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RESEARCH ARTICLE

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

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ABSTRACT

The evolutionary origin and maintenance of discontinuous gas exchange (DGE) in tracheate arthropods are poorly understood and highly controversial. We investigated prioritization of abiotic factors in the gas exchange control cascade by examining oxygen, water and haemolymph pH regulation in the grasshopper *Paracinema tricolor*. Using a full-factorial design, grasshoppers were acclimated to hypoxic or hyperoxic (5% O_2, 40% O_2) gas conditions, or dehydrated or hydrated, whereafter their CO_2 release was measured under a range of O_2 and relative humidity (RH) conditions (5%, 21%, 40% O_2 and 5%, 60%, 90% RH). DGE was significantly less common in grasshoppers acclimated to dehydrating conditions compared with the other acclimations (hypoxia, 98%; hyperoxia, 100%; hydrated, 100%; dehydrated, 67%). Acclimation to dehydrating conditions resulted in a significant decrease in haemolymph pH from 7.0±0.3 to 6.6±0.1 (mean ± s.d., P=0.018) and also significantly increased the open (O)-phase duration under 5% O_2 treatment conditions (5% O_2, 44.1±29.3 min; 40% O_2, 15.8±8.0 min; 5% RH, 17.8±1.3 min; 60% RH, 24.0±9.7 min; 90% RH, 20.6±8.9 min). The observed acidosis could potentially explain the extension of the O-phase under low RH conditions, when it would perhaps seem more useful to reduce the O-phase to lower respiratory water loss. The results confirm that DGE occurrence and modulation are affected by multiple abiotic factors. A hierarchical framework for abiotic factors influencing DGE is proposed in which the following stressors are prioritized in decreasing order of importance: oxygen supply, CO_2 excretion and pH modulation, oxidative damage protection and water savings.

KEY WORDS: Discontinuous gas exchange, Prioritization, Water regulation, Oxidative damage, pH regulation.

INTRODUCTION

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

DGE is thought to have evolved primarily as a means to reduce respiratory water loss (hygric hypothesis) (Buck et al., 1953; Levy and Schneiderman, 1966; Kestler, 1985; Hadley and Quinlan, 1993; Hadley, 1994), because insects can control the O-phase and C-phase of their spiracles. By lengthening the C-phase and shortening the O-phase, insects can reduce the amount of respiratory water loss (Buck et al., 1953; Lighton, 1990; Hadley, 1994; Lighton, 1996; Chown and Davis, 2003). However, several competing hypotheses have also been proposed to explain the origin and maintenance of DGE: (1) the oxidative damage hypothesis – DGE is an adaptation to minimize oxidative damage to tissue by regulating intratracheal oxygen levels, while still ensuring adequate gas exchange, in a respiratory system evolved for high aerobic performance (Hetz and Bradley, 2005; Chown et al., 2006); (2) the chthonic hypothesis – DGE facilitates gas exchange under hypoxic and/or hypercapnic conditions (Lighton, 1996; Lighton, 1998); (3) the chthonic–hygric hypothesis – DGE is an adaptation to reduce respiratory water loss under hypoxic and/or hypercapnic conditions (Lighton and Berrigan, 1995); (4) the strolling arthropod hypothesis – DGE is an adaptation to reduce the risk of parasitic infestation of the tracheae by increasing the frequency of spiracle closure (Chown et al., 2006); and (5) the emergent property hypothesis – DGE is a non-adaptive outcome of interactions between O_2 and CO_2 set-points (Chown and Holter, 2000). Despite much research investigating the evolutionary origin and current maintenance of DGE, there is still no consensus regarding which of the proposed hypotheses is likely to be correct. New hypotheses have also been proposed, such as the neural hypothesis, which states that DGE is the consequence of the downregulation of brain activity (Matthews and White, 2011b; Matthews and White, 2013). Much recent support has been found for both the hygric and oxidative damage hypotheses (e.g. Hetz and Bradley, 2005; White et al., 2007; Terblanche et al., 2008; Schimpf et al., 2009; Williams et al., 2010; Schimpf et al., 2012; but see Boardman et al., 2012), suggesting that both may provide mechanistic explanations for the evolution of DGE. If this is indeed the case, the next logical step is to understand the prioritization of control within the organism, so further elucidating the regulation of gas exchange, and the integration of inputs and homeostatic setpoints in the system.

