individual. The hypC phase was defined as a period of at least 30 s during which mean $\dot{V}_{CO_2}$ was less than the mean $\dot{V}_{CO_2}$ preceding the switch (see Fig. 1D for example), as spiracle closure was not always visible. The anoxic burst was taken as the period (>120 s), after the gas switch to anoxia, during which maximum CO_2 release occurred.

Regression method

In the regression method, $\dot{V}_{CO_2}$ is regressed against $\dot{V}_{H_2O}$ (dependent variable), yielding a positive relationship. A period (>25 min) of the data trace was chosen where the individual was at rest and where high variation in CO_2 and H_2O was visible. The y-intercept of the regression line is equivalent to CWL (Gibbs and Johnson, 2004) and RWL is calculated as the difference between TWL and CWL.

Statistical analysis

Gas exchange patterns and water loss partitioning methods

The three different gas exchange patterns (DGE, CGE and cyclic) were compared for the following variables: $\dot{V}_{CO_2}$, $\dot{V}_{H_2O}$, $\dot{V}_{H_2O}$ per unit $\dot{V}_{CO_2}$, slope of regression of $\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$, CWL (i.e. regression method), RWL and percentage RWL (%RWL; relative to TWL) using restricted maximum likelihood (REML) ANOVA. Individual was considered a random effect, while body mass and gas exchange pattern were taken to be fixed effects. The three gas exchange patterns were coded separately except in analyses where the total number of data points was too small to create three separate categories (e.g. temperature experiments), and in analyses on subsets of the data that did not include any instances of cyclic gas exchange.

To detect bias, the three water loss partitioning methods were compared with each other using Friedman's ANOVA with CWL, RWL and %RWL as dependent variables, water loss partitioning method as the repeated effect, and individual as the categorical predictor. Values were compared only for those runs that exhibited DGE and a hypC phase (N=6 trials for 4 individuals). However, the hyperoxic switch method was designed specifically for insects performing CGE (Lighton et al., 2004), so CWL, RWL and %RWL estimates calculated by the hyperoxic switch method were compared among gas exchange patterns using REML ANOVA with individual as a random effect, and body mass and gas exchange pattern as fixed effects (N=16 trials for 8 individuals).

Among- *versus* within-individual variation was assessed by calculating repeatability of mean $\dot{V}_{CO_2}$, mean $\dot{V}_{H_2O}$, anoxic $\dot{V}_{H_2O}$ burst, CWL and RWL from each of the three methods according to the procedure described in Lessells and Boag (Lessells and Boag, 1987). Repeatability, confidence intervals (Krebs, 1999) and standard error (Becker, 1984) were calculated using values obtained from a one-way ANOVA in STATISTICA, with individual as the categorical predictor.

Effect of temperature on gas exchange

A χ^2 contingency table comparing observed with expected values was used to evaluate the association of gas exchange pattern with temperature (taken as a categorical variable). To assess the relationship of $\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$ with a change in temperature, the mean rate and emission volume of CO_2 and H_2O, and the ratio of $\dot{V}_{H_2O}$ per unit $\dot{V}_{CO_2}$ for the total of all phases together, were compared for CGE and DGE/cyclic gas exchange using Mann–Whitney U-

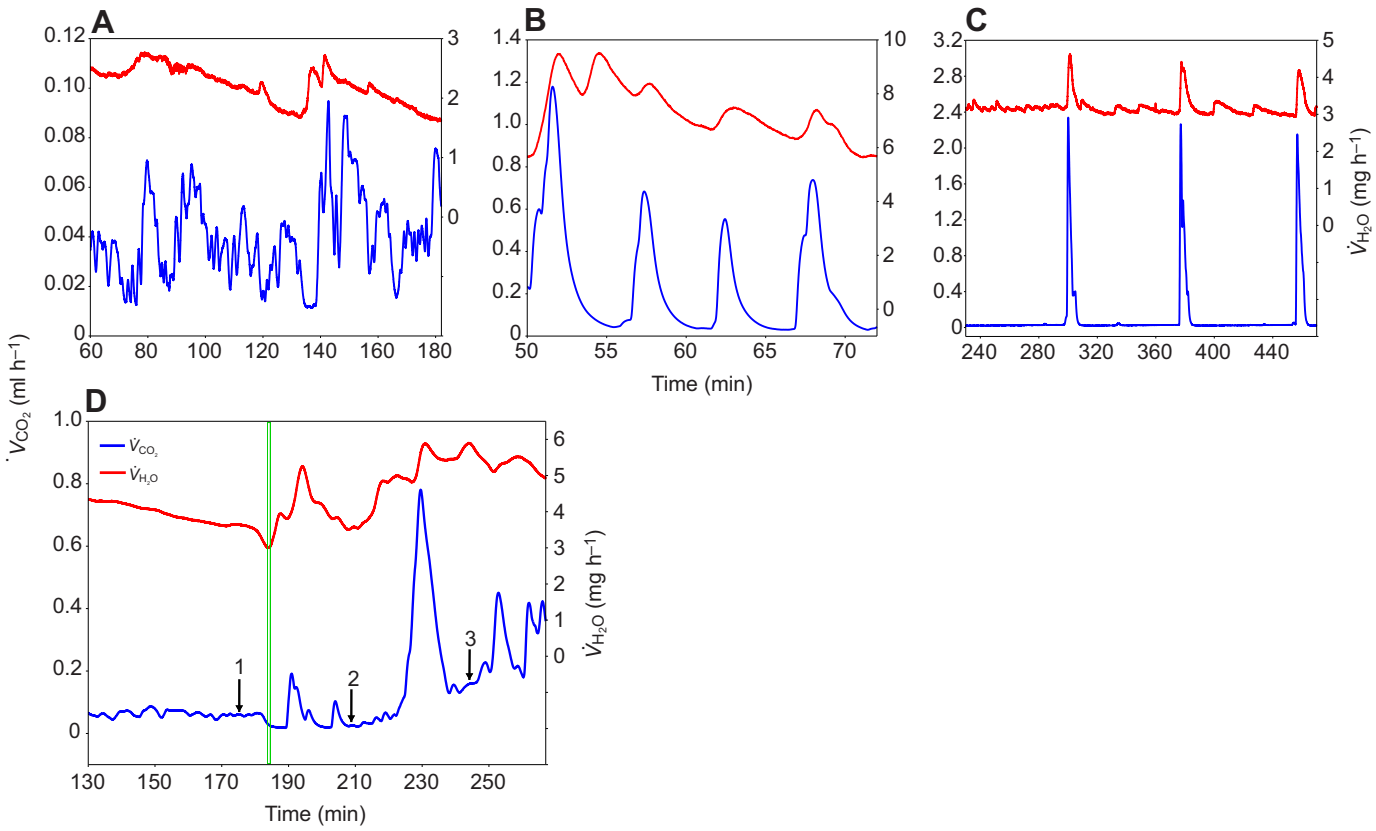

Fig. 1. Representative continuous, cyclic and discontinuous gas exchange patterns for *Aptera fusca*. Measurements were made at 15°C and a flow rate of 200 ml min^{-1}. Examples of the different gas exchange patterns: (A) continuous gas exchange (mass: 1.927 g); (B) cyclic gas exchange (mass: 1.861 g); and (C) discontinuous gas exchange (mass: 1.892 g). For a discussion regarding how the different gas exchange patterns were defined, see Materials and methods. (D) A typical recording of an *A. fusca* individual (mass: 2.179 g) for the hyperoxic switch method for estimation of respiratory water loss. The different gas switches are indicated on the graph: (1) hyperoxic switch (100% O_2); (2) anoxic switch (100% N_2); and (3) switch back to normoxia. A large dip in $\dot{V}_{H_2O}$ (rate of H_2O release) is clearly visible following the switch to hyperoxic gas. A dip in $\dot{V}_{CO_2}$ (rate of CO_2 release) that coincides with the dip in $\dot{V}_{H_2O}$ is also visible. When the gas is switched to nitrogen, a rise in both $\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$ occurs. The period marked with the green bar indicates the part of the data trace that was used for the calculation of respiratory water loss.

tests for each temperature separately (15, 20, 25 and 30°C). Cyclic gas exchange was combined together with DGE for this analysis because there were only three cases of cyclic gas exchange out of 40 trials. Next, Friedman's ANOVAs were performed across the four temperatures to compare mean overall, CF phase and O phase of DGE values for the following variables: duration, $\dot{V}_{CO_2}$, CO_2 emission volume, $\dot{V}_{H2O}$, H_2O emission volume, and $\dot{V}_{H2O}$ per unit $\dot{V}_{CO_2}$. We then performed linear regressions of each gas exchange and water loss variable against temperature. Differences in slopes were compared with a general linear model homogeneity-of-slopes test in STATISTICA. Finally, we performed linear regressions of $\dot{V}_{CO_2}$ with DGE cycle frequency and O-phase CO_2 emission volume to determine whether the observed increase in MR with temperature was due to increased burst frequency or increased emission volume (see Klok and Chown, 2005).

