

## Cyclic gas-exchange in the Chilean red cricket: inter-individual variation and thermal dependence

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### Summary

One of the most puzzling features of respiration in insects is cyclic gas exchange (CGE, the extreme form of discontinuous gas exchange-cycles, DGC), a periodic respiratory pattern that appeared independently several times in the evolution of arthropods. Although it is a striking feature of insects and some non-insect species, to date there is no clear knowledge of how widespread it is, or its adaptive significance. Here we show for the first time that a cricket (*Cratomelus armatus*) from the Stenopelmatidae family exhibits CGE. *C. armatus* shows a conspicuous, convective O-phase, with significantly repeatable ventilatory period and O-phase duration (intraclass correlation coefficients of 0.51 and 0.74, respectively). Also, *C. armatus* exhibits high variation in the CGE patterns, ranging from continuous to highly periodic records, sometimes including the classic F-phase.

No record went to zero and we found significant (inverse) effects of ambient temperature on O-phase duration but not on the ventilatory period. Average  $\dot{V}_{\text{CO}_2}$  and O-phase amplitude (i.e. mean  $\dot{V}_{\text{CO}_2}$  of the peaks) increased with temperature whereas the amplitude of the interburst did not change significantly with ambient temperature. *C. armatus* is a species that lives below ground in humid forests, so our results support the chthonic-hygic hypothesis (i.e. facilitation of gas exchange under hypoxic and hypercapnic conditions, minimizing evaporative water loss), although this assertion needs to be confirmed statistically by a strong inference approach.

Key words: respiration, discontinuous gas-exchange cycles, cyclic gas exchange, Stenopelmatidae.

### Introduction

In animals, energy metabolism represents the ‘intensity’ of functioning in a given situation. The rate of metabolism (MR), usually measured by the rate of consumption of O<sub>2</sub> or production of CO<sub>2</sub>, represents the *in vivo* energy metabolism of an individual animal. Such an informative state variable has long attracted the interest of physiologists, who have measured the rate of metabolism in thousands of animals in the search for adaptive patterns (McNab, 2002). In a single record, whenever the MR pattern has consistently deviated from a relatively constant time-series with non-periodic fluctuations, investigators have wondered about a mechanistic description and have mostly found an adaptive explanation. For instance, sudden reductions in metabolic rate over days or weeks in small birds and mammals were demonstrated to represent periods of physiological dormancy (i.e. torpor and hibernation), which have adaptive significance as an energy-saving strategy (e.g. Barclay et al., 2001). Total interruptions in MR in several invertebrates were suggested to be adaptations for desiccation (Alpert, 2006). Drastic increases in MR capacity during cold exposure in small endotherms were shown to be a significant adaptation for enduring winter (e.g. Klaasen et al., 2002). Sharp

increases in invertebrate MR after feeding were shown to be a direct consequence of digestive energy costs, with adaptive significance for nutrient absorption (Bradley et al., 2003).

The complex patterns of gas exchange in some insects are an exception: their adaptive significance is still unknown. The respiratory patterns of insects have been classified into three main kinds: continuous gas exchange, cyclic gas exchange (CGE) and discontinuous gas-exchange cycles (DGC) (Gibbs and Johnson, 2004). CGE in insects is similar to other animals in that a continuous trace of CO<sub>2</sub> production is recorded, with minor variation due to activity and/or random noise. CGE is characterized by periodic bursts of CO<sub>2</sub> production, followed by valleys of low CO<sub>2</sub> production. In contrast, DGC is characterized by bursts of CO<sub>2</sub> production interspersed by zero exchange. It has been shown that these phases are the response to successive openings of the spiracle (Chown and Nicolson, 2004 and references therein). Hence, the peaks of CO<sub>2</sub> production are named the O- (‘open’) phase, and the valleys are known as the C- (‘closed’) phase. In some cases, before to the O-phase a small CO<sub>2</sub> release is detected, which is known as the F- (‘flutter’) phase, referring to intermittent openings of the spiracle (Chown and Nicolson, 2004).

Interest in CGE and DGC in insects has been considerably unbalanced in terms of insect diversity. According to Chown et al. (Chown et al., 2006), the great majority of studied species are beetles and other holometabolous insects. The interesting view presented by Marais et al. [(Marais et al., 2005), p. 4505] suggests that few species exhibit DGC, and all of these also present CGE and continuous gas exchange. According to this phylogeny, DGC appears to be a recent acquisition in insects, having emerged independently at least five times in distantly related groups. One of these appearances is within the orthoptera clade, mainly represented by orthopters (Marais et al., 2005). Here we describe for the first time the cyclic respiratory patterns of a member of the Stenopelmatidae family (king crickets, Jerusalem crickets, wetas and long-horned grasshoppers), the Chilean red cricket *Cratomelus armatus*, and its inter-individual variation together with its thermal dependence.

