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Cockroaches breathe discontinuously to reduce respiratory water loss

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SUMMARY

The reasons why many insects breathe discontinuously at rest are poorly understood and hotly debated. Three adaptive hypotheses attempt to explain the significance of these discontinuous gas exchange cycles (DGCs), whether it be to save water, to facilitate gas exchange in underground environments or to limit oxidative damage. Comparative studies favour the water saving hypothesis and mechanistic studies are equivocal but no study has examined the acclimation responses of adult insects chronically exposed to a range of respiratory environments. The present research is the first manipulative study of such chronic exposure to take a strong-inference approach to evaluating the competing hypotheses according to the explicit predictions stemming from them. Adult cockroaches (*Nauphoeta cinerea*) were chronically exposed to various treatments of different respiratory gas compositions (O_2, CO_2 and humidity) and the DGC responses were interpreted in light of the *a priori* predictions stemming from the competing hypotheses. Rates of mass loss during respirometry were also measured for animals acclimated to a range of humidity conditions. The results refute the hypotheses of oxidative damage and underground gas exchange, and provide evidence supporting the hypothesis that DGCs serve to reduce respiratory water loss: cockroaches exposed to low humidity conditions exchange respiratory gases for shorter durations during each DGC and showed lower rates of body mass loss during respirometry than cockroaches exposed to high humidity conditions.

Key words: DGC, discontinuous gas exchange, hygric, chthonic, oxidative damage, *Nauphoeta cinerea*.

INTRODUCTION

Since Heller's observation (Heller, 1930), the discontinuous gas exchange cycles (DGCs) exhibited by many quiescent tracheated arthropods have proven to be a source of intrigue and great debate. Insect DGCs are distinguished from continuous and cyclic breathing patterns by regular periods where respiratory gas exchange is essentially prevented due to spiracular closure (Marais and Chown, 2003). Typically, DGCs comprise three phases: closed (C), flutter (F) and open (O), and the patterns of respiratory gas exchange occurring during these cycles has been extensively described in lepidopteran pupae (e.g. Hetz and Bradley, 2005; Levy and Schneiderman, 1966a; Levy and Schneiderman, 1966b; Terblanche et al., 2008). During the C phase the spiracles are tightly occluded and gas exchange with the atmosphere is essentially prevented. Pressure within the tracheae declines as CO_2 is buffered within the haemolymph and O_2 is depleted due to respiration. Once the partial pressure of oxygen (P_{O_2}) within the tracheal system has declined to ~2–4 kPa, the F phase is initiated. During this phase the spiracles open and close with high frequency, facilitating inward convective movement of air, such that a low and stable P_{O_2} is maintained within the tracheal system (Hetz and Bradley, 2005; Levy and Schneiderman, 1966a). Outward movement of H_2O and CO_2 is minimised as a result of the inward convective movement of air, and CO_2 continues to be buffered in the haemolymph (Wobschall and Hetz, 2004). When the partial pressure of CO_2 (P_{CO_2}) within the tracheal system reaches ~5–6 kPa, the spiracles open and respiratory gases are exchanged with the atmosphere. CO_2 is expelled in a burst and O_2 moves inwards until intratracheal P_{CO_2} reaches ~3–4 kPa and the cycle is repeated (Levy and Schneiderman, 1966a).

DGCs are observed in a range of arthropod species (Klok et al., 2002) and are present in at least five insect orders. Species exhibiting DGCs inhabit xeric, mesic, subterranean and non-subterranean environments, and are both winged and wingless. The presence of DGCs in phylogenetically independent groups of insects (Blattodea, Orthoptera, Coleoptera, Ledpidoptera and Hymenoptera) suggests that the breathing pattern is adaptively significant, rather than exists as an ancestral trait (Marais et al., 2005). Three main hypotheses have emerged that attempt to explain the adaptive significance of DGCs (Chown et al., 2006). The hygric hypothesis follows the original suggestions of Buck, Keister and Specht (Buck et al., 1953) that DGCs reduce transpiratory water loss. The chthonic hypothesis (Lighton, 1998) postulates that DGCs are an adaptation to facilitate efficient gas exchange under hypoxic and/or hypercapnic conditions, often characteristic of underground environments. Lighton and Berrigan (Lighton and Berrigan, 1995) originally proposed this hypothesis in combination with the hygric hypothesis, such that DGCs serve to facilitate gas exchange in challenging conditions whilst also avoiding respiratory water loss. In recent literature, however, the pure chthonic hypothesis, irrespective of water loss, has become prominent (Chown et al., 2006). The final hypothesis is the oxidative damage hypothesis (Bradley, 2000), which suggests that DGCs function to limit oxidative damage to tissues. Because the trachae are capable of rapidly delivering oxygen when required (i.e. during flight), when at rest, near-ambient levels of oxygen at the ends of the tracheoles may potentially be harmful to the insects' tissues.