Interspecific comparison

Mean $\dot{V}_{CO_2}$ at 25°C, taken from the temperature experiments (N=9, because one individual was a significant outlier), was converted from ml CO_2 h^{-1} to μW by first converting to units of ml O_2 h^{-1} and then using the oxyjoule conversion factor of (16+5.164×RQ)J ml^{-1} (Lighton et al., 1987), where RQ is the respiratory quotient and is assumed to be 0.84 (Lighton, 2008).

A data set including gas uptake rate (mol O_2 day^{-1}) and WLR (mol H_2O day^{-1}) for 30 insect species was compiled by Woods and Smith (Woods and Smith, 2010). To compare the variation in $\dot{V}_{H2O}$ with gas exchange rate for *A. fusca* with that of the universal model for insects, we added 10 non-independent data points for *A. fusca* to the Woods and Smith (Woods and Smith, 2010) data set: means from gas exchange at 15, 20, 25 and 30°C; mean of pooled individuals showing DGE/cyclic patterns at 15°C; mean of individuals showing CGE at 15°C; maximum steady state rates obtained during the anoxic burst; mean hypC-phase rates; and finally the mean CF- and O-phase rates obtained at 15°C in individuals showing DGE. Although it could be argued that comparison of slopes from intraspecific and interspecific regressions may be of limited value (Heusner, 1991), we reasoned that a reduction in $\dot{V}_{CO_2}$ associated with a transition from CGE to cyclic and/or to DGE, if it were useful for saving water, should result in a decrease in the orthogonal distance to the interspecific relationship, and therefore included a comparison with the intraspecific relationship.

RESULTS

Of the 39 total repetitions, almost two-thirds of all individuals exhibited CGE (62% of trials), while cyclic gas exchange was the rarest pattern (5% of trials; Table 1). Four individuals consistently had the same gas exchange pattern during every trial, and only one of these exhibited DGE consistently. Within the hyperoxic switch trials, almost two-thirds exhibited a hypC phase (63% of trials), and

Table 1. Summary of the number of observations of each gas exchange pattern exhibited by each of the 13 recorded *Aptera fusca* individuals, for each of the trials (three trials per individual)

		Normoxia			Hyperoxic switch		
Individual	Mass (g)	CGE	DGE	Cyclic	Mean cycles	hypC phases	Anoxic bursts
1	2.30±0.09	0	3	0	2.3		
2	1.96±0.07	2	1	0	3.0	3	3
3	1.94±0.04	1	2	0	4.5	3	3
4	1.81±0.07	2	1	0	5.0	2	2
5	2.10±0.09	2	0	1	3.0	3	3
6	2.34±0.07	3	0	0		1	2
7	2.57±0.08	3	0	0			
8	2.09±0.18	2	1	0	2.0	2	3
9	2.37±0.12	1	2	0	3.5	2	2
10	2.52±0.05	3	0	0		1	1
11	2.61±0.08	1	2	0	3.0		
12	2.33±0.09	2	0	1	4.0	0	1
13	2.78±0.17	2	1	0	3.0		
Total		24 (62%)	13 (33%)	2 (5%)		17 (63%)	20 (74%)

The mean number of cycles (where cyclic gas exchange was present), as well as the number of hypC-phases and anoxic bursts that were observed during the hyperoxic switch, are also indicated. Mass data are means ± s.e.m.
CGE, continuous gas exchange; DGE, discontinuous gas exchange; hypC phase, hyperoxic C phase.

in 74% of the trials an anoxic burst was visible in the H_2O trace (Table 1).

None of the measured metabolic or water loss variables differed significantly across gas exchange pattern types when measured over 22 h at 15°C (Table 2). CWL, RWL and %RWL estimates differed significantly among water loss partitioning methods when compared only among individuals that performed DGE and had a hypC phase (Table 2). CWL, as estimated by the hyperoxic switch method, was significantly lower than when measured by the other two methods (χ^2=9.00, d.f.=2, P=0.011). RWL and %RWL were significantly higher when calculated by the hyperoxic switch method than when calculated with the other two methods (χ^2=9.00, d.f.=2, P=0.011 and χ^2=9.00, d.f.=2, P=0.011, respectively). RWL as measured by the hyperoxic switch method was significantly higher in individuals that performed DGE prior to the gas switch than those that performed CGE (REML ANOVA, F=7.63, d.f.=1, 6, P=0.033). Repeatability was low across the tested range of criteria, except for the anoxic H_2O burst, which was significantly highly repeatable (F=39.28, d.f.=8, 11, P<0.0001; Table 3).

Temperature effects on $\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$

There was no significant association between gas exchange pattern and temperature (χ^2=4.31, d.f.=6, P=0.63). $\dot{V}_{CO_2}$, $\dot{V}_{H_2O}$ and $\dot{V}_{H_2O}$ per unit $\dot{V}_{CO_2}$ did not differ significantly among CGE and DGE/cyclic gas exchange at any of the four temperatures. However, at 30°C, CO_2 emission volumes were significantly higher during CGE than during DGE (Table 4). Mean $\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$ increased significantly with an increase in temperature (Fig. 2A, supplementary material Table S1), while $\dot{V}_{H_2O}$ per unit $\dot{V}_{CO_2}$ significantly decreased with increasing temperature. DGE CF-phase $\dot{V}_{CO_2}$ was significantly higher at 25°C than at the other temperatures used in the trials (Fig. 2B, supplementary material Table S1). DGE CF-phase CO_2 emission volume significantly decreased with increasing temperature (Fig. 2B). Closed/flutter and O-phase duration decreased significantly, although at different rates, with an increase in temperature (homogeneity-of-slopes test: F=18.56, d.f.=1, P<0.001; Fig. 3, supplementary material Table S2).

In regression analysis we found a significant positive relationship between trial temperature and log $\dot{V}_{CO_2}$, and a significant negative

Table 2. Comparison of gas exchange and water loss variables among different gas exchange pattern types and methods of partitioning water loss in *A. fusca*

	Gas exchange pattern[a]				Water loss partitioning method[b]				Hyperoxic switch method[c]		
	DGE	CGE	Cyclic	P	Traditional	Hyperoxic switch	Regression	P	DGE	CGE	P
$\dot{V}_{CO_2}$ (ml h^{-1})	0.09±0.01	0.12±0.01	0.08±0.03	0.084							
$\dot{V}_{H_2O}$ (mg h^{-1})	2.94±0.43	3.06±0.45	2.46±0.64	0.498							
$\dot{V}_{H_2O}$ per unit $\dot{V}_{CO_2}$	32.64±3.82	24.95±2.48	36.94±19.83	0.129							
Slope	1.62±0.61	1.75±0.28	1.85±1.58	0.892							
CWL $\dot{V}_{H_2O}$ (mg h^{-1})	2.72±0.36	2.80±0.42	2.10±0.49	0.321	6.29±0.97	2.47±1.20	6.06±1.08	**0.011**	2.47±1.20	2.66±0.49	0.873
RWL $\dot{V}_{H_2O}$ (mg h^{-1})	0.22±0.10	0.26±0.05	0.36±0.14	0.767	0.15±0.11	3.74±0.47	0.38±0.22	**0.011**	3.74±0.47	2.06±0.36	**0.033**
%RWL of TWL	6.49±1.97	8.91±1.34	13.98±2.21	0.255	1.71±1.03	67.81±13.08	6.14±3.50	**0.011**	67.81±13.08	45.67±7.19	0.204

Trials were run at 15°C (values are means ± s.e.m.). DGE, discontinuous gas exchange; CGE, continuous gas exchange; $\dot{V}_{CO_2}$, rate of CO_2 release; $\dot{V}_{H_2O}$, rate of H_2O release; CWL, cuticular water loss; RWL, respiratory water loss; TWL, total water loss. Statistically significant differences are highlighted in bold.
[a]N=39 trials on 13 cockroaches; P-values are from REML ANOVA. CWL and RWL were calculated using the regression method as this method is applicable to all gas exchange patterns.
[b]N=6 trials on 4 cockroaches; P-values are from Friedman's ANOVA. Only experimental trials that exhibited both DGE and a hyperoxic C phase were utilized for this analysis.
[c]N=16 trials on 8 cockroaches; P-values are from REML ANOVA. All experimental trials that exhibited a true hyperoxic C phase were utilized for this analysis, regardless of gas exchange pattern, but there were no instances of cyclic gas exchange which fit these criteria.

