



## The effects of temperature on the gas exchange cycle in *Agathemera crassa*

Mariana Thienel <sup>a</sup>, Mauricio Canals <sup>a</sup>, Francisco Bozinovic <sup>b</sup>, Claudio Veloso <sup>a,\*</sup>

<sup>a</sup> Departamento de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile

<sup>b</sup> Departamento de Ecología and Center of Applied Ecology and Sustainability (CAPES), Facultad de Ciencias Biológicas, Universidad Católica de Chile, Santiago 6513677, Chile

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### ABSTRACT

Insects exhibit three patterns of gas exchange: continuous (CoGE), cyclic (CGE) and discontinuous (DGE). In this work, we present the first record of a DGE in Phasmatodea and its transition to CGE and to CoGE through a thermal gradient. The rate of CO<sub>2</sub> production (VCO<sub>2</sub>) at 10, 20 and 30 °C was examined in adults of *Agathemera crassa*, a high-Andean phasmid of central Chile. Carbon dioxide release was recorded during 24 h with L:D cycle of 12:12 h in order to record both rest and activity periods. At rest, *A. crassa* showed three patterns of gas exchange, highlighting the use of DGE preferably at 10 °C. As the temperature increased, the CoGE pattern was more frequent being the only pattern observed in all individuals at 30 °C. During activity, patterns changed to CoGE with a significant increase in VCO<sub>2</sub>. Our results support the idea that gas exchange patterns in insects are not distinct but correspond to a continuum of responses addressed by metabolic demand and where DGE can be expressed only under an absolute state of rest. Our results support the idea that the presence of the DGE may be underestimated in other insect taxa because they may have been measured under conditions where this pattern not necessarily can be expressed.

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### 1. Introduction

Gas exchange among insects involves a complex process that depends on environmental factors such as temperature, availability of water and the hypoxia/hypercapnia condition (Chown and Nicolson, 2004). Gas exchange patterns among insects have been classified into three main types: continuous gas exchange (CoGE), cyclic gas exchange (CGE) and discontinuous gas exchange (DGE) (Gibbs and Johnson, 2004). According to Chown and Nicolson (2004), in the CoGE pattern the spiracles held open with high carbon dioxide release, while in the CGE pattern the spiracles are partially open generating periodic peaks of CO<sub>2</sub> production followed by valleys of low CO<sub>2</sub> release. By contrast according to Buck et al. (1953) and Schneiderman (1960), DGE is characterized by a dynamic activity of spiracles, where it is possible to distinguish three phases: 1) the O phase (open), which corresponds to the peak of CO<sub>2</sub> release generated by the total opening of the spiracles, 2) the C phase (closed), which is generated by the total closure of the spiracles and 3) the F phase

(flutter), where there is a slight release of CO<sub>2</sub> above the stage as a product of intermittent opening of the spiracles

From a phylogenetic study, Marais et al. (2005) suggested that the CGE is the ancestral character of the group. DGE appears to have evolved independently in few species of five orders, which also show cyclic and continuous patterns. There have been at least seven hypotheses to explain the origin of discontinuous and cyclic gas exchange (see Chown et al., 2006; Chown, 2011 for details and discussions): (1) the water hypothesis (Buck et al., 1953; Levy and Schneiderman, 1966); (2) the water-chthonic hypothesis (Lighton and Berrigan, 1995); (3) the oxidative damage hypothesis (Hetz and Bradley, 2005), (4) the emergent property hypothesis (Chown and Holter, 2000), (5) the 'strolling arthropod' hypothesis (Harrison et al., 2001); (6) the 'continuum hypothesis' (Contreras and Bradley, 2010) and (7) the neural hypothesis (Matthews and White, 2011). Unfortunately, the study of CGE and DGE in insects has been biased in terms of species diversity, mainly holometabolous insects (Chown et al., 2006). Indeed, for the order Phasmatodea there is a lack of information about gas exchange; however, the patterns described so far show the presence of the continuous and cyclic mechanisms (Marais et al., 2005).

Given this background, and based on assumptions regarding the mechanisms of the origin of cyclic and discontinuous gas exchange, in

\* Corresponding author at: Departamento de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile, Santiago, Chile. Tel.: +56 2 29787362.