To date, research examining the adaptive function of DGCs has not been well integrated. A mixture of mechanistic and comparative studies fails to provide unequivocal support for any of the current hypotheses. One possible approach for investigating the function of DGCs involves analysing potential changes in the insects' gas exchange patterns in response to environmental variation. Many organisms can respond to changes in the environment through

morphological or physiological alterations that allow improved function in the new conditions. This process of change in response to environmental variation is known as phenotypic plasticity or acclimation response (Fordyce, 2006). Until now, during examination of DGCs, adult insects have only been subjected to acute changes in respiratory gas conditions, as opposed to being chronically exposed. It has therefore not yet been discovered whether or not insects are capable of modifying their gas exchange patterns in response to prolonged changes in respiratory environments. The potential acclimation response of an insect to a range of environmental conditions could be utilised to differentiate among the three putative adaptive functions of DGCs, as each hypothesis can be used to make distinct predictions regarding the changes in DGC patterns in response to different respiratory environments (Table 1). The literature is largely devoid of prediction-based approaches for understanding the function of DGCs, and such a strong-inference approach would give more credibility to results (Huey et al., 1999). The present research is the first manipulative strong-inference study to address changes in the DGCs of adult insects in response to chronic exposure to varying respiratory environments. This research makes it possible to differentiate among the competing hypotheses and provides insight into the possible selective pressures that may have led to the evolution of DGCs by evaluating the hypotheses according to the explicit predictions stemming from them.

The present study aimed to test among the competing hypotheses for the function of DGCs using the speckled cockroach (*Nauphoeta cinerea*). Cockroaches were chronically exposed to different concentrations of O_2, CO_2 and water vapour [in practice relative humidity (RH)] and DGC responses were examined in light of the *a priori* predictions of the competing hypotheses (Table 1). In the case of the hygric hypothesis, a positive relationship between O phase duration and RH treatment is predicted, as most respiratory water loss occurs during the O phase (Chown et al., 2006). Thus, animals exposed to low levels of ambient RH will have shorter O phases than animals acclimated to high RH. In the case of the chthonic hypothesis, either a positive relationship between CO_2 treatment and the C and F phase durations or a negative relationship between O_2 treatment and the C and F phase durations is predicted, because the CO_2 and O_2 partial pressure gradients required to facilitate efficient gas exchange are generated during these phases. Thus, animals acclimated to low O_2, high CO_2 or both are predicted to have relatively long C and F phases, such that large partial pressure gradients are established to maintain adequate gas exchange under hypoxic or hypercapnic conditions. Finally, in the case of the oxidative damage hypothesis, a negative relationship between O phase duration and O_2 treatment is predicted, because oxidative damage would be greatest during the O phase. Thus, animals exposed to high O_2 are predicted to have shorter O durations that animals acclimated to low O_2.

MATERIALS AND METHODS

Nauphoeta cinerea Olivier 1789 was a suitable study organism for this research as, following preliminary investigations, it was shown to exhibit a conspicuous DGC (Fig. 1). Final instar cockroaches were obtained from The Herp Shop (Ardeer, Victoria, Australia) and maintained as single-sex stock populations in 60 l plastic containers at a constant temperature of 23±1.5°C and a 12 h:12 h L:D cycle. Cockroaches were provided with an *ad libitum* diet of carrots and dry cat food. The stock population was maintained at environmental conditions: 21% O_2, 0.03% (atmospheric) CO_2 and ambient RH (~60–80%). Upon maturation, samples of male cockroaches from the stock population were randomly selected and assigned to acclimation treatments. Females were not used in this study to eliminate changes in metabolism and gas exchange associated with reproduction (Rossolimo, 1982), as female *N. cinerea* are facultatively parthenogenetic (Corely et al., 2001).

In order to elucidate whether or not DGC patterns showed an acclimation response, cockroaches were chronically exposed to a number of different gas conditions. Exposure treatments lasted five weeks, a period adequate to elicit acclimation responses in cockroaches (Dehnel and Segal, 1956). For each of the gases [O_2, CO_2 and water vapour (RH)], a range of treatments from low to high was used. Each treatment population (N~50) was housed in a 7 l polypropylene (Sistema, New Zealand) container under the same temperature and L:D conditions as the stock population. The treatment gases were set and delivered to the acclimation boxes at a flow rate of ~200 ml min^{-1}, measured with a mechanical flow meter (Duff and McIntosh, Sydney, Australia). This ensured constant turnover of the gas within the container and maintained a slight positive pressure inside the container. Gas exited the container *via* a minimum of 1 m of 8 mm outer diameter tubing.

To ascertain whether a change in DGC pattern occurred during the exposure period, cockroach respiratory patterns were characterised at 23±1°C upon completion of acclimation treatments. As such, the rate of CO_2 release of 12–16 randomly selected cockroaches was measured using standard flow-through respirometry (Withers, 2001). Two cockroaches were measured simultaneously using each of the two sample cells of a Li-7000 (Li-Cor, Nebraska, USA) CO_2–H_2O analyser. This precluded simultaneous measurement of CO_2 and H_2O but increased the number of individuals that could be measured. Cockroaches were placed individually in one of two 25 ml respirometry chambers to which gas (see Table 2 and below for details) was delivered at a constant flow rate of 200 ml min^{-1}. Unless explicitly stated otherwise, the incurrent gas was dry (Drierite, Sigma-Aldrich, Steinheim, Germany) and CO_2-free (Soda Lime, Fluka, Steinheim, Germany)

Table 1. Explicit predictions regarding the response of discontinuous gas exchange cycle phase durations following exposure to varying respiratory environments

	Treatments			
	Low		High	
Hypothesis	Factor	Phase response	Factor	Phase response
Hygric	RH	O duration decrease	RH	O duration increase
Chthonic	CO_2	C and F duration decrease	CO_2	C and F duration increase
	O_2	C and F duration increase	O_2	C and F duration decrease
Oxidative damage	O_2	O duration increase	O_2	O duration decrease

RH, relative humidity; O, open phase; C, closed phase; F, flutter phase.