Five hypotheses for the evolutionary origin of CGE and DGC have been proposed; in brief: (1) the 'hygric hypothesis' (CGE and/or DGC represent an adaptation to xeric environments, limiting evaporative water loss), (2) the 'chthonic-hygric' hypothesis (CGE and/or DGC represent an adaptation to hypoxic/hypercapnic environments, at the same time limiting evaporative water loss), (3) the 'oxidative damage' hypothesis (CGE and/or DGC represent a strategy to reduce the oxidative damage during periods of reduced oxygen demand), (4) the 'emergent property' hypothesis (CGE and/or DGC represent a non-adaptive epiphenomenon of the interaction of two feedback system regulating gas exchange with minimal demand) and (5) the 'strolling arthropod' hypothesis (CGE and/or DGC are an adaptation to increase the frequency of spiracle closure to reduce the risk of parasitic infestation) (for details, see Chown et al., 2006), but none of these hypotheses appear to have received consistent experimental support and the debate seems to be focussed on the first two (Chown and Nicolson, 2004; Gibbs and Johnson, 2004; Marais et al., 2005).

In the present work, we have studied the respiratory patterns of the Chilean red cricket *Cratomelus armatus*, a fossorial species that inhabits humid forests in the Southern hemisphere. According to the 'chthonic-hygric' hypothesis, we predict that this species will exhibit DGC and/or CGE in order to survive the hypercapnic/hypoxic environments that it inhabits. To be a target of natural selection, a trait should exhibit inter-individual variation or repeatability. Consequently, we predict that the metabolic patterns of this species will exhibit significant repeatabilities (see also Chappell and Rogowitz, 2000; Marais et al., 2005; Chown et al., 2006).

## Materials and methods

### Insects

The Chilean red cricket *Cratomelus armatus* Blanchard is a robust endemic cricket that lives in central-south Chile, in humid forests. It is found below dead logs, where it builds galleries in moist soil with its strong mandibles, feeding on a

great variety of vegetal and animal material (i.e. it has an omnivorous diet). It is of solitary and nocturnal habits, exhibiting conspicuous aggressiveness and cannibalism when living together, especially individuals of the same sex and size. Adults have small (non-functional) wings and do not have a stridulating mechanism. According to Angulo (Angulo, 2001), the growth of the Chilean red cricket consists of five instars with considerable variation in adult size (ca. 20–30 mm long, 1.5–2.5 g), males being smaller than females.

We captured 35 crickets ranging from 0.2 to 2 g (representing all stages) in Valdivia (39°48'S, 73°14'W) and housed them individually in plastic cages filled with 3 cm of humid litter. Crickets were fed daily with rabbit chow and water *ad libitum*, and maintained at 18°C under a natural photoperiod.

### Respirometry

Our respirometry system was similar to that described elsewhere (Lighton and Turner, 2004; Chappell and Rogowitz, 2000). In brief, CO<sub>2</sub> production was measured continuously using an infra-red CO<sub>2</sub> analyzer (LI-COR LI6262, Lincoln, NV, USA) capable of resolving differences of 1 p.p.m. of CO<sub>2</sub> in air. The analyzer was calibrated periodically against a precision gas mixture. Although there was almost no drift between calibrations, we performed baseline measurements before and after each recording. Flow rates of dry, CO<sub>2</sub>-free air (ambient air scrubbed free of water vapour and CO<sub>2</sub> using a Drierite/soda lime column) were maintained at 100 ml min<sup>-1</sup> ±1% by a Sierra mass flow controller (Henderson, NV, USA). We used cylindrical Sable Systems (Las Vegas, NV, USA) precision metabolic chambers (8 ml) covered by metal paper, associated with an AD-1 linear activity unit (Sable Systems) to record movement of the crickets by monitoring fluctuations in reflected infrared light at ca. 900 nm. Additionally, crickets were visually monitored at intervals of ca. 5 min during measurements. Each cricket was measured over at least a 1 h period. Each record was automatically transformed by a macro program recorded in the Expe Data software (Sable Systems), in order to (1) correct the 8-s lag introduced by the distance between the analyzer and the chamber and then to match the activity record with the  $\dot{V}_{\text{CO}_2}$  record, (2) to transform the measure from p.p.m. to ml CO<sub>2</sub> h<sup>-1</sup>, taking into account the flow rate, (3) to eliminate the first 5 min records (300 samples). To evaluate the effect of ambient temperature on  $\dot{V}_{\text{CO}_2}$  and CGE, metabolic rate was measured at 15°C, 20°C and 25°C (using three independent groups of crickets). To determine inter-individual variation in  $\dot{V}_{\text{CO}_2}$  and CGE (i.e. its time-consistency or repeatability), we repeated the measurements at 20°C, 15 days later (with the same crickets).