E-mail address: [cveloso@uchile.cl](mailto:cveloso@uchile.cl) (C. Veloso).

this work we tested the effect of ambient temperature on the appearance of different gas exchange patterns in *Agathemera crassa* (Phasmatodea). Thus with an increase in temperature, it is expected a transition between patterns associated to the variation of cycle components (period, frequency and amplitude), from DGE, going by CGE and finally to CoGE. It is also expected that DGE will be more pronounced during rest periods, and disappear or be modified to CoGE or CGE during activity periods. We studied this problem in the hemimetabolous phasmid *A. crassa* (Phasmatodea: Agathemeridae) from the Andes range

## 2. Material and methods

### 2.1. Capture and maintenance

*A. crassa* use microhabitats under and between rocks, which are generally located near their main food source, the plant *Acaena splendens* (Rosaceae) (C. Veloso personal observations). *A. crassa* possesses sexual dimorphism. Indeed, body mass of males and females reaches as maximum of 4 and 13 g, respectively. This species inhabits the Andes in central Chile at 1900–3000 m a.s.l. (Camousseight, 1995; Vera et al., 2012). In this habitat, climate is dry and warm during summer and cold/snowy during winter (Hoffmann et al., 1998).

During early austral spring of 2010, we captured a total of 20 adults (10 females and 10 males) from the Andean locality of Lagunillas ( $33^{\circ}25'S$ ;  $71^{\circ}28'W$ , at 2400 m a.s.l.) in central Chile. Once transferred to the laboratory, animals were placed in plastic boxes separated by sex within thermocontrolled chambers with daily photoperiods L:D = 12:12 at fluctuating temperatures ranging from 20 to 5 °C (these fluctuations are similar to natural conditions) and fed ad libitum with fresh food (*A. splendens*). Body mass ( $m_b$ ) for males was on average  $2.4 \pm 0.34$  and for females it was  $5.68 \pm 0.84$  g.

### 2.2. Respirometry

We followed the respirometric protocol proposed for insects by Chappell and Rogowitz (2000), Lighton and Turner (2004) and Nespolo et al. (2007), consisting of an open continuous flow system ( $200 \text{ ml min}^{-1}$ ), in which  $\text{CO}_2$  and water were removed from the air before the metabolic chamber through columns of barium hydroxide lime (Baralyme®) and calcium sulfate anhydride (Drierite®), respectively. Air entered the metabolic glass chamber (120 ml) located within a photo- and thermo-regulated cabinet. Subsequently, the air flowed through a water vapor analyzer Sable Systems International® (Las Vegas, Nevada, USA), model RH-300, after which the air was filtered with Drierite®, and then passed through a Sable  $\text{CO}_2$  Systems International® FOXBOX model, and monitored every 5 s. An average of ten minimum values of  $\text{CO}_2$  production were selected. Data were corrected by STP and drift (baseline). Finally, data was analyzed using the ExpeData software (Sable Systems International®) version 1.0.3.

Measurements of  $\text{VCO}_2$  were made for 24 h (in order to record periods of activity and rest) at three contrasting ambient temperatures (10, 20 and 30 °C) with a photoperiod L:D = 12:12 h. Each individual was measured only once at one temperature in a random order. Activity period was determined by visual inspection, as the abrupt and sustained increase of  $\text{CO}_2$  production during dark conditions, while rest period was identified as all the remaining trace during both dark and light conditions.

### 2.3. Determining patterns of gas exchange

Records of  $\text{CO}_2$  production were categorized as continuous, cyclic or discontinuous first by inspection, and then according to the method proposed by Marais et al. (2005): from the raw  $\text{VCO}_2$  trace, the gas exchange pattern was termed CGE or DGE when <30% of data points lay above the average  $\text{VCO}_2$  line, then DGE is readily identified due to

the presence of phases C and F. In contrast, when >30% of data points are above the average line, the gas exchange pattern termed was CoGE. In those individuals in whose measurement was observed more than one gas exchange pattern, we used the simple majority rule proposed by Terblanche et al. (2008). Evaluated components of those gas exchange patterns that showed cyclicity (DGE and CGE), were  $\text{CO}_2$  emission rate ( $\text{VCO}_2$ ), frequency, period and duration of each phase (O, F and C in DGE, valleys and peak in CGE). The CoGE pattern was only analyzed based on  $\text{VCO}_2$ . We used 10 samples for calculating mean  $\text{VCO}_2$

### 2.4. Statistical analysis

Statistical analyses were performed using parametric and nonparametric tests, depending on the type of data and compliance with the assumptions of normality, homoscedasticity and independence. The effect of temperature on mass-specific  $\text{VCO}_2$  was studied by repeated measures analysis of variance (ANOVA), which was applied after an analysis of covariance (ANCOVA) using body mass ( $m_b$ ) as covariate. To assess the proportion of gas exchange patterns between temperatures we used the Chi-square test. To compare  $\text{VCO}_2$  between the period of activity and rest, we applied the Wilcoxon test (for pattern and heat treatment). All statistical analyses were conducted using Statistica 6.0 software (Statsoft Inc., Tulsa, OK).