Table 3. Repeatability values for mean $\dot{V}_{CO_2}$, mean $\dot{V}_{H_2O}$, CWL, RWL, anoxic burst, and slope of the regression method

	F-ratio	d.f.	Repeatability	Lower CI	Upper CI	n_o
Mean $\dot{V}_{CO_2}$	1.71	12, 26	0.191±0.10	–0.12	0.58	3.0
Mean $\dot{V}_{H_2O}$	2.01	12, 26	0.252±0.11	–0.07	0.63	3.0
CWL						
Traditional method	1.72	7, 5	0.312±0.45	–0.90	0.84	1.6
Hyperoxic method	1.23	7, 9	0.101±0.35	–0.51	0.70	2.1
Regression method	2.03	12, 26	0.256±0.19	–0.07	0.63	3.0
RWL						
Traditional method	2.32	7, 5	0.454±0.38	–0.72	0.88	1.6
Hyperoxic method	1.16	7, 9	0.071±0.36	–0.53	0.69	2.1
Regression method	1.08	12, 26	0.025±0.17	–0.23	0.43	3.0
Anoxic H_2O burst	**39.28***	8, 11	**0.946±0.04**	**0.82**	**0.99**	**2.2**
Slope (regression method)	0.98	12, 26	–0.008±0.17	–0.25	0.39	3.0

Cuticular water loss (CWL) and respiratory water loss (RWL) were calculated by using the three different methods.
n_o is a weighted mean representing the number of data points used per individual in the ANOVA out of a maximum of three. If all three experimental runs were used in analysis for all individuals (balanced design), then n_o=3 (Lessells and Boag, 1987).
*P<0.0001. Statistically significant repeatability is highlighted in bold. $\dot{V}_{CO_2}$, rate of CO_2 release; $\dot{V}_{H_2O}$, rate of H_2O release.

relationship between trial temperature and log $\dot{V}_{H_2O}$ per unit $\dot{V}_{CO_2}$ (supplementary material Table S2). Additionally, frequency of DGE cycles, CF-phase $\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$, and O-phase $\dot{V}_{H_2O}$ all increased significantly with elevated temperature (supplementary material Table S2). $\dot{V}_{CO_2}$ was positively and significantly correlated with frequency, but not with O-phase CO_2 emission volume. This indicates that increased $\dot{V}_{CO_2}$ with increased temperature is a function of increased frequency of bursts, and not increased burst volume (supplementary material Table S2).

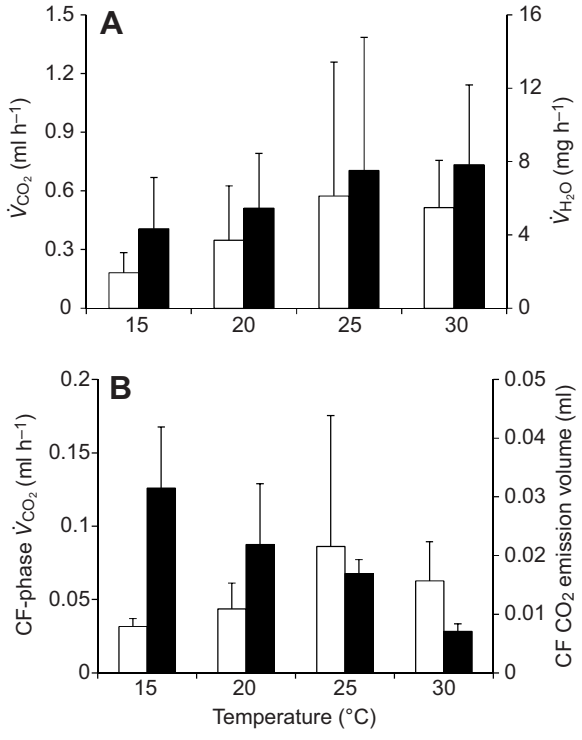

Fig. 2. Results of Friedman's ANOVA to compare means of $\dot{V}_{CO_2}$, $\dot{V}_{H_2O}$ and closed/flutter (CF)-phase CO_2 emission volume among temperature trials (A) averaged across several discontinuous gas exchange (DGE) cycles, and (B) averaged for a number of CF phases of the DGE cycle. Open bars correspond to the left *y*-axis, and solid bars correspond to the right *y*-axis. Data are means ± s.d.

Relationship of water loss to metabolic rate

Mean mass-specific MR for *A. fusca* at 25°C was 144.017 µl CO_2 g^{-1} h^{-1}, and observed WLR was 5.085 mg h^{-1} (data from temperature switch trials). The ratio of log MR to log WLR was not significantly different between DGE and CGE (mean ± s.e.m.=1.62±0.02, 1.57±0.03, respectively; one-way ANOVA, F=0.55, d.f.=2, P>0.05). WLRs of *A. fusca* were higher than those expected based on MR of any of the other insect species included in the Woods and Smith (Woods and Smith, 2010) data set. Further, most WLR values that were measured under a range of experimental conditions and states for *A. fusca* fall outside of the 95% prediction interval of the WLR–gas-uptake-rate relationship for all insect species. Only three measured WLR values (obtained from maximum $\dot{V}_{CO_2}$ during anoxic burst, mean O-phase $\dot{V}_{CO_2}$ at 15°C and mean $\dot{V}_{CO_2}$ at 25°C) fell within the 95% prediction interval (Fig. 4).

DISCUSSION

From this study we have established a basic understanding of gas exchange variation and concomitant water loss for *A. fusca*. In addition to adding new data to a growing global data set of gas exchange characteristics of diverse terrestrial organisms (see White et al., 2007; Terblanche et al., 2008; Woods and Smith, 2010), this baseline understanding allows us to contribute to the debate

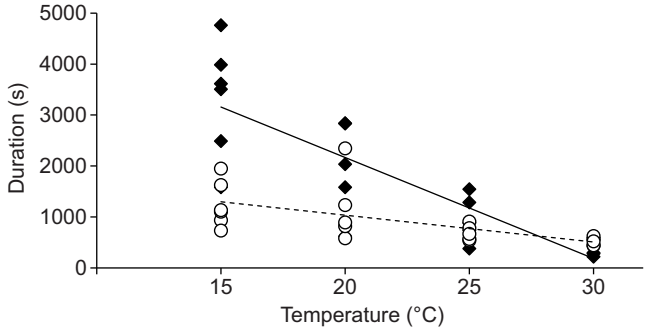

Fig. 3. Linear regressions of closed/flutter (CF)-phase duration (filled diamonds, solid line) and open (O)-phase duration (open circles, dashed line) with temperature. Regression equation for CF phase duration: y=–198.48x+6136.308; r^2=0.699; P<0.0001. Regression equation for O phase duration: y=–52.689x+2087.951; r^2=0.369; P<0.0035.

Table 4. Comparison of $\dot{V}_{CO_2}$, $\dot{V}_{H_2O}$ and emission volume at different temperatures within different gas exchange patterns (results from Mann–Whitney *U*-test)

Temperature (°C)	$\dot{V}_{CO_2}$ (ml h^{-1})				$\dot{V}_{H_2O}$ (mg h^{-1})				$\dot{V}_{H_2O}$ per unit $\dot{V}_{CO_2}$				CO_2 emission volume (ml)				H_2O emission volume (mg)			
	CGE	*N*	DGE*	*N*	CGE	*N*	DGE*	*N*	CGE	*N*	DGE*	*N*	CGE	*N*	DGE*	*N*	CGE	*N*	DGE*	*N*
15	0.22±0.07	4	0.15±0.02	6	5.65±1.75	4	3.15±1.00	4	27.01±2.48	4	25.60±8.28	4	0.29±0.07	4	0.19±0.03	6	7.49±1.44	4	3.90±1.32	4
20	0.26±0.07	5	0.40±0.16	5	5.21±1.52	5	6.71±1.98	3	19.14±3.10	5	24.85±11.01	3	0.47±0.20	5	0.27±0.06	5	8.97±3.69	5	5.91±1.78	3
25	0.29±0.11	3	0.69±0.30	7	3.64±1.50	3	9.62±3.85	5	12.39±3.97	3	16.55±4.39	5	0.74±0.42	3	0.35±0.10	7	8.15±5.27	3	5.48±1.07	5
30	0.41±0.09	4	0.58±0.11	6	5.34±0.90	4	10.30±2.47	4	13.82±2.20	4	21.67±4.44	4	**0.81±0.17**	4	**0.20±0.09**	6	10.54±1.71	4	4.06±1.75	4

Values are means ± s.e.m. Bold type indicates significant difference at the 95% level (P=0.04).
*DGE includes DGE and cyclic gas exchange patterns. $\dot{V}_{CO_2}$, rate of CO_2 release; $\dot{V}_{H_2O}$, rate of H_2O release; CGE, continuous gas exchange; DGE, discontinuous gas exchange.

regarding the evolutionary origins and potential benefits of gas exchange pattern variation, and in particular DGE, in the context of water savings.