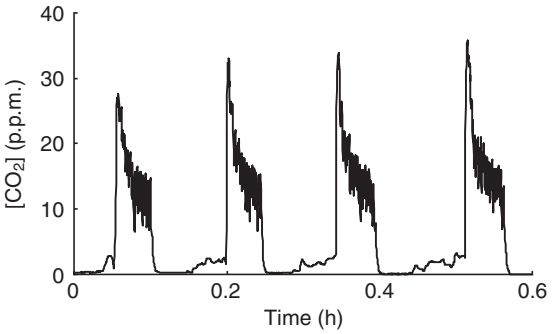

Fig. 1. Measurement of carbon dioxide release over time from *Nauphoeta cinerea*, demonstrating a conspicuous discontinuous gas exchange cycle. Measurements were taken at a flow rate of 200 ml min^{-1}.

to maximise the accuracy of the analyser. The fractional CO_2 content of the excurrent gas from each chamber was recorded to a computer at a sampling frequency of 1 Hz.

All respirometry was performed during the inactive phase of the circadian cycle (daytime), and food was withdrawn at least 24 h prior to measurements. After being placed in the respirometry chamber, cockroaches were allocated a one-hour 'settling in' period. The gas exchange patterns of the animals were then measured under the appropriate gases, which were presented sequentially in a random order during a single respirometry session. The chamber was darkened to encourage resting behaviour (and hence initiation of DGCs). The mass of each cockroach was also recorded to 0.001 g before and after respirometry measurements.

Oxygen exposure comprised four treatments: 5, 10, 21 and 40±1.1% O_2, and carbon dioxide and relative humidity each comprised three treatments (0.03, 3±0.03 and 6±0.3% CO_2, and 25±0.1, 45±0.3 and 90±1.4% RH, respectively). Compressed mixes of O_2, CO_2 and N_2 obtained from and certified by a commercial supplier (BOC gases, Brisbane, Australia) were used for the O_2 and CO_2 acclimations. Desired levels of RH were produced by equilibrating saturated air with water vapour at a range of temperatures (2, 10 and 21°C for 25, 45 and 90% RH at 23°C) using constant temperature cabinets, and were verified using a RH-300 Water Vapour Analyser (Sable Systems, Las Vegas, NV, USA). Table 2 provides an overview of the nominal levels of acclimation treatments ('acclimation gas' hereafter) and the gas conditions under which DGCs were measured ('measurement gas' hereafter). Following acclimation treatments, cockroaches were measured under the conditions to which they were chronically exposed, as well as under the conditions of the other treatments for a particular gas where possible. Thus, animals acclimated to 5, 10, 21 or 40% were measured at each of these O_2 concentrations in dry air, animals acclimated to 0, 3 or 6% CO_2 were measured at 0% CO_2 in dry air (measurement at higher levels of CO_2 was not possible because the analyser saturated at 50 p.p.m. CO_2), and animals acclimated to 25, 45 or 90% RH were measured at 25 and 45% RH (due to the risk of condensation in the analyser at 90% RH).

The recorded data were used to characterise respiratory gas exchange patterns in Microsoft Excel (Redmond, WA, USA), and only individuals exhibiting DGCs were used for analysis. For each DGC, total DGC (O+CF), O and CF phase durations were recorded and metabolic rates were calculated according to Withers (Withers, 2001):

$$\dot{V}_{CO_2} = (\dot{V}_I \times F_eCO_2) / [1 + (\{1/RE\} - 1) \times F_eCO_2],$$

where $\dot{V}_{CO_2}$=rate of CO_2 production, $\dot{V}_I$=carbon dioxide concentration, F_eCO_2=excurrent fraction of CO_2 and RE=respiratory exchange ratio, which was assumed to be 0.8. Rate of CO_2 production was used as a proxy for metabolic rate.

C and F phases were combined due to the difficulty of unambiguously differentiating the F phase in all individuals, and because F phase may commence before CO_2 release is detected using flow-through respirometry (Hadley and Quinlan, 1993; Harrison et al., 1995; Wobschall and Hetz, 2004). Mixed model analysis of variance (ANOVA) and analysis of covariance (ANCOVA) were used to test for an effect of acclimation treatment on total DGC, O and CF phase durations. The individual identification number of cockroaches was included as a random effect to account for the measurement of multiple cycles per individual, and in the cases of O_2 and RH, to account for the measurement of individuals in multiple gas conditions. In initial analyses, the following variables were included: acclimation treatment, time (am or pm), chamber, resting (settling in) gas, measurement gas, measurement order, mass, metabolic rate and identification number. In subsequent analyses, non-significant variables were eliminated and any significant variables were analysed for an interaction with acclimation treatment. Final models always included acclimation treatment, measurement gas, mass, metabolic rate and identification number regardless of their significance. An interaction between acclimation treatment and measurement gas was always tested for, and any other significant covariates or interactions were also included. Data were tested for normality using Shapiro–Wilk tests, and non-normal data were transformed to improve normality ($\log_{10}$ or square root). In one quarter of the cases, data did not reach normality. In these circumstances the transformation that rendered the data closest to normal distribution was accepted, as according to the Central Limit Theorem, the distribution of means tends toward normality

Table 2. A summary of acclimation treatment conditions and nominal gas compositions in which discontinuous gas exchange patterns were measured