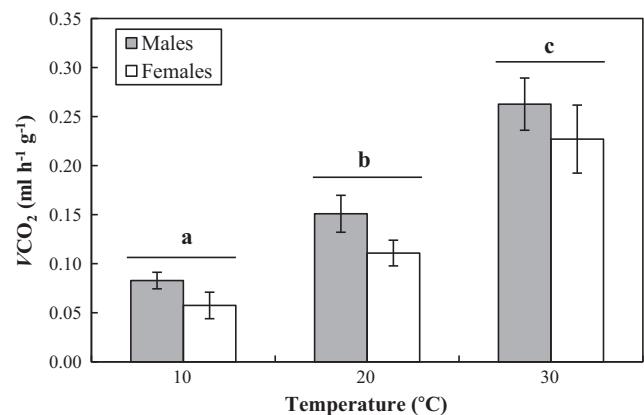
## 3. Results

### 3.1. $\text{CO}_2$ production

Increasing temperature caused a significant increase in mass-specific  $\text{VCO}_2$  (ANOVA:  $F_{2,30} = 430.83, P < 0.0001$ ). By controlling for body mass in each temperature treatment, it was observed that both males and females release the same amount of  $\text{CO}_2$  (ANCOVA at 10 °C:  $F_{1,14} = 0.026, P = 0.87$ . At 20 °C:  $F_{1,14} = 0.505, P = 0.49$ . At 30 °C:  $F_{1,14} = 0.006, P = 0.94$ ) (Fig. 1).

### 3.2. Gas exchange patterns and temperature

Among all thermal treatments we observed a dissimilar proportion of gas exchange patterns (Table 1). At 10 °C, we observed only gas exchange patterns with cyclicity: 85% of 20 individuals presented DGE while the remaining 15% exhibited CGE. At 20 °C, insects showed three patterns: 60% of 20 individuals had CoGE, 25% exhibited CGE and 15% showed DGE. Finally, at 30 °C we observed no presence of cyclical patterns—i.e. all twenty individuals exhibited CoGE. We observed significant differences between the proportion of gas exchange patterns



**Fig. 1.** Temperature effect on  $\text{CO}_2$  production rate. Values are indicated as mean  $\pm$  SD. Different letters correspond to significant differences between thermal treatments given by repeated measurements ANOVA.

**Table 1**

Percentage of the presence of gas exchange patterns in males and females of *Agathemera crassa* in the measured temperatures.

	10 °C (n = 20)			20 °C (n = 20)			30 °C (n = 20)		
	DGE	CGE	CoGE	DGE	CGE	CoGE	DGE	CGE	CoGE
Males	80%	20%	0%	20%	20%	60%	0%	0%	100%
Females	90%	10%	0%	10%	30%	60%	0%	0%	100%

among treatments ( $X^2 = 48.45$ ,  $df = 4$ ,  $P < 0.0001$ ). At 10 °C there was a significant presence of cyclical patterns (DGE and CGE) in comparison to 20 °C and even more to 30 °C (Table 2). Also, these patterns were more frequent at 20 °C than at 30 °C. There were no significant differences of the proportion of gas exchange patterns between males and females in any temperature ( $X^2 = 0.925$ ,  $df = 5$ ,  $P = 0.97$ ).

The presence of DGE was observed at 10 and 20 °C only at rest, where we identified the three phases that characterize this breathing pattern (Table 3). However, although VCO<sub>2</sub> values were closed to zero during the C phase, CO<sub>2</sub> values never reached absolute zero. Fig. 2A and B shows two records representing DGE at 10 and 20 °C, respectively. In terms of the effect of temperature on the DGE components, we observed a direct and significant effect over amplitude (Mann–Whitney test  $Z = -2.268$ ,  $P < 0.023$ ) and cycle frequency ( $Z = -2.646$ ,  $P < 0.008$ ) with an increase of 1.5- and 2.5-fold, respectively.