One potential way to investigate prioritization in the gas exchange control cascade is by the explicit manipulation of potentially

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List of symbols and abbreviations

C	closed phase
CF	closed/flutter phase
DGE	discontinuous gas exchange
GLZ	generalized linear model
O	open phase
P_{O_2}	partial pressure of O_2
RH	relative humidity
$\dot{V}_{CO_2}$	rate of CO_2 release
WLR	water loss rate

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

We aimed to investigate the prioritization of factors that influence discontinuous gas exchange. We did so by assessing a suite of *a priori* predictions for whole-organism responses (see Table 1) against empirical data collected from the grasshopper *Paracinema tricolor* (Thunberg 1815), which readily shows DGE at rest (Fig. 1). These predictions assume that tracheal and spiracle conductance is not a limiting factor and that morphology is not adjusted rapidly within the experimental time frame. The predictions were: (1) if water saving is the key, short-term priority of DGE, then DGE should be absent under conditions of high RH, but present under conditions of low RH, as it can clearly contribute to respiratory water conservation. Under conditions of low RH, O-phase duration should also decrease, while C-phase duration should increase; (2) if the prevention of oxidative damage is the key priority of DGE, the alteration of ambient RH conditions should have little or no effect on the presence or absence of DGE; however, at high ambient P_{O_2}, DGE should be present and cycle frequency should decrease (i.e. by increasing C-phase duration, while perhaps also decreasing O-phase duration) to lower the amount of oxidative damage to tissue; and (3) if haemolymph pH regulation is the key priority of DGE, DGE should be absent in dehydrated individuals because dehydration will lead to a decrease in haemolymph volume, which is likely to be problematic for the animal's acid–base status. Spiracles would therefore need to be kept open for longer so that the animal can release as much CO_2 as possible. Key to this idea is that respiratory acidosis is the main driver of pH change, though other changes due to haemolymph volume reduction may also be influential.

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

RESULTS

DGE was present under all experimental treatments in *P. tricolor* from both the hyperoxia and hydrated status acclimation conditions. For the hypoxia acclimation, only 1/40 individuals did not show DGE, while for the dehydration acclimation condition, only 24/36 (67%) of individuals maintained DGE (Table 2), differing significantly from all other acclimation treatments. Within this dehydration acclimation group, significantly fewer individuals in the

Table 1. Predictions for discontinuous gas exchange responses after acclimation to different conditions and exposure to varying environments

	Treatment		Oxidative damage hypothesis		Hygric hypothesis		Haemolymph pH
Acclimation	O_2	H_2O	DGE present?	Phase regulation	DGE present?	Phase regulation	DGE present?
Dehydrated	Low		No		Yes[a]	Shorter O and/or longer CF phase	No[f]
	High		Yes	Shorter O and/or longer CF phase	Yes[a]	Shorter O and/or longer CF phase	No[f]
		Low	No		Yes[a]	Shorter O and/or longer CF phase	No[f]
		High	No		No		No[f]
Hydrated	Low		No[d]		No		
	High		Yes[d]	Shorter O and/or longer CF phase[D]	No		
		Low	No		Yes[e]	Shorter O and/or longer CF phase[b,c]	
		High	No		No[e]		
Hypoxia	Low		No		No		
	High		Yes	Shorter O and/or longer CF phase	No		
		Low	No		Yes	Shorter O and/or longer CF phase	
		High	No		No		
Hyperoxia	Low		No		No		
	High		Yes	Shorter O and/or longer CF phase	No		
		Low	No		Yes	Shorter O and/or longer CF phase	
		High	No		No		

DGE, discontinuous gas exchange; O, open phase; CF, closed/flutter phase. Responses include presence/absence of DGE, as well as modulation of the different phases. Responses are explained as predicted by the different hypotheses or abiotic factors influencing DGE (O_2, H_2O and haemolymph pH). Hypoxia, 5% O_2; hyperoxia, 40% O_2.

Superscript letters indicate example references where the effect has been documented: [a]Schimpf et al., 2012; [b]Lighton, 1990; [c]Chown and Davis, 2003; [d]Terblanche et al., 2008; [e]Sláma et al., 2007; [f]Hadley and Quinlan, 1993.