### Statistics

Common statistical analyses were performed with Statistica 6.1 (Statsoft Inc 2004). *C. armatus* did not exhibit classical DGC since the records never reached zero, so in order to characterize the cyclic pattern of metabolism into informative continuous variables, we operationally define the records as

composed of an interburst, at the valleys of the records, and an O-phase, corresponding to the peaks of  $\text{CO}_2$  release. The O-phase was characterized by regular ventilatory cycles, which were also detected by the infrared motion device when the cricket was the right position (i.e. resting longitudinally in the chamber). As judged by its standard deviation (s.d.), the ventilatory cycles were remarkably regular within each record and for each individual (Fig. 1), and we could compute their period and variance directly from the records using the Expe Data software. However the O-phase was less regular and required a less ambiguous estimation procedure, namely spectrum analysis (Shumway, 1988). Spectrum analysis is concerned with the exploration of cyclical patterns of data, the purpose of the analysis being to decompose a complex time-series with cyclical components into a few underlying sinusoidal functions of particular wavelengths. Similar to multiple regression analysis, the dependent variable is the time series and the independent variables are the sine function of all possible frequencies. For 'q' different sine and cosine functions, with k ranging from 1 to q, this type of linear multiple regression model may be written as:

$$X_t = a_0 + \sum [a_k \cos(\lambda t) + b_k \sin(\lambda t)],$$

where  $\lambda$  is the frequency in radians per unit of time ( $t$ ), and  $a_k$  and  $b_k$  are the regression coefficients, indicating the goodness of fit to the data. However, the procedure of fitting this function to data is considerably more complex than standard regression methods. It is performed by fast Fourier transformation and spectral density estimation (SDE), which finds the frequency (or period) with the greatest spectral density in the time-series (i.e. the frequency regions consisting of many adjacent frequencies that contribute most to the periodical behaviour of the series). Then, by SDE it is possible to adjust the function and extract the period (the inverse of  $\lambda$ ) with higher probability in the cyclic series.

Given that all metabolic variables were correlated among them, the effect of ambient temperature on metabolic variables was assessed by multivariate analysis of covariance (MANCOVA) using body mass  $M_b$  as covariate, and Tukey *post-hoc* test in each response variable. To fulfil ANOVA assumptions, data were  $\log_e$ -transformed. Repeatability was computed by the intraclass correlation coefficient ( $\tau$ ) by calculating the between- and within-individual variance component from one-way ANCOVAs ( $M_b$  as covariable). Then,  $\tau = (\text{between individuals variance component}) / (\text{within individual variance component})$ . In some cases (ventilatory period, O-phase and interburst), several measurements were taken within each record, which were included as a nested factor. Hence, in addition to the between- and within-individual variance components, for these variables we also computed the between records-within individual variance, component.