The presence of CGE was observed at 10 and 20 °C, only at rest. It was possible to identify both valleys and peaks of CO<sub>2</sub> production, regularly distributed over the record (Table 4). Fig. 2C and D shows two representative records of CGE, at 10 and 20 °C, respectively. We observed a direct and significant dependence of temperature on the cycle frequency ( $Z = -2.23$ ,  $P < 0.025$ ), with a 3-fold increase in value between 10 and 20 °C. Inversely, the cycle period dropped with temperature ( $Z = 2.23$ ,  $P < 0.025$ ), decreasing approximately 2.5-fold from 10 to 20 °C. No differences were found in peak amplitudes between 10 and 20 °C in this pattern ( $Z = -1.64$ ,  $P = 0.1$ ).

The CoGE pattern was recorded both at rest as well during activity periods. During rest, CoGE was only observed at 20 and 30 °C, while during activity, this pattern was always present in all thermal treatments. Table 5 describes mean values of VCO<sub>2</sub> at 20 and 30 °C, and Fig. 2E and F shows two representative records of CoGE patterns at rest.

Regarding CO<sub>2</sub> production during activity, it was observed that at 10 °C, 14 of 17 individuals who exhibited respiratory discontinuity (DGE) at rest changed their trace to a continuous one (CoGE); disappearing the cycle and its phases (Fig. 3). The 3 individuals remaining showed no activity period, keeping DGE throughout all measurement. Also, all those individuals who exhibited CGE at rest changed their CO<sub>2</sub> production to CoGE in activity. Metabolic rate during activity at 10 °C in individuals who had DGE was significantly greater than at rest (Wilcoxon test:  $Z = 3.30$ ,  $P < 0.001$ ). Nevertheless a note of caution is necessary here. The idea that CO<sub>2</sub> did not reach zero between peaks should also be a result of the low flow rate that was used.

Because no significant differences in metabolic rates between individuals with different gas exchange patterns (DGE, CGE and CoGE) were found, we performed an analysis that considered all individuals measured at 20 °C in which it was possible to distinguish their activity period. The Wilcoxon test for paired samples showed significant differences in metabolic rate between the two periods ( $Z = 3.82$ ,  $P = 0.0001$ ), with a total increase in metabolic rate of 65%. Finally, individuals measured at 30 °C (all showed CoGE) whose periods of activity could be

**Table 3**

Descriptive results (mean ± SD values) of the discontinuous gas exchange pattern.

DGE	10 °C		20 °C	
	Males (n = 8)	Females (n = 9)	Males (n = 2)	Females (n = 1)
M <sub>b</sub> (g)	2.33 ± 0.23	5.75 ± 0.86	2.33 ± 0.18	5.49 ± (-)
VCO <sub>2</sub> ((ml h <sup>-1</sup> )	0.16 ± 0.02	0.29 ± 0.07	0.31 ± 0.03	0.61 ± (-)
Amplitude (ml h <sup>-1</sup> )	0.55 ± 0.23	1.05 ± 0.48	0.77 ± 0.16	2.13 ± (-)
Frequency (mHz)	0.24 ± 0.14	0.19 ± 0.07	0.92 ± 0.44	0.88 ± (-)
Period (h)	1.98 ± 1.20	1.78 ± 0.56	0.38 ± 0.15	0.35 ± (-)
C phase				
Duration (min)	21.90 ± 7.95	28.14 ± 11.07	6.89 ± 1.96	6.34 ± (-)
VCO <sub>2</sub> (ml h <sup>-1</sup> )	0.08 ± 0.02	0.14 ± 0.06	0.12 ± 0.01	0.18 ± (-)
F phase				
Duration (min)	82.48 ± 55.40	74.58 ± 49.40	12.14 ± 4.74	10.91 ± (-)
VCO <sub>2</sub> (ml h <sup>-1</sup> )	0.10 ± 0.02	0.19 ± 0.08	0.18 ± 0.03	0.28 ± (-)
O phase				
Duration (min)	16.53 ± 2.59	19.04 ± 1.59	11.32 ± 2.82	7.59 ± (-)
VCO <sub>2</sub> (ml h <sup>-1</sup> )	0.40 ± 0.10	0.76 ± 0.24	0.56 ± 0.08	1.22 ± (-)

detected showed significant differences in metabolic rate between the period of activity and resting (Wilcoxon:  $Z = 2.67$ ,  $P = 0.008$ ), with a total increase in metabolic rate of 53%.