Treatments	Low acclimation	Medium acclimation		High acclimation	DGC measurement conditions
Oxygen	**5% O_2**	**10% O_2**	**21% O_2**	**40% O_2**	**5, 10, 21 and 40% O_2**
	0% CO_2	0% CO_2	0% CO_2	0% CO_2	0% CO_2
	90% RH	90% RH	90% RH	90% RH	0% RH
Carbon dioxide	21% O_2	21% O_2		21% O_2	21% O_2
	0% CO_2	**3% CO_2**		**6% CO_2**	**0% CO_2**
	90% RH	90% RH		90% RH	0% RH
Relative humidity (RH)	21% O_2	21% O_2		21% O_2	21% O_2
	0% CO_2	0% CO_2		0% CO_2	0% CO_2
	25% RH	**45% RH**		**90% RH**	**25 and 45% RH**

for large sample sizes despite a non-normal population distribution (Quinn and Keough, 2002; Zar, 1974).

Additionally, to determine if RH acclimation had an effect on water loss, rates of mass loss during respirometry were compared for animals acclimated to 25, 45 and 90% RH using ANCOVA with body mass as a covariate. All statistical tests were conducted using JMP v.7.0.1 (SAS Institute Inc., Cary, NC, USA), and α was set at 0.05 for all tests. For clarity, adjusted means are presented in figures, and are shown ±s.e.m.

RESULTS

The effect of acclimation treatment on DGC duration is always reported regardless of significance. There was never a significant interaction between acclimation treatment and measurement gas ($P>0.05$ in all cases). Other covariates and interactions are only reported if their effects were significant, except in cases where a significant covariate did not have a significant interaction with acclimation treatment, in which case the non-significant interactions are also reported. In addition, Table 3 provides a summary of the mean initial mass and mean metabolic rates for each acclimation treatment at the conclusion of the chronic exposure period.

Carbon dioxide

Mass had a significant effect on total DGC duration (ANOVA $F_{1,32}=7.6$, $P=0.01$) but there was no significant interaction between mass and CO_2 acclimation treatment (ANOVA $F_{2,26}=0.84$, $P=0.44$). There was a significant effect of acclimation treatment on total DGC duration (ANOVA $F_{2,29}=7.52$, $P=0.002$), and 6% CO_2 exposure resulted in significantly shorter DGC durations compared with 0% and 3% (Tukey's HSD) (Fig. 2).

There was a significant effect of mass and acclimation treatment on O phase duration (ANOVA $F_{1,29}=4.58$, $P=0.04$; $F_{2,27}=8.03$, $P=0.002$) but there was no significant interaction between mass and treatment (ANOVA $F_{2,24}=0.56$, $P=0.58$). O phase duration was significantly shorter following 3% and 6% CO_2 treatments when compared with 0% (Tukey's HSD) (Fig. 2).

There was a significant effect of acclimation treatment on CF phase duration (ANOVA $F_{2,30}=6.7$, $P=0.004$). CF phase duration was significantly shorter following exposure to 6% CO_2 than following exposure to 3%, and neither were significantly different from 0% (Tukey's HSD) (Fig. 2).

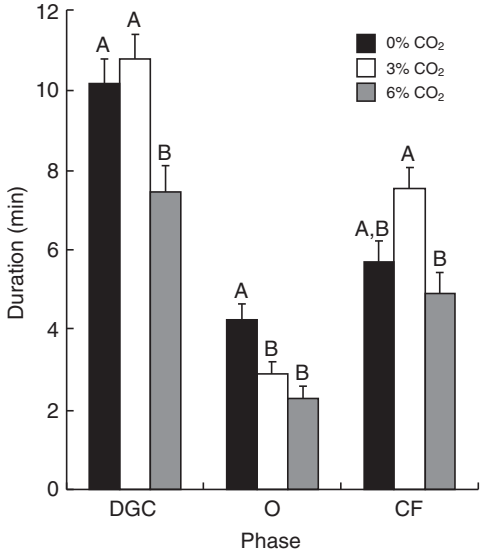

Fig. 2. Adjusted least square means of total discontinuous gas exchange cycles (DGC), open (O) and closed-flutter (CF) phase durations following exposure to CO_2 treatments, shown +s.e.m. Exposure to 6% consistently resulted in shorter phase durations than lower levels of CO_2. Total sample size: $N=144$ measurements from 36 individuals (0%: $N=47$ measurements from 15 individuals; 3%: $N=47$ measurements from 11 individuals; 6%: $N=50$ measurements from 10 individuals). Columns within a phase that share a letter (A or B) are not significantly different from each other (Tukey's HSD).

Relative humidity

Metabolic rate had a significant effect on total DGC duration (ANOVA $F_{1,138}=6.8$, $P=0.01$) but there was no significant interaction between metabolic rate and RH acclimation treatments (ANOVA $F_{2,118}=2.7$, $P=0.07$). There was a significant effect of treatment (ANOVA $F_{2,24}=6.1$, $P=0.007$), with exposure to 90% RH resulting in significantly longer total DGC duration compared with 25% (Tukey's HSD) (Fig. 3).

There was no effect of metabolic rate on O phase duration, so metabolic rate was excluded from subsequent analyses of O phase. There was a significant effect of acclimation treatment on O phase duration (ANOVA $F_{2,23}=8.9$, $P=0.001$). O phase duration was significantly longer following exposure to 90% compared with 25% RH (Tukey's HSD) (Fig. 3).