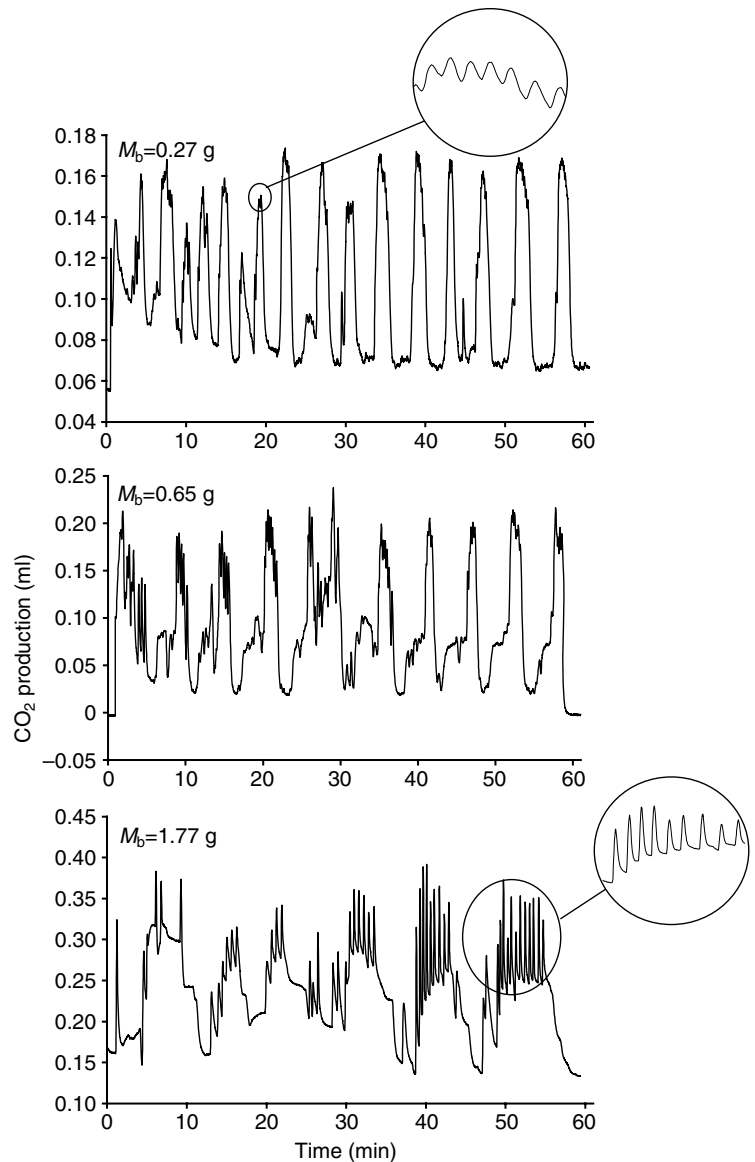


Fig. 1. Three representative  $\dot{V}_{\text{CO}_2}$  records in individuals of different body masses, exhibiting the ventilatory cycles (enlarged) and the characteristic low-frequency cycles of cyclic gas exchange (CGE).

## Results

The pattern of  $\dot{V}_{\text{CO}_2}$  observed in the Chilean red cricket was cyclic in 90% of the records, (Fig. 1, Fig. 2). During CGE, no cricket exhibited any movement within the chamber other than ventilatory movements, most of which (especially in large individuals) were also detected by the motion infrared device. According to the general pattern of  $\dot{V}_{\text{CO}_2}$ , in order to characterize CGE, five variables were extracted from the records: the O-phase period or duration (from the spectral analysis), the ventilatory period, average  $\dot{V}_{\text{CO}_2}$ , O-phase amplitude and interburst amplitude  $\dot{V}_{\text{CO}_2}$  (Table 1). The only variable that did not exhibit body mass dependence was O-phase duration (Fig. 3).

Table 1. Descriptive results of cyclic gas exchange (CGE) and ambient temperature in *Cratomelus armatus*

Variable	Ambient temperature (°C)		
	15	20	25
$M_b$ (g)	0.58±0.05 (6)	0.74±0.15 (20)	0.72±0.12 (8)
O-phase period (s)	1147.2±147.1 (7)	424.8±33.2 (20)	319.2±44.0 (8)
Ventilatory period (s)	15.2±2.9 (3)	14.0±1.4 (20)	12.9±1.3 (7)
Mean $\dot{V}_{CO_2}$ (ml CO <sub>2</sub> min <sup>-1</sup> )	0.061±0.008 (7)	0.149±0.019 (20)	0.128±0.025 (8)
O-phase amplitude (ml CO <sub>2</sub> min <sup>-1</sup> )	0.674±0.591 (7)	0.196±0.024 (20)	0.183±0.027(8)
Interburst amplitude (ml CO <sub>2</sub> min <sup>-1</sup> )	0.019±0.003 (6)	0.093±0.015 (20)	0.070±0.024 (8)

Values are means ± s.e.m. (number of individuals in parentheses).

Computed variables (O-phase period, ventilatory period, mean  $\dot{V}_{CO_2}$ , O-phase and interburst amplitude) are defined in Materials and Methods and in Fig. 1.