Gas exchange pattern

For *A. fusca*, gas exchange pattern is highly variable across individuals, and even over time within a single individual. Only four out of the total of 13 recorded individuals showed the same gas exchange pattern during every trial (Table 1). This variability in pattern type within and between individuals has also been observed in other cockroach species (Miller, 1973; Marais and Chown, 2003; Gray and Chown, 2008; but see Schimpf et al., 2012). Of the total of 39 trials that were run, DGE was exhibited in 33% of the trials, CGE in 62% and cyclic gas exchange in 5% (Table 1).

Comparing methods for partitioning water loss

Calculating CWL using the traditional method is precise (Chown et al., 2006a; Gray and Chown, 2008) and can be used as a benchmark to compare the precision of the newer, alternative methods. For the hyperoxic switch method, all variables estimated had low repeatability, except for the anoxic burst. A true hypC phase and anoxic burst occurred in 63 and 74% of the runs, respectively, although complete spiracle opening and closure were still not achieved in all runs. This may indicate an overall insensitivity of the species to oxygen variation, which may be of further interest itself in light of the oxidative damage hypothesis of DGE (e.g. Hetz and Bradley, 2005; Boardman et al., 2012; Matthews et al., 2012).

Gray and Chown (Gray and Chown, 2008) found the regression method to be the least accurate and obtained negative values for RWL in some cases. We found that the results obtained from this method were highly reliant upon appropriate data selection. Once the data were suitably extracted, however, when comparing the absolute and percentage values of CWL and RWL obtained across the three different methods, the regression method resulted in values similar to those of the traditional method. The values obtained from the hyperoxic switch method were higher, although not always significantly so, for RWL and %RWL (relative to TWL), and significantly lower for CWL. In the case of %RWL, the estimate obtained from the hyperoxic switch method was very different from that of the traditional and regression methods. This result is largely in keeping with other studies. For example, Gray and Chown (Gray and Chown, 2008) found that the hyperoxic switch method yielded significantly higher estimates for %RWL than the other two methods. However, the difference in their estimates was smaller than that found in this study [present study: 45–67% RWL in hyperoxic switch *versus* 2–14% RWL in traditional and regression methods, respectively; Gray and Chown (Gray and Chown, 2008): 21% RWL in hyperoxic switch *versus* 13–14% RWL in traditional and regression methods, respectively] and may be a consequence of the fact that the hyperoxic switch method was developed for estimation of RWL on continuous, rather than discontinuous, gas exchange patterns (Lighton et al., 2004). Mellanby (Mellanby, 1934) found that if an insect's spiracles are forced open (because of anoxia or hypercapnia), WLR increases at a rate that is independent of MR. This increased water loss because of forced spiracular opening in our trials could therefore have contributed to the different values that were obtained by the hyperoxic switch method in comparison with values obtained from the other two methods. This indicates the need for additional comparisons of these three methods under varying experimental conditions and in different taxa, although

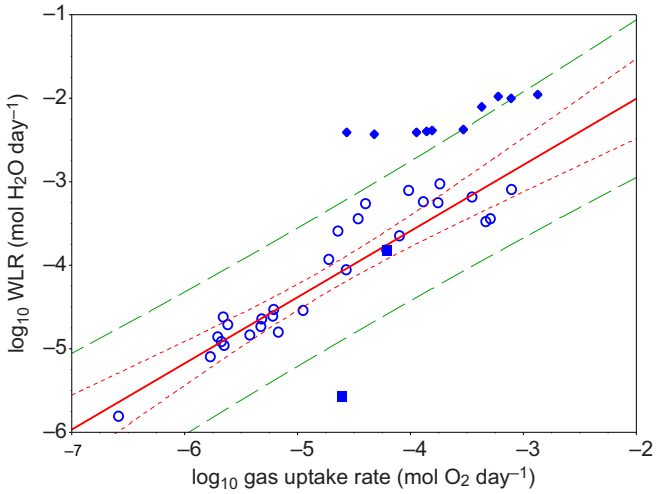

Fig. 4. Water loss rate (WLR) as a function of oxygen uptake rate (MR, metabolic rate) for 31 species of insects. Results obtained from our study for *Aptera fusca* are included with data for 30 species obtained from Woods and Smith (Woods and Smith, 2010). Regression equation for all insects (N=30): logWLR=0.792×logMR–0.421 (indicated by solid line; red stippled lines indicate 95% confidence intervals; green dashed lines indicate 95% prediction intervals). Open circles indicate values for 28 non-cockroach insect species and solid squares indicate values for two cockroach species from Woods and Smith (Woods and Smith, 2010). Solid diamonds indicate values obtained in this study for *A. fusca* under a range of experimental conditions and states measured in our experiments (see Materials and methods for details).

clearly gas exchange pattern type and the use of forced spiracular opening may be major factors influencing the outcomes of the three methods.

DGE as a water saving strategy

There was no significant difference in any gas exchange or water loss variables among the three gas exchange patterns at 15°C, suggesting limited support for pattern variation being associated with a RWL reduction (cf. Williams et al., 2010). At 15°C, we found %RWL (relative to TWL) to be 6.5% for DGE, 8.9% for CGE, and 14.0% for cyclic gas exchange, although this variation was not significant and did not follow the expected rank order (DGE <cyclic <CGE). The factors underlying this lack of difference are unclear. On the one hand, this may be a consequence of unusually high cuticular WLR. The cuticular permeability for *A. fusca* was calculated as being 12.3 $\mu g\,h^{-1}\,cm^{-2}\,Torr^{-1}$. Compared with the cuticular permeabilities for other mesic [e.g. *Periplaneta americana*, 55 $\mu g\,h^{-1}\,cm^{-2}\,Torr^{-1}$; *P. fulginosa*, 57; *Diploptera punctata*, 20.9; *Pycnoscelus surinamensis*, 38.7; *Blatta orientalis*, 48 (Edney, 1977; Hadley, 1994)] and xeric cockroach species [e.g. *Arenivaga investigata*, 30 $\mu g\,h^{-1}\,cm^{-2}\,Torr^{-1}$; *Arenivaga apache*, 80.6 (Edney, 1977; Hadley, 1994)] this value is relatively low, although these measurements from the literature were made under a range of different, typically hotter, conditions. However, cuticular permeability estimated for *Blattella germanica* [3.42 $\mu g\,h^{-1}\,cm^{-2}\,Torr^{-1}$ (Dingha et al., 2005)] and *Perisphaeria* sp. [3.88 $\mu g\,h^{-1}\,cm^{-2}\,Torr^{-1}$ (Gray and Chown, 2008)] is much lower.

On the other hand, the species may have an unusually high MR relative to what might be expected for its size. However, this was also unlikely to be the case for *A. fusca*. On a mass-specific basis, MR for *A. fusca* is similar to that of another cockroach, *Perisphaeria* sp., from South Africa [MR for all gas exchange patterns: *Perisphaeria*, 73.9 $\mu l\,CO_2\,g^{-1}\,h^{-1}$ *versus A. fusca*, 107.1 $\mu l\,CO_2\,g^{-1}\,h^{-1}$ (our data from temperature switch trials at 20°C)] (Marais and Chown, 2003). The temperature coefficient (Q_{10}) for MR of this species is 2.1, which is also close to the assumed consensus value of 2.0 (Lighton, 2008), and influenced all pattern types similarly (supplementary material Table S1). For DGE, we found that this increase was due to an increase in cycle frequency and not an increase in burst volume, as was found in weevils (Klok and Chown, 2005) and grasshoppers (Chappell et al., 2009). Mean $\dot{V}_{H_2O}$ increased with higher temperature for DGE, but not for CGE (Table 4). At 30°C, however, the emission volume of H_2O was higher during CGE than during DGE, although not significantly so (P=0.06; Table 4). It may be argued that this outcome lends some support to the hygric hypothesis, as water loss is expected to be lower during DGE than during other gas exchange patterns, especially under more desiccating conditions (higher temperatures/lower humidity) (Schimpf et al., 2009). However, given the mesic habitat occupied by the species, one would have expected a shift between pattern types at all temperatures, and an overall reduction in $\dot{V}_{H_2O}$ wherever possible, especially if atmospheric moisture was low, as was the case in all our trials.