#### 4. Discussion

*A. crassa* exhibited the 3 gas exchange patterns described in the literature. Ambient temperature and activity period determined when these patterns were displayed by these insects. The presence of the DGE pattern was associated with low and moderate temperatures (10 and 20 °C), with great predominance at 10 °C but only at rest (Figs. 1, 2 and 3). We identified this gas exchange pattern although CO<sub>2</sub> release values never reached zero, because it is assumed that part of this molecule is released via cuticle, above all considering the large body size of this species.

The CGE pattern was exhibited at the same temperatures as DGE, also at rest; however, it was not dominant. The appearance of the CGE was associated with moderate and high temperatures (20 and 30 °C) and both during rest and activity periods. DGE (and CGE) was displayed only during the resting phase (daytime). During the period of activity (nighttime) all cyclicity disappeared, modifying production to a continuous CO<sub>2</sub> release, with a consequent increase in metabolic rate. The foregoing agrees with Chown (2011), indicating that patterns with cycles have always been observed at rest.

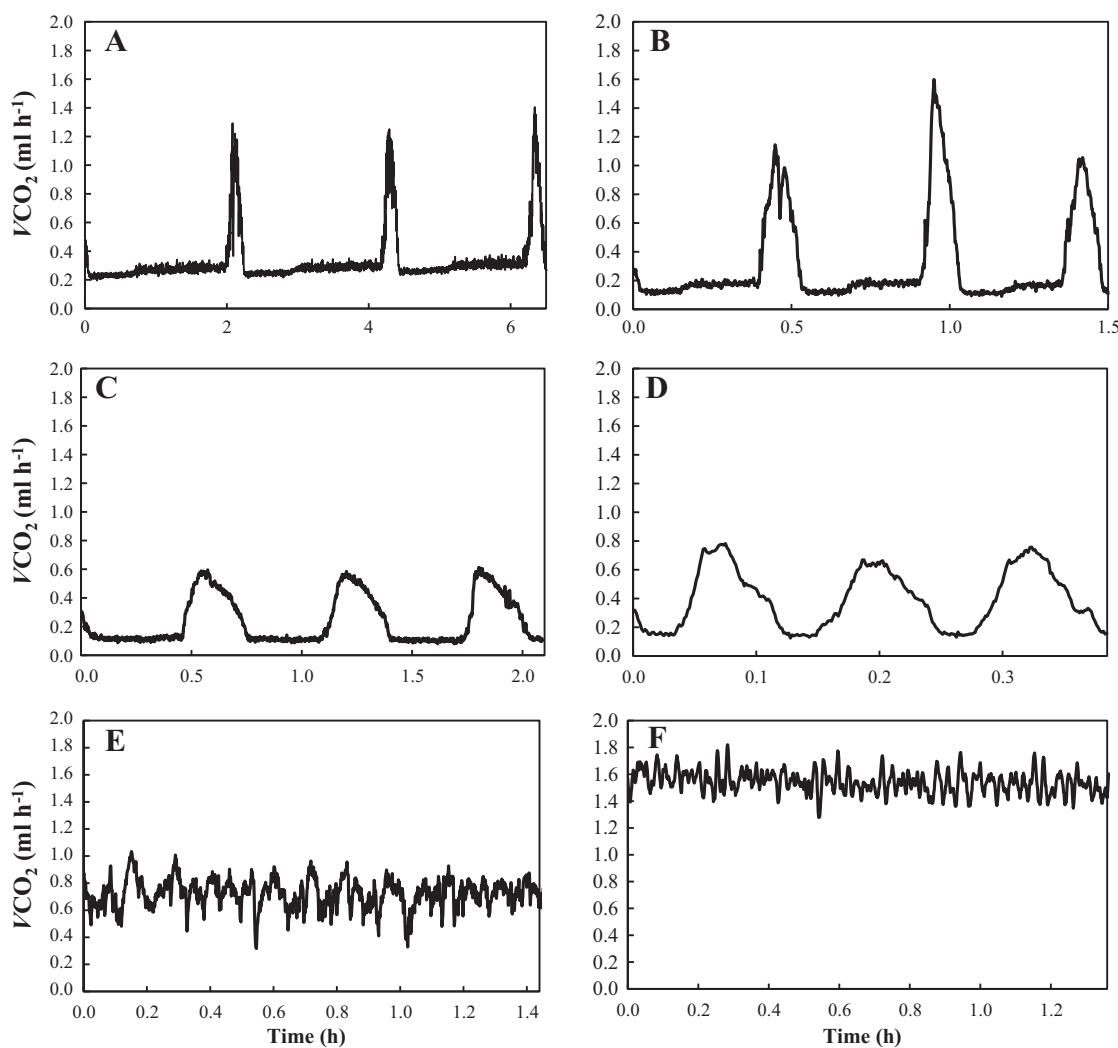
The influence of temperature on the components of the gas exchange cycles in insects has been well documented (Davis et al., 1999; Chappell and Rogowitz, 2000; Lighton and Joos, 2002; Chown and Davis, 2003; Contreras and Bradley, 2010, among others). As expected for ectotherms, they all agree that an increase in temperature, with a consequent increase in metabolic rate, is accompanied by an increase in frequency and a decrease in the duration of phases. In *A. crassa* the behavior of the components of the DGE and CGE cycles according to temperature is quite similar to those described in the literature. In particular, the cycle duration of this phasmid DGE was determined mainly by phase C + F, corresponding to 85 and 66% of the total cycle at 10 and 20 °C respectively, while phase O contributed to a low ratio. However, although the O phase occupied a small fraction of the DGE, values of VCO<sub>2</sub> production rate were always high, thus showing that it is during this phase that the greatest gas exchange cycle occurs, in agreement with the reports of Lighton (1996), Chown et al. (2006) and Quinlan and Gibbs (2006).