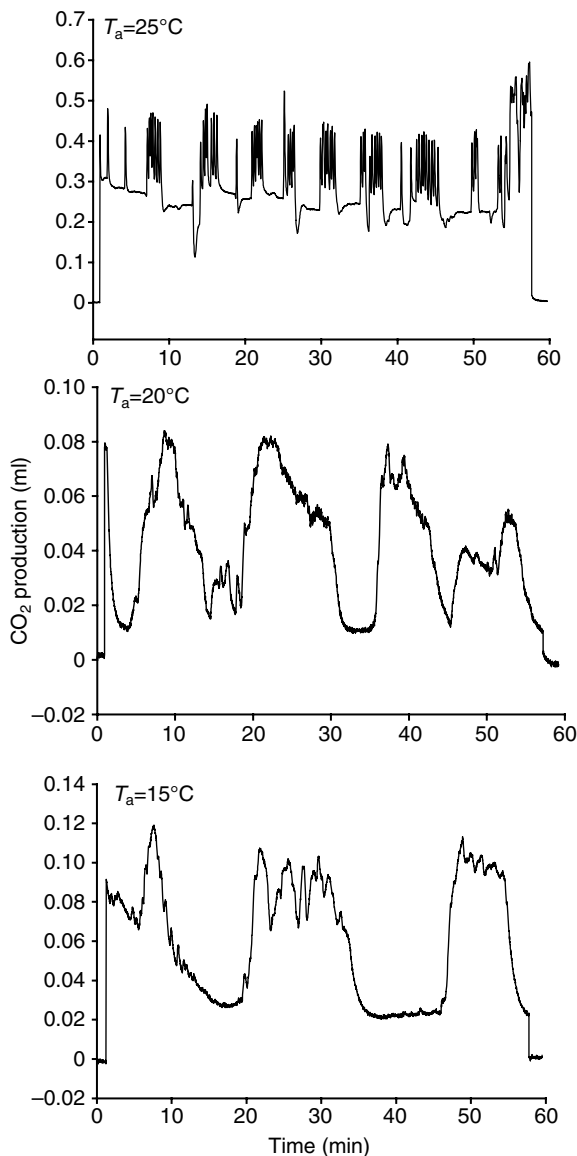


Fig. 2. Three representative  $\dot{V}_{CO_2}$  records at different ambient temperatures ( $T_a$ ).

There were significant effects of ambient temperature on metabolic variables (Wilk's lambda=0.166;  $F_{10,20}=2.91$ ;  $P=0.020$ ). Tukey's *post-hoc* test revealed that the O-phase duration was inversely affected by ambient temperature (Fig. 4). There were non-significant effects of temperature on ventilatory period (Fig. 4). The amplitude of average and O-phase increased with temperature whereas the amplitude of the interburst changed only between 15° and 20°C (Fig. 5).

Both ventilatory and O-phase periods presented significant repeatabilities (Table 2). Also, the within-record component was significant for the ventilatory period, indicating that this variable is remarkably consistent over time. On the other hand, average, O-phase amplitude and interburst amplitude did not exhibit significant repeatability at the individual level, although the mean  $\dot{V}_{CO_2}$  and the interburst amplitude showed moderate repeatability (Table 2). The amplitude of the O-phase exhibited significant within-individual component of variation, however (Table 2).

## Discussion

Historically, the most studied aspects of king crickets, Jerusalem crickets, wetas, long-horned grasshoppers and other Stenopelmatidae are the population biology (Leisnham and Jamieson, 2002; McCartney et al., 2006), taxonomy/phylogeography (Trewick et al., 2000; Morgan-Richards and Wallis, 2003; Jost and Shaw, 2006), reproduction (Bateman, 2000; Gwynne, 2004), stridulating mechanisms (Field and Roberts, 2003) and physiology of freezing tolerance (Wharton et al., 2000). Thus it is surprising to find that this is not only the first report of cyclic gas exchange (CGE) in this family, but the first study of any aspect of its metabolic physiology.

### *Cyclic gas exchange and discontinuous gas exchange-cycles*

The pattern of CGE exhibited by *C. armatus* in many cases presents the C-, F-, O-phases with considerable intraspecific variation. Actually shifts to continuous gas exchange in the same individuals and even in the same record are common, as in most insects that exhibit DGC or CGE (Marais et al., 2005).