Although individual phases of the DGE cycle may in some cases be altered for improved water conservation (e.g. longer C and shorter O phases, Terblanche et al., 2010) or to ensure oxygen supply meets demand (e.g. Contreras and Bradley, 2011), DGE may have originally evolved in association with reduced energy consumption states (Lighton, 1996; Matthews and White, 2011a; see Chown, 2011). Indeed, low MR is characteristic of DGE relative to other patterns (Marais and Chown, 2003; Gibbs and Johnson, 2004), and is typically observed in quiescent individuals only. It is therefore possible that DGE arises at low MR, and once the central nervous system relinquishes control to the pattern generators in the peripheral nervous system (Matthews and White, 2011a) in order to save energy. It is also possible that the interaction between P_{O_2} and P_{CO_2} set-points determine the opening and closing of the spiracles (Chown, 2011). In *A. fusca*, as temperature increased both CF- and O-phase duration decreased significantly, while burst frequency increased (supplementary material Tables S1, S2). This alteration in phase duration could be due to the increased metabolic demand (due to increasing temperature), leading to modified spiracular opening/closing, and simultaneously avoiding acidic pH accumulation in the haemolymph.

On the basis of the hygric cost of gas exchange (Woods and Smith, 2010), however, *A. fusca* is unusual because it shows a high WLR per unit energy consumption (expressed as mol O_2 uptake) compared with the mean predicted for other insects, which in turn is higher than the universal model prediction for all organisms. This suggests that *A. fusca* loses more water than expected for its MR, indicating that it is likely under considerable pressure to conserve water, especially if exposed to xeric conditions. It is well established that insects can sense changes in their environment (e.g. variation in atmospheric RH and temperature), and may respond by means of a range of behavioural and physiological changes (reviewed in Chown et al., 2011). For *A. fusca*, however, changes in the pattern of gas exchange do not seem to be one of these responses, as desiccating conditions did not result in the increased presence of DGE, nor an alteration in gas exchange pattern more generally. One potential reason why *A. fusca* does not respond to low RH by altering its gas exchange pattern might be because, when compared with other insects or cockroach species, the relationship of WLR to MR for *A. fusca* does not differ with gas exchange pattern, a result that is inconsistent with several other studies that suggest DGE is useful

for conserving water (e.g. Chown and Davis, 2003; Schimpf et al., 2009; Williams et al., 2010). This observation is further supported by the fact that depression of $\dot{V}_{CO_2}$ (evidenced by our temperature or gas switching experiments) (Fig. 4) does not allow *A. fusca* to reduce its WLR in a manner that brings it closer to the interspecific relationship of the universal model (i.e. the orthogonal distance is not reduced). Indeed, the slope of the interspecific relationship is significantly shallower than the slope of the interspecific relationship for other organisms (*t*-test of slopes: t=5.78, d.f.=29, P<0.0001), indicating that variation in gas exchange pattern, or a reduction in MR in particular, in this insect may ultimately not be capable of serving a water conservation function. This outcome stands in stark contrast to other work on gas exchange and water loss in cockroaches published to date (e.g. Schimpf et al., 2009; Schimpf et al., 2012) and therefore warrants further investigation.

LIST OF SYMBOLS AND ABBREVIATIONS

C phase	closed phase
CF phase	closed/flutter phase
CGE	continuous gas exchange
CWL	cuticular water loss
DGE	discontinuous gas exchange
F phase	flutter phase
hypC phase	hyperoxic C phase
MR	metabolic rate
O phase	open phase
RH	relative humidity
RQ	respiratory quotient
RWL	respiratory water loss
TWL	total water loss
$\dot{V}_{CO_2}$	rate of CO_2 release
$\dot{V}_{H_2O}$	rate of H_2O release
WLR	water loss rate

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AUTHOR CONTRIBUTIONS

J.S.T., C.P.P. and C.S.B. designed the research. B.G., C.S.B. and C.P.P. executed the research. This work served as part of C.P.P.'s Honour's project. B.G. and C.S.B. undertook additional experimental work, data extraction and analyses. All authors contributed to analyses and writing.

COMPETING INTERESTS

No competing interests declared.

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Table S1. Comparison of $V_{\cdot CO_2}$ and $V_{\cdot H_2O}$ across different temperatures (results from Friedman's ANOVAs; values are mean±SE).

	Mean[a]					Closed/Flutter-phase[b]					Open-phase[b]				
Temp (°C)	$V_{\cdot CO_2}$ ($ml.h^{-1}$)	CO_2 emission volume (ml)	$V_{\cdot H_2O}$ ($mg.h^{-1}$)	H_2O emission volume (mg)	$V_{\cdot H_2O}$ per unit $V_{\cdot CO_2}$	Duration (s)	$V_{\cdot CO_2}$ ($ml.h^{-1}$)	CO_2 emission volume (ml)	$V_{\cdot H_2O}$ ($mg.h^{-1}$)	H_2O emission volume (mg)	Duration (s)	$V_{\cdot CO_2}$ ($ml.h^{-1}$)	CO_2 emission volume (ml)	$V_{\cdot H_2O}$ ($mg.h^{-1}$)	H_2O emission volume (mg)
15	0.18±0.03	0.23±0.04	4.40±1.04	5.70±1.13	26.30±4.01	3670±368	0.05±0.01	0.06±0.02	3.50±1.30	3.52±1.36	1169±207	0.54±0.09	0.16±0.02	3.56±1.40	1.07±0.34
20	0.33±0.09	0.33±0.10	5.77±1.15	7.82±2.35	21.28±4.18	1981±416	0.07±0.02	0.04±0.01	5.92±1.44	2.97±0.94	1171±311	0.83±0.20	0.23±0.07	7.24±2.20	2.94±1.22
25	0.57±0.22	0.45±0.14	7.37±2.60	6.48±1.91	14.99±3.03	963±205	1.04±0.03	0.02±0.00	10.76±5.96	2.11±0.41	689±69	1.27±0.57	0.25±0.13	11.92±6.65	2.54±1.45
30	0.51±0.08	0.40±0.13	7.82±1.54	7.30±1.67	17.75±2.73	385±76	0.12±0.04	0.01±0.00	8.72±2.91	0.94±0.11	516±36	0.76±0.10	0.11±0.01	9.16±2.88	1.43±0.56
p-value	**<0.001*****	0.896	**0.008****	0.789	**0.006****	**0.002****	**0.003****	**0.003****	0.145	0.086	**0.003****	0.241	0.077	0.072	0.241

[a] Mean values were calculated across all gas exchange patterns.
[b] Closed/flutter and open-phase values were calculated for those individuals that performed DGE only.

***p*<0.01. Statistically significant differences are highlighted in bold type.
****p*<0.001

Temp, temperature; $V_{\cdot CO_2}$, rate of CO_2 release; $V_{\cdot H_2O}$, rate of H_2O release; DGE, discontinuous gas exchange.

Table S2. Relationship of gas exchange parameters with temperature, as well as $V\dot{C}O_2$ with frequency and O-phase CO_2 emission volume (results from linear regression analysis).

Gas exchange pattern	Regression	Intercept±SE	Slope±SE	r^2	F	d.f.	*p*-value
All patterns	Log_{10} $V\dot{C}O_2$ vs. temp	**-1.21±0.18**	**0.03±0.01**	**0.30**	**15.90**	**(1,38)**	**<0.001***
	Log_{10} $V\dot{H}_2O$ vs. temp	0.29±0.22	0.02±0.01	0.11	3.61	(1,30)	0.067
	CO_2 emission volume vs. temp	0.07±0.23	0.01±0.01	0.03	1.44	(1,38)	0.238
	H_2O emission volume vs. temp	4.93±3.99	0.05±0.17	0.00	0.07	(1,30)	0.789
	Log_{10} ($V\dot{H}_2O$/$V\dot{C}O_2$) vs. temp	**1.55±0.15**	**-0.01±0.01**	**0.13**	**4.40**	**(1,30)**	**0.044**
DGE only	Log_{10} frequency (Hz) vs. temp	**-5.03±0.21**	**0.09±0.01**	**0.84**	**98.75**	**(1,19)**	**<0.001***
	Log_{10} CF-phase $V\dot{C}O_2$ vs. temp	**-1.72±0.24**	**0.02±0.01**	**0.22**	**5.38**	**(1,19)**	**0.032***
	Log_{10} CF-phase $V\dot{H}_2O$ vs. temp	**0.03±0.29**	**0.03±0.01**	**0.35**	**6.02**	**(1,11)**	**0.032***
	CF-phase duration (s) vs. temp	**6136.31±683.94**	**-198.48±29.92**	**0.70**	**44.01**	**(1,19)**	**<0.001***
	Log_{10} O-phase $V\dot{C}O_2$ vs. temp	-0.54±0.23	0.02±0.01	0.13	2.76	(1,19)	0.113
	Log_{10} O-phase $V\dot{H}_2O$ vs. temp	**0.04±0.31**	**0.03±0.01**	**0.34**	**5.79**	**(1,11)**	**0.035***
	O-phase duration (s) vs. temp	**2087.95±361.52**	**-52.69±15.81**	**0.37**	**11.10**	**(1,19)**	**0.004***
	CF-phase CO_2 emission volume vs. temp	**0.09±0.02**	**-0.00±0.00**	**0.29**	**7.75**	**(1,19)**	**0.012***
	CF-phase H_2O emission volume vs. temp	5.05±1.62	-0.13±0.07	0.22	3.13	(1,11)	0.104
	O-phase CO_2 emission volume vs. temp	0.22±0.14	-0.00±0.01	0.00	0.06	(1,19)	0.815
	O-phase H_2O emission volume vs. temp	1.28±1.87	0.03±0.08	0.01	0.12	(1,11)	0.732
	Log_{10} $V\dot{C}O_2$ vs. log_{10} frequency (Hz)	**0.46±0.18**	**0.35±0.06**	**0.69**	**37.25**	**(1,17)**	**<0.001***
	Log_{10} $V\dot{C}O_2$ vs. log_{10} O-phase CO_2 emission volume	-0.67±0.45	-0.09±0.51	0.00	0.03	(1,17)	0.865

*p<0.05. Statistically significant differences are highlighted in bold type.