Our results support the idea that gas exchange patterns are not distinct but correspond to a transition of respiratory responses as a

**Table 2**

Chi-square for gas exchange patterns proportion presented in the temperatures measured.

Treatment (°C)	Chi-square ( $X^2$ )	df	p
10–20	22.3	2	<<0.0001
10–30	40.0	2	<<0.0001
20–30	10.0	2	0.007



**Fig. 2.** Representative examples of gas exchange patterns at three temperatures: DGE at 10 °C (A), DGE at 20 °C (B), CGE at 10 °C (C), CGE at 20 °C (D), CoGE at 20 °C (E) and CoGE at 30 °C (F).

continuum that starts from DGE, goes by CGE and finishes in CoGE, which is driven by the increase of metabolic demand experienced by the insect and whose values would vary so that it would not be possible to consider them as a discrete feature (Bradley, 2007; Contreras and Bradley, 2010; Contreras and Bradley, 2011). Thus, gas exchange pattern transition would be explained by spiracular dynamics, which are mediated by ambient temperature (Lehmann and Schützner, 2010)

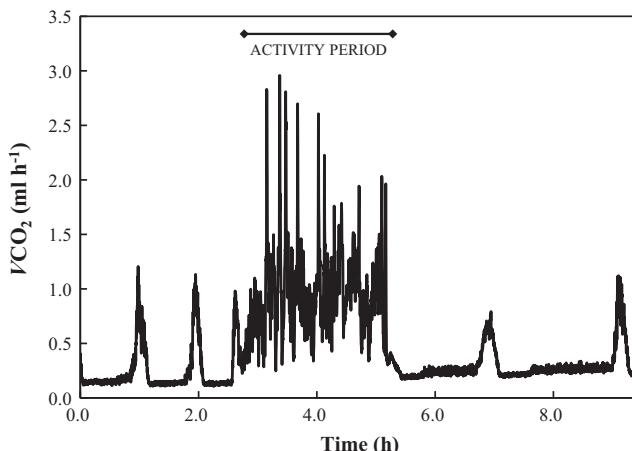
**Table 4**  
Descriptive results (mean  $\pm$  SD values) of the cyclic gas exchange pattern.