Metabolic rate had a significant effect on CF phase duration (ANOVA $F_{1,101}=13.0$, $P=0.0005$) but there was no significant effect of acclimation treatment (ANOVA $F_{2,24}=3.2$, $P=0.06$, Tukey's HSD) (Fig. 3).

Mass loss was significantly affected by RH acclimation ($F_{2,23}=24.0$, $P<0.0001$) and correlated with body mass ($F_{1,23}=12.7$, $P=0.002$). Rate of mass loss was significantly reduced following exposure to 25% RH compared with 45% and 90% RH (Tukey's HSD) (Fig. 4).

Table 3. A summary of mean initial mass and mean metabolic rate of cockroaches in each acclimation treatment

Acclimation treatment				
O_2 (%)	CO_2 (%)	RH (%)	Mass (g) ±s.e.m. (*N*)	Metabolic rate ($\dot{V}_{CO_2}$) ±s.e.m. (*N*)
5	0	90	0.450±0.014 (8)	0.090±0.003 (8)
10	0	90	0.530±0.013 (11)	0.104±0.008 (11)
21	0	90	0.480±0.014 (16)	0.094±0.005 (16)
40	0	90	0.529±0.014 (9)	0.106±0.016 (9)
21	3	90	0.430±0.008 (11)	0.088±0.006 (11)
21	6	90	0.430±0.014 (10)	0.100±0.006 (10)
21	0	25	0.485±0.018 (10)	0.076±0.006 (10)
21	0	45	0.423±0.02 (6)	0.053±0.004 (6)

Measurements were taken at the conclusion of the chronic exposure treatments. RH, relative humidity.

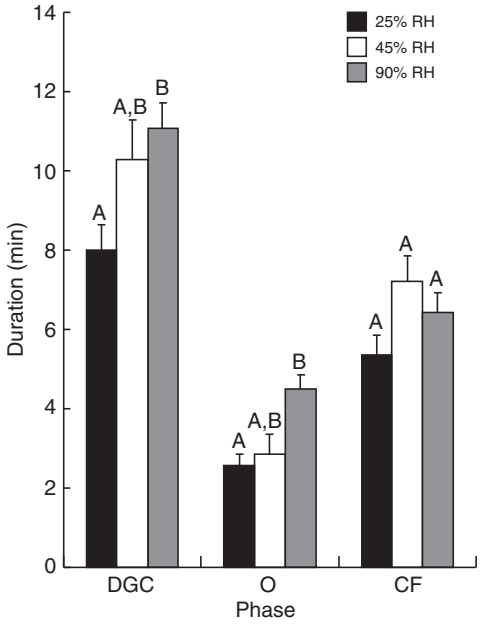

Fig. 3. Adjusted least square means of total discontinuous gas exchange cycles (DGC), open (O) and closed-flutter (CF) phase durations following exposure to relative humidity (RH) treatments, shown +s.e.m. Exposure to 90% RH resulted in longer DGC and O durations than exposure to lower levels of RH. Total sample size: N=184 measurements from 27 individuals (25%: N=72 measurements from 10 individuals; 45%: N=38 measurements from 6 individuals; 90%: N=70 measurements from 10 individuals). Columns within a phase that share a letter (A or B) are not significantly different from each other (Tukey's HSD).

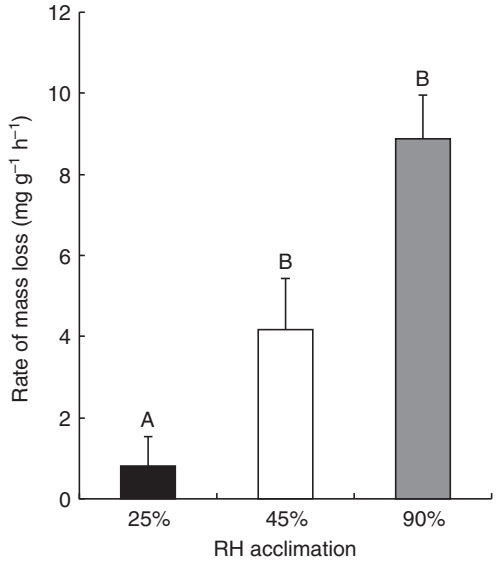

Fig. 4. Mean rates of mass loss for the three relative humidity (RH) treatments, shown +s.e.m. Exposure to 25% RH (N=10) resulted in a decreased rate of mass loss compared with mass loss rates following exposure to 45 and 90% RH (N=6 and 10, respectively). Columns that share a letter (A or B) are not significantly different from each other (Tukey's HSD).

Oxygen

Initial analyses revealed that O_2 acclimation treatment, measurement gas and measurement order had significant effects on total DGC duration (ANOVA $F_{3,66}$=4.2, P=0.008; $F_{3,431}$=13.0, P<0.0001; $F_{3,424}$=4.6, P=0.004). There was also a significant interaction between treatment and measurement order (ANOVA $F_{9,420}$=3.3, P=0.0007).

There was no effect of acclimation treatment on CF phase duration (ANOVA $F_{3,65}$=0.38, P=0.77) but there was a significant interaction between acclimation treatment and measurement order (ANOVA $F_{9,420}$=2.1, P=0.03).