Temp (°C); $V\dot{C}O_2$ ($ml.h^{-1}$); $V\dot{H}_2O$ ($mg.h^{-1}$); CO_2 emission volume (ml); H_2O emission volume (mg).

$V\dot{C}O_2$, rate of CO_2 release; $V\dot{H}_2O$, rate of H_2O release; temp, temperature; CF-phase; closed/flutter phase; O-phase; open phase; DGE, discontinuous gas exchange.

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SHORT COMMUNICATION

High metabolic and water-loss rates in caterpillar aggregations: evidence against the resource-conservation hypothesis

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SUMMARY

Several hypotheses have been proposed for explaining animal aggregation, including energy or water conservation. However, these physiological hypotheses have not been well investigated. Here, we report the effects of aggregation on metabolic ($\dot{V}_{CO_2}$) and evaporative water-loss rates ($\dot{V}_{H_2O}$) of the gregarious caterpillar *Eutricha capensis*, by comparing individuals and groups of individuals (*N*=10–100). Contrary to findings from previous physiological studies, we did not find an advantage to aggregation: unexpectedly, $\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$ did not decrease with increasing group size. $\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$ generally remained constant or increased in larger groups relative to individuals. The amount of water lost per unit of CO_2 exchanged ($\dot{V}_{H_2O}$:$\dot{V}_{CO_2}$ ratio) showed a marked increase in grouped caterpillars, particularly in larger groups. Other benefits of aggregation (e.g. reduced predation or increased growth rates) likely outweigh these potential costs, because individuals of *E. capensis* aggregate voluntarily despite no obvious energetic or hygric advantage, and other potentially confounding group effects (e.g. increased thermoregulatory advantage or whole-animal activity) are inconsequential. The results of this study provide an important exception to physiological studies reporting enhanced energy or water conservation in animal groups.

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Key words: grouping, respiratory metabolism, desiccation, scaling.

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INTRODUCTION

Aggregation of individuals within species is a common biological phenomenon. The reasons proposed for aggregation are wide-ranging, including a reduction in predation risk (e.g. Ruxton and Sherratt, 2006), increased sexual signalling or mating success (e.g. Sullivan, 1981), enhanced foraging success, increased growth rates (e.g. Knapp and Casey, 1986) and improved energetic or hygric efficiency (e.g. Benoit et al., 2007; Killen et al., 2012).

However, for terrestrial animals, scaling of energetic and/or hygric efficiency with experimental manipulation of group size (i.e. number of individuals), termed herein the 'resource-conservation hypothesis', has only been examined in a handful of studies and species to date, with most reporting marked benefits of aggregation (e.g. Benoit et al., 2007; Waters et al., 2010; Modlmeier et al., 2013). These studies have mainly focused on Hymenoptera and other highly social insects (e.g. Cao and Dornhaus, 2008; Waters et al., 2010; Modlmeier et al., 2013) and their generality is therefore unclear. Based on metabolic scaling theories, varying group size could alter metabolic or hygric efficiency during inactivity in at least four possible ways. First, increasing group size may change the surface area-to-volume relationship and thereby influence physiological rates in a predictable, geometric manner. One general geometric prediction is that metabolic rate should scale as $m^{0.67}$, where m=body mass, which is unlikely to change with variation in aggregation size. However, for evaporative water loss rates the geometric expectation of changing group size is less clear and depends, at least partly, on the physical arrangement of the aggregation (see Materials and methods, Fig. 1). Second, the metabolic theory of ecology (MTE) predicts a $m^{0.75}$ scaling relationship for metabolic rate irrespective of group size (both within and between individuals), unless the assumptions underlying the MTE are violated in some way (reviewed in Sibly et al., 2012). Third, variation in group size may have no effect, or be balanced by increases in some rates and reductions in others, resulting in isometric scaling ($m^{1.0}$) across groups varying in mass. Finally, increasing group size could entail metabolic or hygric costs, resulting in scaling of rates greater than isometry ($m^{>1.0}$). Two general predictions can be made for the resource-conservation hypothesis of grouped individuals. First, grouped animals should have lower physiological rates per individual than individuals measured in isolation, and second, as groups get larger the benefits should increase (i.e. rates should be reduced even further when calculated on a *per capita* basis).

Here, we examined the impact of group size on metabolic and water-loss rates ($\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$) in an insect species that aggregates voluntarily in nature (Fig. 2A,B). Using Cape Lappet moth caterpillars (*Eutricha capensis* Linnaeus 1767) collected during an outbreak, we measured $\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$ across a range of group sizes. Using an experimental approach, we tested the resource-conservation hypothesis and the two general predictions which expect different

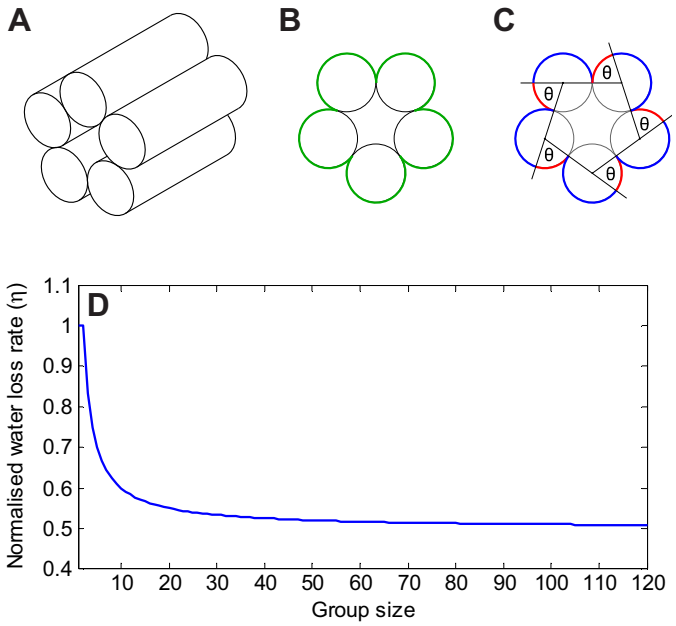

Fig. 1. (A) Model of caterpillars stacked in a cylindrical configuration, (B) view of cylinder ends with exposed sections coloured green and (C) diagram of cylinder ends used in the calculation of normalised exposed area. θ is the angular change in contact point between cylinders (as viewed in an anti-clockwise direction for subsequent adjacent cylinders). (D) Normalised water loss rate (η) as a function of group size.

effects of group size on energetic or hygric efficiency, while attempting to eliminate temperature and activity as potential confounding factors.

MATERIALS AND METHODS

Mid-developmental stage (4th or 5th instar) Cape Lappet moth caterpillars (*N*=212) were collected from a home garden in Stellenbosch, Western Cape, South Africa. At the start of laboratory rearing, caterpillars had a mean mass of 0.6 g (total group mass 136.7 g). During the experiments, caterpillars grew ninefold to 5.4±0.4 g before pupating after a period of *ca*. 2 months. During rearing, animals were maintained at a mean temperature of 20.3±0.03°C and were kept in the dark to avoid the potential confounding effects of diurnal photoperiod fluctuations. Caterpillars were fed *Acacia saligna* leaves and given water *ad libitum*.