CGE	10 °C		20 °C	
	Males (n = 2)	Females (n = 1)	Males (n = 2)	Females (n = 3)
$M_b$ (g)	2.70 $\pm$ 0.64	5.02 $\pm$ (—)	2.40 $\pm$ 0.20	5.75 $\pm$ 0.72
$VCO_2$ (ml h $^{-1}$ )	0.06 $\pm$ 0.01	0.07 $\pm$ (—)	0.10 $\pm$ 0.04	0.13 $\pm$ 0.07
Amplitude (ml h $^{-1}$ )	0.35 $\pm$ 0.17	0.35 $\pm$ (—)	0.36 $\pm$ 0.03	1.27 $\pm$ 0.74
Frequency (mHz)	0.56 $\pm$ 0.01	0.44 $\pm$ (—)	2.30 $\pm$ 0.34	1.83 $\pm$ 0.01
Period (h)	0.57 $\pm$ 0.02	0.63 $\pm$ (—)	0.13 $\pm$ 0.01	0.15 $\pm$ 0.01
<i>Valley</i>				
Duration (min)	15.53 $\pm$ 6.90	19.79 $\pm$ (—)	2.96 $\pm$ 0.95	2.37 $\pm$ 0.56
$VCO_2$ (ml h $^{-1}$ )	0.10 $\pm$ 0.01	0.11 $\pm$ (—)	0.16 $\pm$ 0.006	0.22 $\pm$ 0.06
<i>Peak</i>				
Duration (min)	18.03 $\pm$ 4.24	20.19 $\pm$ (—)	5.67 $\pm$ 0.23	6.91 $\pm$ 0.34
$VCO_2$ (ml h $^{-1}$ )	0.31 $\pm$ 0.07	3.58 $\pm$ (—)	0.48 $\pm$ 0.02	0.85 $\pm$ 0.40

and where DGE predominates at low demand, when closed spiracles are the norm. Then, as metabolic demand increases, gas exchange would become cyclical, with the spiracles not completely closed. Finally, CoGE occurs during high metabolic demand when the spiracles are open. In addition, Basson and Terblanche (2011) proposed that variation in gas exchange patterns at short time scales would be given by the interaction between cellular metabolic demand and the structural and physiological design of the system of gas exchange (spiracular tracheal system and behavior) and by the buffering capacity of CO<sub>2</sub>. However, we also observed the presence of two or three different patterns at the same temperature. Hadley and Quinlan (1993), Williams and Bradley (1998) and Williams et al. (2010) postulated that marginal differences in some cycle components, as well in metabolic rate, do not necessarily differ between gas exchange patterns, making it difficult to determine a pattern (especially between DGE and CGE).

**Table 5**  
Descriptive results (mean  $\pm$  SD values) of the continuous gas exchange pattern.

CoGE	20 °C		30 °C	
	Males (n = 6)	Females (n = 6)	Males (n = 10)	Females (n = 10)
$M_b$ (g)	2.28 $\pm$ 0.24	5.68 $\pm$ 1.03	2.40 $\pm$ 0.34	5.68 $\pm$ 0.84
Mean $VCO_2$ (ml h $^{-1}$ )	0.37 $\pm$ 0.06	0.72 $\pm$ 0.22	0.67 $\pm$ 0.11	1.54 $\pm$ 0.39



**Fig. 3.** Representative example of DGE loss during the activity period of *A. crassa*.

When the activity period starts cycles despair. This suggests that during this period insect requires a higher ventilation due to an increase in metabolic demand. When the activity period finishes and the insects are at metabolic rest then, gas exchange returns to cyclicity. This phenomenon seems to be associated with locomotion cost (Chown and Nicolson, 2004; Harrison et al., 1991).

Support for this hypothesis does not mean that other hypotheses are not acting, because there are multiple independent evolutionary origins explaining the emergence of DGE and not a single function underlying their genesis (Matthews and White, 2012). Thus, it remains open the interesting possibility to test multiples ambient factors that can be acting in gas exchange patterns transition in *A. crassa*, as for instance, hypoxia and hypercapnia, given the underground Andean environment that this species inhabit.

Finally, the display of DGE in *A. crassa* mainly at both conditions low ambient temperature and at rest could imply that this pattern is underestimated in other taxa including insects and arthropods, because studies are conducted under conditions where DGE not necessarily would be expressed. Based in the phylogeny proposed by Marais et al. (2005) concerning the origin of DGE, this is the first empirical record of DGE in the order Phasmatodea.

#### List of abbreviations

CGE	cyclic gas exchange
CoGE	continuous gas exchange
DGE	discontinuous gas exchange
M <sub>b</sub>	body mass
VCO <sub>2</sub>	rate of CO <sub>2</sub> production

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