Acclimation treatment had a significant effect on O phase duration (ANOVA $F_{3,60}$=16.9, P=0.0001) but there was a significant interaction between acclimation treatment and metabolic rate, and between acclimation treatment and measurement order (ANOVA $F_{1,218}$=23.6, P<0.0001; $F_{3,217}$=4.3, P=0.006, respectively). Exploratory examination of these effects suggested a difference in acclimation response between hypoxic and hyperoxic conditions, and subsequent analyses were conducted on hypoxic (5, 10 and 21% O_2, measured at each of these levels) and hyperoxic (21 and 40% O_2, measured at each of these levels) groups separately.

Hypoxic group

Acclimation treatment had a significant effect on total DGC duration (ANOVA $F_{2,49}$=5.4, P=0.007) and there was a significant interaction between treatment and measurement order (ANOVA $F_{6,247}$=2.6, P=0.02). Only the 21% treatment measured in the first hour was significantly different from that measured in the third hour (Tukey's HSD) (Fig. 5A).

Both acclimation treatment and measurement gas had a significant effect on O phase duration (ANOVA $F_{2,40}$=7.1, P=0.002; $F_{2,236}$=50.8, P<0.0001, respectively) and there was a significant interaction between acclimation treatment and measurement order (ANOVA $F_{6,237}$=3.1, P=0.006). Only the 21% treatment measured in the first hour was significantly different to the measurement in the second hour (Tukey's HSD) (Fig. 5B).

Measurement gas had a significant effect on CF phase duration (ANOVA $F_{2,252}$=27.4, P<0.0001) but there was no significant effect of acclimation treatment (ANOVA $F_{2,26}$=0.31, P=0.73) (Fig. 5C).

Hyperoxic group

There was no significant effect of acclimation treatment on total DGC duration (ANOVA $F_{1,23}$=0.49, P=0.49), nor a significant effect of any other variable.

Metabolic rate had a significant effect on O phase duration (ANOVA $F_{1,80}$=4.8, P=0.01) but there was no significant effect of acclimation treatment (ANOVA $F_{1,20}$=3.2, P=0.09).

Both resting gas and metabolic rate had a significant effect on CF phase duration (ANOVA $F_{3,15}$=3.8, P=0.03; $F_{1,41}$=4.8, P=0.03). Acclimation treatment had no significant effect on CF phase duration (ANOVA $F_{1,17}$=0.5, P=0.49).

Total sample size of N=137 measurements from 24 individuals (21%: N=82 measurements from 15 individuals, 40%; N=55 measurements from 9 individuals).

DISCUSSION

The present research is the first of its kind to demonstrate that adult insects alter their respiratory gas exchange patterns in response to chronic exposure to varying environments. Cockroaches showed a significant acclimation response to each of the O_2, CO_2 and RH treatments. These responses are compared with the explicit predictions based on the three

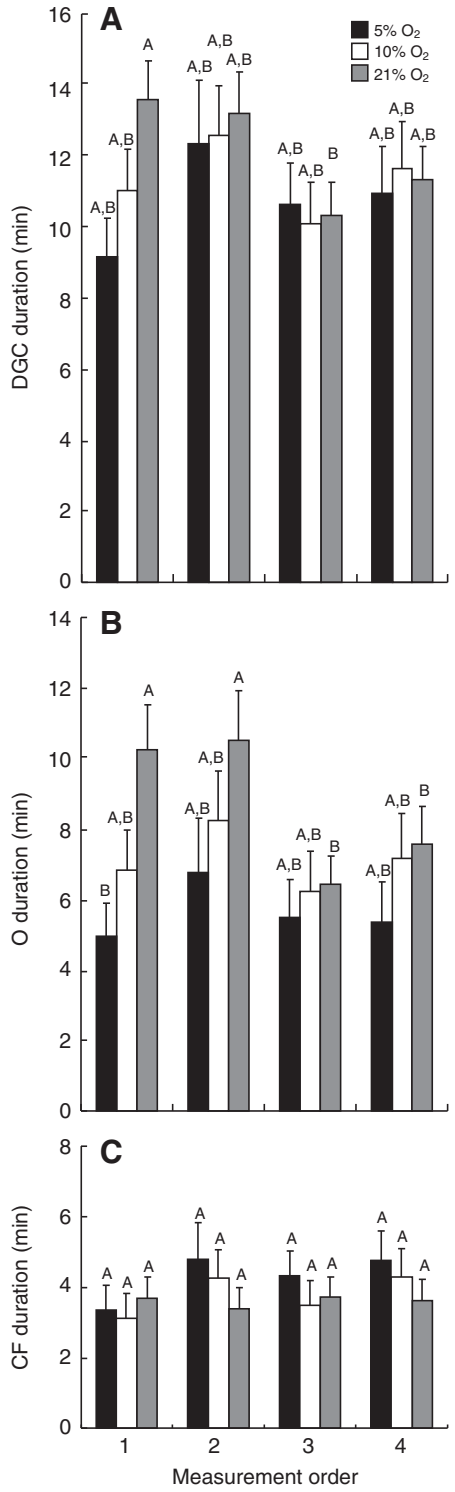

Fig. 5. Adjusted least square means of phase durations in the hypoxic group of O_2 treatments according to measurement order (hour), shown +s.e.m. A positive relationship between acclimation and total discontinuous gas exchange cycles (DGC) and open (O) phase durations is apparent but only in the first two hours of measurement (A and B). There was no effect of acclimation treatment on the closed-flutter (CF) phase duration (C). Total sample size: *N*=275 measurements from 34 individuals (5%: *N*=69 measurements from 8 individuals; 10%: *N*=81 measurements from 11 individuals; 21%: *N*=125 measurements from 15 individuals). Columns that share a letter (A or B) are not significantly different from each other (Tukey's HSD).

adaptive hypotheses in order to elicit support for any number of these hypotheses.