Rates of CO_2 and H_2O release ($\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$, respectively) by caterpillars in groups of varying size (*N*=1, 10, 15, 25, 50 and 100) were estimated using flow-through respirometry. A calibrated infrared CO_2/H_2O analyser (Li-7000, Li-Cor, Lincoln, NE, USA) was set up as follows: an aquarium pump (Hailea, RaoPing County, GuangDong Province, China) fed atmospheric air into scrubber columns containing soda lime (MERCK, Gauteng, RSA) and silica gel/Drierite (ratio 1:1) (WA Hammond Drierite Company Ltd, Xenia, OH, USA) to remove CO_2 and H_2O vapour, respectively, from the airstream. This airstream was controlled at a constant flow rate of 250 ml min^{-1} by a flow control valve (Model 840, Side-Trak, Sierra Instruments, Monterey, CA, USA) connected to a mass flow control unit (Sable Systems, MFC-2, Las Vegas, NV, USA). Thereafter, air was fed through the zero channel of the CO_2/H_2O analyser and through a custom-built cuvette (each designed to accommodate different caterpillar group sizes), which was placed in a cooler box to minimise disturbance. Cuvettes had a wooden dowel suspended inside to allow the caterpillars to aggregate as in their natural environment. Only hydrophobic Bev-A-Line tubing was used for plumbing throughout the whole system, as this tubing minimises water vapour adsorbance. Calibration span gas concentrations varied among group sizes to ensure that $\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$ were recorded accurately within the analyser's measurement range. For all trials, baseline recordings were undertaken with a cuvette containing only the dowel. Thereafter, animals were introduced and allowed to settle before recordings began. Caterpillars were counted and a group mass was measured (±0.1 mg) with an electronic microbalance (MS104S, Mettler Toledo, Greifensee, Switzerland) prior to and after each trial. For each group size, the smallest possible cuvette was used to minimise analyser response times. The time constant for the largest cuvette was calculated to be 6.6 min (1650 ml/250 ml min^{-1}), therefore taking 33 min (6.6×5) for 99% of CO_2 to be read by the analyser. In all cases, the durations of data used for analysis greatly exceeded the maximum time constant (mean selected data periods were 288 min and 278 min for $\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$, respectively).

Each recording was performed overnight at a mean temperature of 20.2±0.3°C. The activity of individual caterpillars was recorded using an infrared activity detector (AD2, Sable Systems). Activity of groups (*N*=10, 15, 25 and 50) was monitored using a webcam (Logitech QuickCam Pro 9000) with an imaging frequency of 30 s, which was subsequently converted into a video (Yawcam version 0.3.9). A thermocouple (T-type, 36 standard wire gauge) was attached to the dowel inside the cuvette to record the temperature inside the aggregated group (T_{Agg}). A second thermocouple was secured against the outside of the cuvette to measure ambient chamber temperature (T_a). Thermocouples were connected to a datalogger (TC-08, Pico Technology, St Neots, UK) and recorded at 1 Hz sampling frequency with PicoLogger software.

Respirometry data were extracted using ExpeData (version 1.1.25, Sable Systems). Only periods of resting $\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$ (confirmed with activity detection and video analysis, Fig. 2C) were used for analyses. Data were corrected for baseline drift at standard temperature and pressure and converted to ml h^{-1} for $\dot{V}_{CO_2}$ and mg h^{-1} for $\dot{V}_{H_2O}$.

As the analyser's H_2O channel response times were slow for the largest group sizes (*N*=50 and 100), $\dot{V}_{H_2O}$ was estimated using two different methods. The first method was based on $\dot{V}_{H_2O}$ data obtained from respirometry trials calculated for group sizes of *N*=25 and smaller. The second method involved determining the $\dot{V}_{H_2O}$ gravimetrically as the difference between mass before and after a respirometry run divided by the duration of the run. There was a strong positive correlation between these two methods of determining $\dot{V}_{H_2O}$ (r^2=0.969) and therefore, to increase the size of the dataset, all analyses were performed using the gravimetric $\dot{V}_{H_2O}$ estimate and included groups of up to *N*=100 individuals.

Calculation of expected $\dot{V}_{H_2O}$ as a function of group size

To calculate the expected $\dot{V}_{H_2O}$ as a function of group size, we modelled the caterpillars as cylinders with constant length (*l*) and radius (*r*) arranged in a cylindrical configuration (Fig. 1A). We did not expect the surfaces on the inside of the cylindrical configuration to contribute to the $\dot{V}_{H_2O}$ of the group of caterpillars and assumed that the combined $\dot{V}_{H_2O}$ is proportional to the exposed surface area of the group of caterpillars. The surface area of a cylinder (excluding the surface area of the ends) is given by the product of the circumference of the circular end and the cylinder length, or:

$$A_c = (2\pi r) \times l\,, \quad (1)$$

where *r* is the radius and *l* the length of the cylinder. For cylinders arranged in a cylindrical configuration, the exposed surface area is given by:

$$A_{c,exp} = \eta(2\pi r) \times l\,, \quad (2)$$

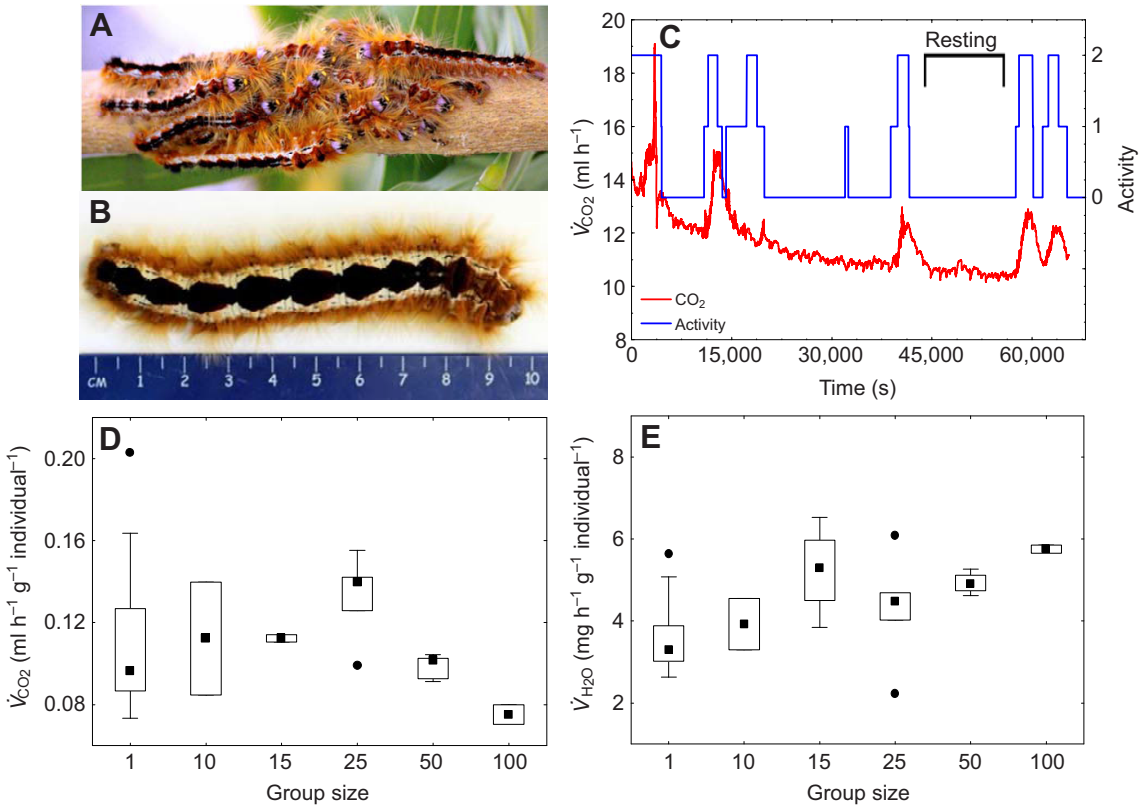

Fig. 2. Rates of metabolism and water loss for (A) aggregated and (B) individual *Eutricha capensis*. (C) Metabolic rate recorded as $\dot{V}_{CO_2}$ (red line, left axis) for a group of 50 caterpillars matches activity patterns (blue line, right axis) recorded with a webcam. Activity was scored as 2=high activity (majority of individuals moving), 1=low activity (one or two individuals moving) and 0=no activity (see also supplementary material Movie 1). The period of rest where data were extracted is indicated. Metabolic rate measured as $\dot{V}_{CO_2}$ (D) and water loss rate $\dot{V}_{H_2O}$ (E) did not decrease as group size increased, as predicted by the resource-conservation hypothesis. There was no significant difference between groups for $\dot{V}_{CO_2}$ ($\dot{V}_{CO_2}$: $H_{5,34}$=9.04, P>0.10). For $\dot{V}_{H_2O}$, there was a significant increase with group size ($\dot{V}_{H_2O}$: $H_{5,36}$=14.96, P<0.05). Box plots represent median (squares) with 25–75 percentiles, and whiskers (errors) are non-outlier range (minimum and maximum). Circles denote outliers.

where η is the fraction of the circumference of the cylinder ends that is exposed. The ratio of the exposed surface area to the total surface area is therefore given by:

$$\frac{A_{c,exp}}{A_c} = \eta . \quad (3)$$

For a group of N caterpillars, the expected $\dot{V}_{H_2O}$ for the group is given by:

$$\dot{V}_{H2O,total} = \eta \times N \times \dot{V}_{H2O,individual} , \quad (4)$$

where $\dot{V}_{H2O,individual}$ is the $\dot{V}_{H_2O}$ of an individual caterpillar with its surface area fully exposed. The expected $\dot{V}_{H_2O}$ therefore requires the calculation of η, which is the sum of the exposed arcs (shown in green in Fig. 1B) divided by the sum of the cylinder circumferences. The total exposed arc length can then be calculated as the sum of N half-circle arcs (shown in blue) and N smaller arcs (shown in red) of which the combined length of the latter is equal to the circumference of a single cylinder end (as $N\times\theta$=360 deg) (Fig. 1C). The value of η can therefore be calculated for individuals as:

$$\eta = \frac{\frac{1}{2}N \times 2\pi r + 2\pi r}{N \times 2\pi r} = \frac{1}{2} + \frac{1}{N} . \quad (5)$$

For N=1, the whole caterpillar surface area is exposed and therefore, η=1. Consequently, the normalised $\dot{V}_{H_2O}$ is expected to decrease as group size increases (Fig. 1D).