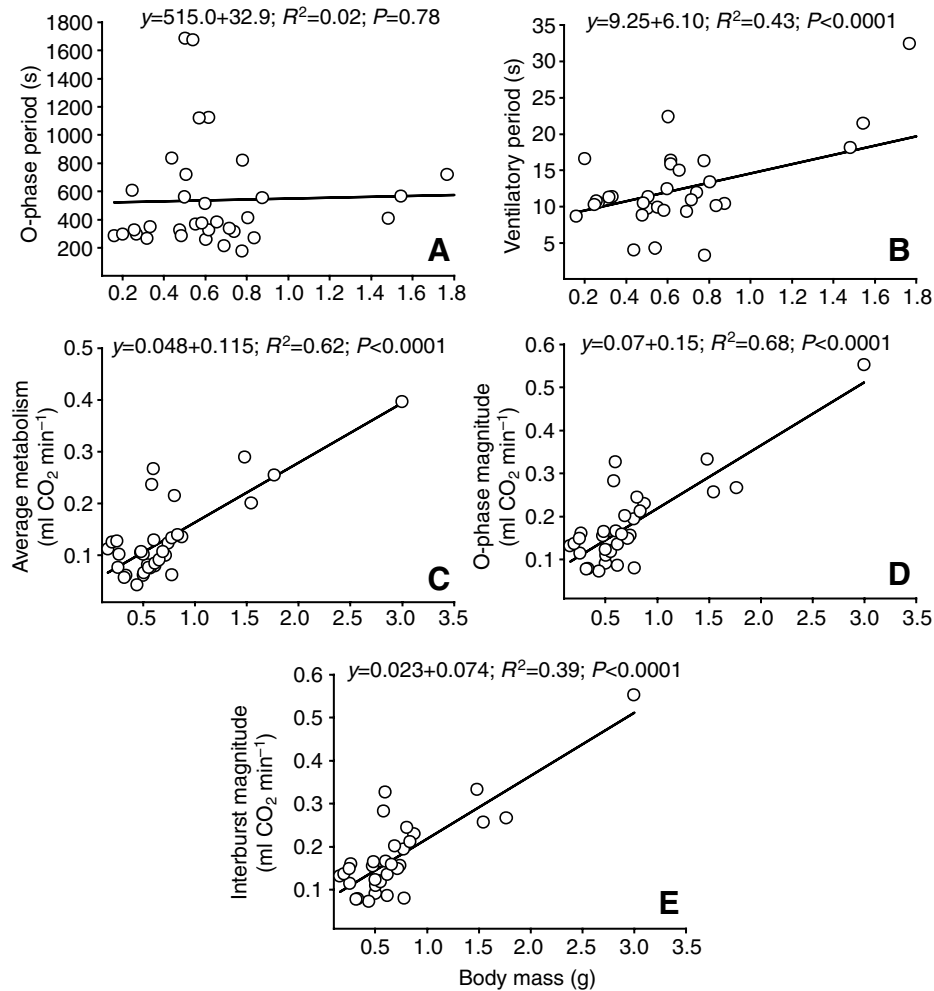


Fig. 3. Regressions between metabolic variables and body mass. (A) O-phase period; (B) ventilatory period; (C) average metabolism; (D) O-phase magnitude; (E) interburst magnitude. All relationships are significant except for O-phase period (A).

Table 2. Repeatability as intraclass correlation coefficient ( $\tau$ ), obtained from variance components in a one-way ANCOVA using body mass as covariable

Variable	Variance component (statistics in parentheses)			$\tau$
	Between individuals	Between repetitions, within individuals	Within repetitions, within individuals (=Error)	
O-phase period	<b>20850.2</b> ( $F_{10,10}=6.00$ ; $P=0.004$ )	–	7511.0	<b>0.74</b>
Ventilatory period	<b>6.43</b> ( $F_{12,12}=3.81$ ; $P=0.014$ )	<b>3.35</b> ( $F_{12,65}=4.97$ ; $P<0.0001$ )	2.95	<b>0.51</b>
Mean $\dot{V}_{CO_2}$ (ml $CO_2$ min $^{-1}$ )	0.0011 ( $F_{10,10}=1.92$ ; $P=0.16$ )	–	0.0022	0.34
O-phase amplitude (ml $CO_2$ min $^{-1}$ )	0.0005 ( $F_{10,10}=1.15$ ; $P=0.41$ )	<b>0.0055</b> ( $F_{10,57}=18.9$ ; $P<0.0001$ )	0.0011	0.048
Interburst (ml $CO_2$ min $^{-1}$ )	0.0010 ( $F_{10,10}=2.01$ ; $P=0.15$ )	0.00068 ( $F_{10,57}=1.67$ ; $P=0.11$ )	0.0036	0.18

In some cases (ventilatory period, O-phase period and interburst period) several measurements were taken within each record, which were included as a nested factor (i.e. the 'between repetitions, within individuals' column).

Significant components are highlighted in bold type.