Chthonic hypothesis

CF phase duration was shortest following exposure to high levels of CO_2 and longer when exposed to lower levels (Fig. 2). This response runs counter to the predictions set out by the chthonic hypothesis, which suggests that C and F phase duration will increase in hypercapnia to facilitate adequate gas exchange *via* a steep respiratory gas gradient. Similarly, there was no significant effect of O_2 acclimation treatments on C and F phase duration. This further refutes the chthonic hypothesis, which proposes an increase in the C and F duration as O_2 levels decrease, again to facilitate adequate gas exchange. Unfortunately, however, we were only able to measure animals in conditions of 0% CO_2, and so it remains unknown how animals acclimated to high levels of CO_2 will exchange respiratory gases in hypercapnia. The few species that have been measured have been shown to cease DGCs in hypercapnia (Harrison et al., 1995; Terblanche et al., 2008), and it would be interesting to determine if this is also the case for cockroaches acclimated to hypercapnia.

Oxidative damage hypothesis

The significant interaction between acclimation and measurement order demonstrates that the effects of oxygen acclimation are dependent on measurement order. Although Tukey's HSD does not identify any significant pair-wise differences between O_2 treatments (Fig. 5A,B), there is an apparent positive relationship between hypoxic O_2 treatments and the DGC and O phase durations. This relationship is only apparent in the first one to two hours of measurement (i.e. the second and third hours of total respirometry session), after which it appears to be obscured. Nevertheless, regardless of whether O phase duration increases with O_2 treatments or remains unchanged, both responses are clearly inconsistent with the predictions made by the oxidative damage hypothesis, which states that the O phase should decrease in duration following exposure to higher levels of oxygen in order to protect tissues from oxidative damage. The lack of an acclimation response to hyperoxia further suggests that DGCs are not required to limit oxidative damage. Similarly, although intratracheal P_{O_2} is regulated at 4–5 kPa during the CF phase in atmospheres of up to 50 kPa O_2 in atlas moths and silkworm pupae (Hetz and Bradley, 2005; Levy and Schneiderman, 1966a), this regulation is not maintained at higher P_{O_2}s (Levy and Schneiderman, 1966b).

Hygric hypothesis

The hygric hypothesis recently received support from work by Marais et al. (Marais et al., 2005) and White et al. (White et al., 2007) and the present research lends further credence to the original explanation for the adaptive function of DGCs (Buck et al., 1953; Buck and Keister, 1955; Burkett and Schneiderman, 1974a; Kestler, 1985; Levy and Schneiderman, 1966a; Lighton, 1990; Lighton et al., 1993). Exposure to low levels of RH results in a reduction in DGC duration, as well as a reduction in the duration of the O phase whereas the duration of the CF phase was unaffected (Fig. 3). The change in O duration is consistent with the explicit predictions that stem from the hygric hypothesis (Table 1). O phase durations were longest following acclimation to high levels of humidity where the saturation deficit between the respiratory surfaces and that atmosphere is likely to be small, and rates of water loss are likely to be low. Following exposure to low humidity, O phase durations were shorter, which presumably acted to reduce respiratory water

loss. This finding is further supported by the fact that mean rates of mass loss were 5–10-fold higher following acclimation to 45 and 90% RH treatments than when compared with 25% RH acclimation (Fig. 4). It is acknowledged that mass loss alone is not a definitive measure of respiratory water loss as it does not discriminate between mass lost *via* defecation, cuticular or respiratory transpiration. Further work examining only respiratory water loss would provide an improved point of comparison.

Given that cockroaches show an acclimation response to altered ambient humidity, it is surprising that measurement humidity did not have a significant effect on phase durations. Cockroaches therefore appear unable to detect acute changes in ambient humidity. It is possible that the acute exposure is too short a time for an observable response to occur but it is nevertheless clear that cockroaches do not respond to RH immediately as they do to changes in O_2 and CO_2. Potentially, cockroaches chronically exposed to low levels of humidity have lower levels of body hydration than those chronically exposed to high levels of humidity. Thus, the acclimation response to humidity may actually represent a response to varying levels of hydration. Such a desiccated state is likely to alter the haemolymph P_{CO_2} and pH (Chown, 2002), leading to a change in ventilation rate (Snyder et al., 1980). However, while DGC frequency does increase following acclimation to low RH, CF phase duration remains unchanged. If desiccation-associated changes in haemolymph pH were responsible for the acclimation response to RH, one might expect to see a decrease in the CF phase duration as internal CO_2 would reach the O phase trigger more quickly, because the volume, and therefore presumably the CO_2 buffering capacity, of the haemolymph is reduced. This however is not what is observed for the CF phase duration. Alternatively, the level or concentration of buffers could increase as a consequence of desiccation, and therefore total buffer capacity would remain constant, in which case CF duration would be expected to be independent of hydration status. Clearly, chronic exposure to varying levels of ambient humidity offers exciting opportunities to gain further insight into the mechanistic basis of DGCs. At this stage, however, the mechanism by which cockroaches sense and respond to altered humidity remains unclear.