Statistical analyses

Data were checked for normality and homogeneity of variance, and where these assumptions were violated, non-parametric tests were used. In preliminary analyses, a Type I general linear model was performed to assess the effects of age (number of days from initiation of laboratory holding) and number of individuals independently of individual mass on $\dot{V}_{CO_2}$. This analysis showed that the number of individuals and start mass had a significant effect (P<0.01) on $\dot{V}_{CO_2}$, whereas age did not (P=0.569). Because age did not have a distinct effect on $\dot{V}_{CO_2}$, it was not incorporated in further analyses. We report $\dot{V}_{CO_2}$ in ml h^{-1} g^{-1} individual^{-1}, which was calculated by dividing the average $\dot{V}_{CO_2}$ recorded during a respirometry run ($\dot{V}_{CO_2}$ divided by group size) by the average mass per individual in the group (using the start mass before respirometry divided by group size). $\dot{V}_{H_2O}$ was calculated in the same way and is presented in mg h^{-1} g^{-1} individual^{-1}. We tested for normality using a Shapiro–Wilk tests after three extreme outliers had been removed (two extremes removed from the $\dot{V}_{H_2O}$ dataset, and one removed from the $\dot{V}_{H_2O}$:$\dot{V}_{CO_2}$ ratio dataset) and found that the data for most groups were not normally distributed. Therefore, a non-parametric approach (Kruskal–Wallis test) was used to compare physiological rates among groups.

The temperature inside the respirometry cuvette was estimated and compared between grouped and individual caterpillars to ensure the temperature remained constant across all trials (T_{Agg} 20.5±2.1°C; T_{Ind} 19.8±1.2°C; Mann–Whitney U_{33}=129.5, P>0.44). Furthermore,

these temperature estimates most likely approximate the body temperature of individuals, but owing to potential aggregation-related heating may not necessarily approximate a group's body temperature. Therefore, we also estimated differences between cuvette air temperature during measurement and the inside of the group's core temperature and compared these between grouped and isolated individuals, assuming a zero difference between air and body temperature of singletons (*t*-test, t_{30}=–1.73, P>0.09).

RESULTS AND DISCUSSION

At rest, $\dot{V}_{CO_2}$ did not decrease significantly as group size increased (Fig. 2D) and there were no statistically significant differences between groups ($\dot{V}_{CO_2}$: $H_{5,34}$=9.04, P>0.10). At rest, $\dot{V}_{H_2O}$ increased as group size increased (Fig. 2E) and there was a significant effect of group size ($\dot{V}_{H_2O}$: $H_{5,36}$=14.96, P<0.05), suggesting a hygric penalty to increasing group size.

The ratio of $\dot{V}_{H_2O}$ to $\dot{V}_{CO_2}$, indicating the hygric cost of gas exchange, did not decrease as group size increased, as predicted by the resource-conservation hypothesis (Fig. 3). By contrast, there was a non-significant positive trend suggesting that aggregated caterpillars lost more water per ml CO_2 exchanged than did smaller groups or solitary individuals ($H_{5,33}$=9.93, P>0.07). Therefore, all of our measurements of the above physiological parameters contradict the resource-conservation hypothesis.

In insects, the benefits of aggregation are relatively well established and include reduced predation risk and increased mating success (e.g. Sullivan, 1981; Ruxton and Sherratt, 2006). From a physiological perspective, reported benefits have mainly involved energetic, hygric or thermal advantages. Several previous physiological studies have reported marked, group-related reductions in rates of resource loss or consumption (so-called 'group effects'), by using indirect calorimetric or gravimetric approaches (e.g. Bartholomew et al., 1988; Benoit et al., 2007; Waters et al., 2010). At low ambient temperatures, groups of insects may show elevated body temperatures, which can provide growth and development advantages that would not be present in solitary, more ectothermic individuals (Knapp and Casey, 1986). The results of our study on Cape Lappet Moth caterpillars are unique because they suggest no obvious physiological benefit to aggregation, as $\dot{V}_{CO_2}$, $\dot{V}_{H_2O}$ and $\dot{V}_{H_2O}$:$\dot{V}_{CO_2}$ did not decrease with increasing group size, while $\dot{V}_{H_2O}$ even showed a significant increase. Furthermore, in the case of $\dot{V}_{H_2O}$, the change in rate with increasing group size is in the opposite direction to what might be expected based on changes in surface area/volume relationships (Fig. 1D).

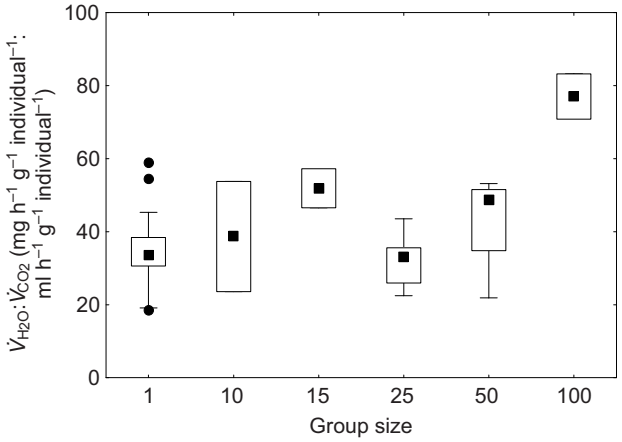

Fig. 3. The ratio between water loss rate ($\dot{V}_{H_2O}$) and metabolic rate ($\dot{V}_{CO_2}$) does not differ among groups of varying size ($H_{5,33}$=9.93, P>0.07). Box plots represent median (squares) with 25–75 percentiles, and whiskers (errors) are non-outlier range (minimum and maximum). Circles denote outliers.

Several potential factors may explain this lack of group-related resource conservation in *E. capensis*. First, increased costs may occur if groups experience temperatures that are elevated above ambient conditions. However, our measurements of the temperature inside and outside aggregations in the laboratory showed this not to be true. Furthermore, an endothermic response seems unlikely given the moderate temperatures experienced during the growing and activity season of *E. capensis*. Most species showing thermal aggregation benefits inhabit Arctic or polar environments where low temperatures may be a limiting factor for population growth.

Second, groups of insects may be more active than solitary individuals, thereby increasing gas-flux rates. However, differential whole-animal activity cannot explain our results because our use of activity detectors and video monitoring ensured that our data only came from resting animals.

Third, aggregation may directly or indirectly increase resting metabolic rates and by association water-loss rates. For example, the immediate presence of other individuals may increase sensory inputs, thus stimulating neural activity, which is known to be energetically expensive (Niven et al., 2007). Alternatively, grouping behaviour may foster higher growth rates, as observed in gypsy moth and eastern tent caterpillars (Knapp and Casey, 1986). Higher costs of biosynthesis may then result in elevated $\dot{V}_{CO_2}$ (and associated $\dot{V}_{H_2O}$). Both of these latter two explanations require further testing. Although we are presently unable to offer a conclusive explanation for the lack of support for the resource-conservation hypothesis – and therefore the relatively high energy and water costs associated with aggregations of *E. capensis* – the frequent occurrence of this aggregation behaviour under natural conditions suggests that it must have some significant counterbalancing benefits. These benefits may include increased growth rates (Knapp and Casey, 1986) or reduced predation risk (Ruxton and Sherratt, 2006). Regardless, our results clearly demonstrate that the resource-conservation hypothesis is not a generally applicable explanation for aggregation behaviour.

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AUTHOR CONTRIBUTIONS

J.S.T. conceived the study; all authors contributed to designing the experiments; R.E.S., B.G., L.B. and C.E.v.D. gathered the data; all authors analysed and interpreted the data; all authors contributed to writing the paper.

COMPETING INTERESTS

No competing interests declared.

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