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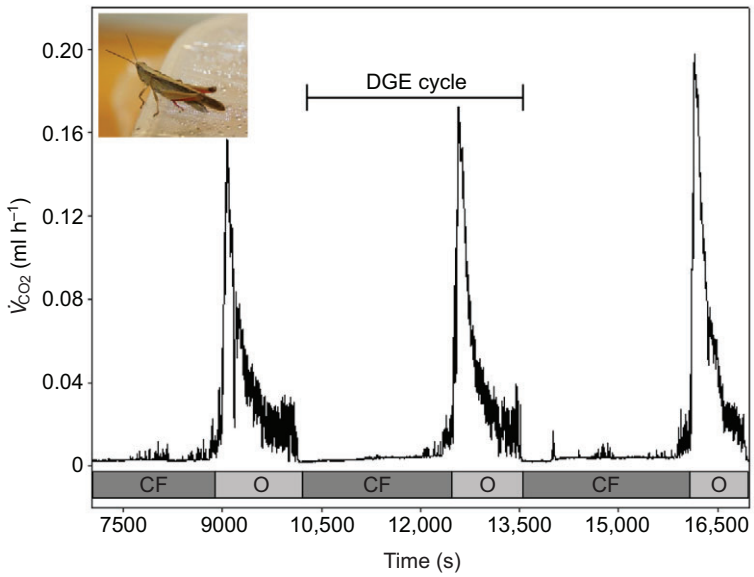

Fig. 1. Typical CO_2 emission trace of a 0.2442 g male *Paracinema tricolor* at 15°C. The individual (shown in the inset) was acclimated to 40% O_2 and recorded under treatment conditions of 21% O_2 and 60% RH. Flow rate was 200 ml min^{-1}. The different phases of the DGE cycle that were used for data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

5% RH and 5% O_2 treatment group maintained DGE than in the other treatment groups (Table 2).

Modulation of DGE

Both acclimation and treatment had significant effects on O-phase duration (Table 3). Assessed across all acclimation groups the 5% O_2, 60% RH treatment resulted in a significantly longer O-phase duration when compared with the 21% O_2, 5% RH; 21% O_2, 90% RH; and 40% O_2, 60% RH treatments (P<0.05 in all cases). Acclimation significantly affected the mean $\dot{V}_{CO_2}$ during a DGE cycle (Table 3; supplementary material Fig. S1), with acclimation at 5% O_2 (taken across all treatment groups) resulting in a significantly lower $\dot{V}_{CO_2}$ than the hydration acclimation (P=0.004). For the mean $\dot{V}_{CO_2}$ during a DGE cycle at 5% O_2 acclimation, all treatment groups had negative deviations from the overall mean (Table 4).

For the experimental treatment of 5% O_2, the duration of the O-phase was significantly longer for the dehydration acclimation than for any of the other acclimation conditions (Fig. 2). Across all individuals from all treatment and acclimation groups, both the mean duration of the O-phase and the mean duration of the CF-phase increased with the mean duration of a DGE cycle (Fig. 3; r=0.233, N=138, P=0.006 and r=0.227, N=138, P=0.008, respectively). Acclimation to dehydrating conditions resulted in a significant decrease in haemolymph pH from 7.0±0.3 to 6.6±0.1 (mean ± s.d.; Mann–Whitney U: U=7, Z=2.372; P=0.018).

DISCUSSION

The insect respiratory system has evolved to facilitate rapid oxygen delivery during periods of activity, but during periods when the insect's metabolic rate is low, these high supplies of O_2 could be harmful to tissues (Hetz and Bradley, 2005). The oxidative damage hypothesis is based on this fact and is supported in moth pupae, which regulate their internal P_{O_2} at low levels during the F-phase, even when exposed to hyperoxic atmospheres (Hetz and Bradley, 2005; see also Terblanche et al., 2008; Boardman et al., 2012). However, for *Locusta migratoria*, internal P_{O_2} is dependent on ambient O_2 concentrations when exposed to hyperoxic conditions (Matthews et al., 2012). According to our *a priori* predictions, there should be a decrease in O-phase and/or an increase in CF-phase duration under hyperoxic conditions to lend support to the oxidative damage hypothesis (Table 1). We did not, however, find any significant decrease in O-phase duration (Fig. 2) or increase in CF-phase duration (supplementary material Table S1) in *P. tricolor*. Therefore, the predictions of the oxidative damage hypothesis were not supported here.