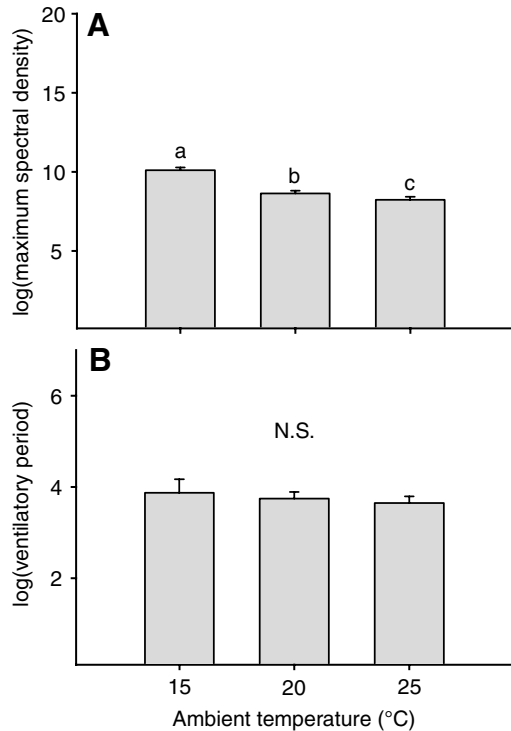


Fig. 4. Logarithms of O-phase duration (A) and ventilatory period (B) (see Materials and methods for details) in individuals of *C. armatus* at three different temperatures. Values are adjusted means  $\pm$  s.e.m. *N* values, see Table 1. Different letters denote significant differences (Tukey *post-hoc* tests); N.S., not significant.

It has been suggested that DGC should be a lot more common in insects than actually reported because low flow rates combined with large metabolic chambers would prevent records falling to zero during the C-phase due to incomplete washout (Gray and Bradley, 2006). We believe this is not the case with our data, since the combination of chamber size (8 ml) and flow rate ( $100 \text{ ml min}^{-1}$ ) that we used gives a time constant of 0.08 min. The time required for the partial pressure of  $\text{CO}_2$  in the chamber to be diluted to 1% of its peak value is 5 times its time constant (i.e. 24 s) (Gray and Bradley, 2006). Considering that in most cases the duration of the interburst in our CGE records ranged from 5 to 25 min, and in no case did  $\dot{V}_{\text{CO}_2}$  reach zero, it is unlikely that *C. armatus* would be experiencing DGC and we were not capable of detecting it.

The components of CGE that we studied were similar to those reported in previous studies of DGC/CGE in insects (Chappell and Rogowitz, 2000; Marais and Chown, 2003) since although cyclic records were clearly identified, they exhibited considerable variation, from showing the three phases, to only showing peaks and valleys that never reached zero (i.e. the spiracle was never completely closed). The O-phase was characterized by conspicuous ventilatory movements (i.e. the O-phase is predominantly convective) that produced high frequency cycles, which yielded highly consistent results over time across individuals.

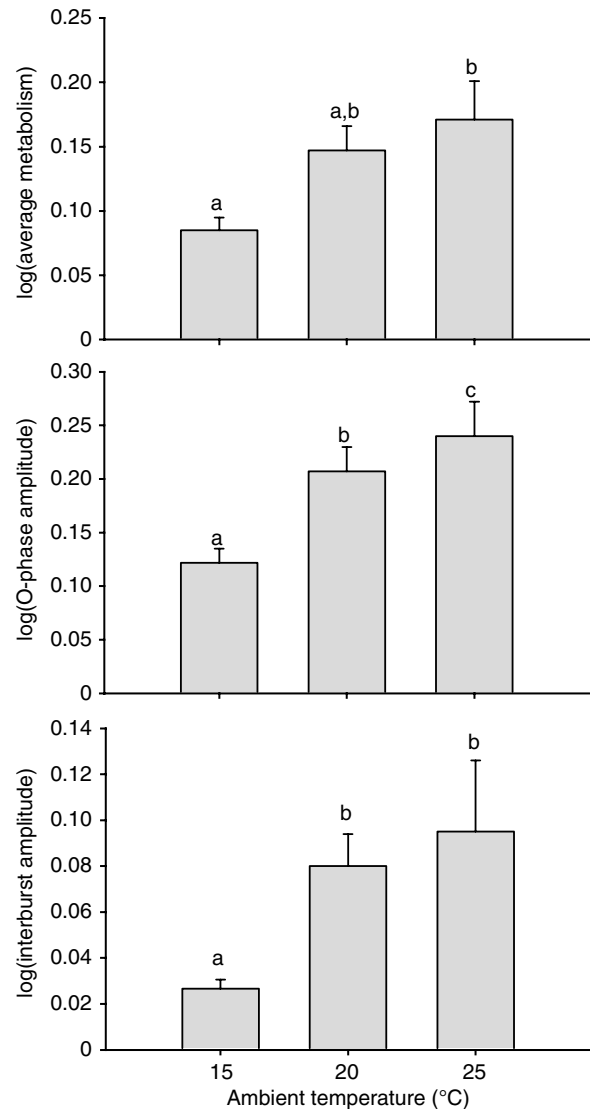


Fig. 5. Logarithms of (A) average, (B) O-phase and (C) interbursts (see Materials and methods for details) in individuals of *C. armatus* at three different temperatures. Values are adjusted means  $\pm$  s.e.m. *N* values, see Table 1. Different letters denote significant differences (Tukey *post-hoc* tests).