Conclusion

The present study has answered calls in the literature for a single-species, strong-inference manipulative approach to examine the evolutionary significance of DGCs (Chown, 2002; Chown et al., 2006; Lighton, 2007; Lighton and Turner, 2008; Marais et al., 2005; Quinlan and Gibbs, 2006; Terblanche et al., 2008). The present research provides support for the hygric hypothesis and disputes both the chthonic and oxidative damage hypotheses. This is in contrast with a recent study by Terblanche et al., which provided support for the oxidative damage hypothesis and limited support for the hygric hypothesis (Terblanche et al., 2008). Terblanche et al. exposed diapausing moth pupae *Samia cynthia* to a range of levels of O_2, CO_2 and humidity and interpreted the responses of the animals in light of the explicit predictions of the competing hypotheses (Terblanche et al., 2008). However, Terblanche et al. examined only the effect of acute exposure on immature insects (Terblanche et al., 2008). It is well documented that low O_2 and high CO_2 levels cause insect spiracles to open (Beckel and Schneiderman, 1957; Burkett and Schneiderman, 1967; Burkett and Schneiderman, 1974b), so it is to be expected that DGCs will cease in hypoxia or hypercapnia. Studies that only examine the DGC responses of insects to acutely altered levels of respiratory gases are therefore of limited value when distinguishing between the various hypotheses for the evolution of DGCs.

Testing among the predictions that stem from the three adaptive hypotheses explaining the evolution of DGCs demonstrates a clear support for the hygric hypothesis. However, implicit in this approach is the assumption that the best model is included in the candidate set (Johnson and Omland, 2004; Quinn and Dunham, 1983). It remains to be seen whether future studies continue to find support for water loss as the driving force for the evolution and maintenance of discontinuous ventilation or whether new hypotheses need to be considered. It has also been suggested that several factors are likely to work together to influence the expression of DGCs (Chown, 2002). It would be advantageous if further research were to be conducted examining the effect on DGCs of combinations of the gas conditions reported here. Such an approach would aid in revealing possible interactive effects of the gas variables that may not be detected when variables are examined in isolation. Indeed in reality, insects encounter microclimates of low O_2 and high CO_2 rather than the individually manipulated gas variable of the present study (Anderson and Ultsch, 1987). Furthermore, the level of intratracheal O_2 influences the level of CO_2 at which spiracles open, and *vice versa* (Burkett and Schneiderman, 1967; Burkett and Schneiderman, 1974b), so it is possible that a combination of hyperoxia and hypercapnia may elicit acclimation responses different to those observed in the present study. Such an approach could also reveal whether or not DGC expression is prioritised according to the most costly variable in the immediate respiratory environment. For example, for animals experiencing water loss stress, DGCs may become important in terms of the oxidative damage hypothesis. Wigglesworth (Wigglesworth, 1935) documented the presence of fluid in the ends of the tracheae under hyperoxic conditions and Kestler (Kestler, 1985) proposed that this fluid functioned to restrict tracheal conductance and hence decrease potential damage resulting from high levels of O_2. If animals become dehydrated, they may be unable to fill the tracheae with water, leaving them vulnerable to oxidative damage. In such instances, O_2 levels may become an important factor for the exhibition of DGCs.

The support garnered for the hygric hypothesis from the research presented here suggests that reducing respiratory water loss was a significant factor in the evolution of DGCs, at least in *N. cinerea*. The hygric hypothesis is the first of the three adaptive hypotheses to be supported by a variety of studies: two broad scale comparative studies (Marais et al., 2005; White et al., 2007) that examined a wide range of species from a diverse range of habitats, many mechanistic studies dealing with acute exposures of respiratory gases (e.g. Chown and Davis, 2003; Duncan et al., 2002a; Duncan et al., 2002b; Duncan and Dickman, 2001; Lighton et al., 1993), and now a mechanistic study that has examined the effect of chronic exposure to various respiratory environments. A thorough understanding of the evolution of physiological traits and their ecological implications is aided by the strength of a number of complementary approaches such as these (Huey and Kingsolver, 1993). Nevertheless, it is important that further studies of acclimation to chronic exposure are conducted on a variety of species, particularly from other orders (such as Hymenoptera, Lepidoptera, Orthoptera and Coleoptera), as it has been suggested that DGCs may have evolved for different reasons in different species (Chown and Nicholson, 2004; Chown et al., 2006). Such acclimation studies will reveal whether or not other factors, such as O_2 or CO_2, were important for the evolution of DGCs in other species or whether in fact support is shown for a single important evolutionary factor.

Finally, it is important that future research addresses the intriguing findings of the present study. Cockroaches responded to all treatments by altering their respiratory gas exchange patterns but the responses to CO_2 and O_2 are not congruent with the predictions stemming from the chthonic and oxidative damage hypotheses. Both the CO_2 and O_2 responses are opposite to what is predicted. Careful consideration needs to be given as to why the DGCs are responding to these factors in this manner, and exploratory analyses of these new observations might lead to new theories for the evolution of DGCs. It remains to be seen if such theories supplant Buck, Keister and Specht's (Keister and Specht, 1953) original hypothesis and the results of the present study, which suggest that DGCs function to reduce respiratory water loss.

LIST OF ABBREVIATIONS

C	closed
DGC	discontinuous gas exchange cycle
F	flutter
O	open
P_{CO_2}	partial pressure of CO_2
P_{O_2}	partial pressure of O_2
RH	relative humidity

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