#### Temperature

As reported in other species (and expected in an ectotherm), the amplitude of the variables that we determined are strongly temperature-dependent (Chappell and Rogowitz, 2000; Terblanche et al., 2004a; Terblanche et al., 2004b). However, there is variation in the duration of the O-period. In most species, a higher ambient temperature induces an increase in the frequency of cycles (or a reduction in the O-period) (Lighton, 1988; Quinlan and Lighton, 1999; Voght and Appel, 2000; Chappell and Rogowitz, 2000). In many species, however, there is no dependence of the O-period or volume on temperature (Davis et al., 1999; Shelton and Appel, 2001). Our results suggest an intermediate response since the O-period was

considerably reduced at the lowest temperature (15°C), but changed little between moderate to high temperatures (20–25°C). It is striking to note that no study has determined the thermal dependence of the ventilatory period in those cases of convective O-phase, especially considering that convective gas-exchange is a necessity for large insects (Kestler, 1985). This variable would be an indication of the effort required by the insect to flush out excess CO<sub>2</sub> during the open phase, and hence could be important for survival. In addition, as with other convective systems such as vertebrate lungs, as the size of the chamber increases (i.e. the volume of the tracheal system), the duration of the cycle is also extended, which is consistent with the significant correlation with body mass that we found.

#### Repeatability

Although many authors have demonstrated that insect standard metabolic rate is repeatable (Chappell and Rogowitz, 2000; Nespolo et al., 2003; Marais and Chown, 2003; Terblanche et al., 2004a), we could not do so for three of its components (i.e. average  $V_{CO_2}$ , O-phase and interburst amplitudes). However, our low sample size precluded any statistically significant conclusion regarding the absence of repeatability (i.e. repeatabilities <0.4 are not detectable with a sample size of  $N=7$ ). In contrast, and even with our relatively low sample size, the repeatability of both ventilatory and O-phase duration was high and significant. Our O-period (estimated by spectral density analysis) was comparable to the O-phase duration computed (from visual inspection) in cerambycid beetles (Chappell and Rogowitz, 2000), or the several variants of the O-period reported for a cockroach (Marais and Chown, 2003) using a simple visual test (Marais et al., 2005). In all three cases, repeatabilities were the largest among the computed metabolic variables in each study, which suggests some generality regarding the inter-individual variation in the O-phase and perhaps its potential to respond to natural selection (Dohm, 2002; Chown et al., 2006).

#### Inferring adaptation

According to Chown et al., there are three requisites necessary to demonstrate the adaptive significance of a trait: (1) that it exhibits significant repeatability; (2) that there exists a consistent relationship between the trait and fitness; and (3) that it exhibits high heritability (Chown et al., 2006).

In the present study, we show that the first condition is met, since at least two of the components of metabolism in *C. armatus* were highly repeatable (see also Chappell and Rogowitz, 2000; Nespolo et al., 2003; Marais and Chown, 2003). Given that *C. armatus* is fossorial and lives in humid soils of temperate forests in the Southern hemisphere (Angulo, 2001), our results would support the concept of adaptation to a hypercapnic/hypoxic environment [the ‘chthonic-hygic’ hypothesis (see Lighton, 1996; Chown et al., 2006)]. This statement could be a sign that the putative relationship between the trait and fitness could exist, and hence provide some support for the second requisite to infer adaptation. However, there are actually no data with which to assess the third

requisite (i.e. the trait presents high heritability). So it is clear that although the many published studies to date have been useful in characterizing the cyclic mode of respiration in insects and other arthropods, its phylogenetic relationships and its mechanistic causes, further research is needed in order to answer the simple question: is CGE/DGC an adaptive feature of insects?

#### List of abbreviations

CGE	cyclic gas exchange
C-phase	‘closed’ phase
DCG	discontinuous gas-exchange cycle
F-phase	‘flutter’ phase
$M_b$	body mass
MR	rate of metabolism
O-phase	‘open’ phase
SDE	spectral density estimation
$t$	time
$\dot{V}_{CO_2}$	rate of CO <sub>2</sub> production
$\tau$	intraclass correlation coefficient
$\lambda$	frequency

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