After oxygen regulation, the next major abiotic factor that could influence the presence/absence and modulation of DGE is a need to conserve respiratory water. This is typically studied under the rubric of the hygric hypothesis, or some derivation thereof (reviewed in Chown et al., 2011). During a DGE cycle, O_2 consumption and CO_2 production within the animal is a continuous process, while the external exchange of gases between the animal and the atmosphere is discontinuous. While the spiracles are kept closed (CF-phase), there is no exchange of respiratory gases (CO_2, H_2O, O_2) with the atmosphere, thereby resulting in a decrease in respiratory water loss. Insects may therefore face a trade-off between the need to support aerobic metabolism and the need to conserve water (Woods and Smith, 2010). For the hygric hypothesis to be supported, a decrease in O-phase duration and/or an increase in CF-phase duration needs to be present under low RH conditions, and/or when an individual is dehydrated, the latter of which is assumed could gain the most benefit from further reductions in water loss (Table 1). In *P. tricolor*, we found no significant decrease in O-phase or increase in CF-phase duration under low RH conditions or in dehydrated individuals (Fig. 2; supplementary material Table S1). If DGE functions simply to reduce respiratory water loss, then a positive relationship should exist between DGE cycle frequency and ambient RH, because in conditions of low RH the C-phase duration should increase to reduce respiratory water loss (e.g. White et al., 2007). However, we did not find a significant difference between DGE cycle frequency for the different RH treatments (5%, 60% and 90% RH) or acclimation conditions (5%, 40% O_2, dehydrated and hydrated). While this prediction makes use of comparative responses to predict mechanistic responses by the organism to a certain environmental variable, this approach may be confounded by short-term responses (White et al., 2007). The acute responses expressed by the organism to a specific environmental condition may be opposite to the evolutionary response (Arendt and Wilson, 1999; Marcil et al., 2006). However, the approach we adopted and the associated inference of mechanistic responses remains an essential step at the experimental level for understanding how organisms function in a given environment.

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Table 2. Contingency table for individuals that abandoned DGE under the various experimental treatments conditions (5% RH, 90% RH, 5% O_2, 40% O_2, control) within each acclimation (5% O_2, 40% O_2, hydrated, dehydrated)

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

DGE, discontinuous gas exchange; RH, relative humidity. Hypoxia, 5% O_2; hyperoxia, 40% O_2.
Numbers in parentheses indicate the total number of individuals recorded for the specific treatment. Bold indicates significant differences.
Chi-square (χ^2) values were calculated with Pearson's Chi-squared test (see Crawley, 2007) for individuals that abandoned DGE. Total values of rows indicate χ^2 values for the different treatment groups, while total values of columns indicate χ^2 values for the different acclimation groups.

With no support found for the predictions of either the hygric or oxidative damage hypotheses, we reasoned that neither protecting tissues against oxidative damage nor minimizing respiratory water loss is the main factor driving DGE in this species. The last factor hypothesized for influencing DGE presence/absence or modulation is haemolymph pH regulation (Table 1). Here, we found that dehydrated individuals had a longer O-phase (although not always significantly so) than individuals from any of the other acclimations at all treatments except the 40% O_2 treatment. For dehydrated individuals, the only treatment that had a significant effect on O-phase duration was 5% O_2, which resulted in a significantly longer O-phase than any of the other acclimations or treatments. For the dehydration acclimation, the number of individuals that abandoned DGE was significantly higher than for the other acclimation groups (Table 2), supporting the prediction that within dehydrated individuals the DGE cycle will be abandoned to ensure adequate haemolymph pH regulation (Table 1). Hadley and Quinlan also found that the occurrence of DGE decreased if the Eastern lubber grasshopper (*Romalea guttata*), a relatively mesic species, was dehydrated (Hadley and Quinlan, 1993).

Dehydrating an insect should affect the acid–base status of the insect's haemolymph and lead to a decrease in haemolymph volume (Wharton, 1985). Dehydration, probably primarily through a reduction in available free water, or possibly also through a reduction in the capacity to buffer free H^+ ions and more rapid build-up of other ions to toxic levels, could therefore also affect haemolymph pH and its associated buffer capacity and could influence the occurrence and/or modulation of DGE. Indeed, ion homeostasis is a critical aspect of cell and tissue survival post-dehydration or thermal stress in insects (Hadley, 1994; Nation, 2002; Chown and Terblanche, 2006; Boardman et al., 2011). The abolition of DGE or the increase in O-phase duration that we observed could be due to the animal attempting to regulate its haemolymph pH. If the O-phase duration is increased, spiracles are kept open for longer, leading to longer periods in which gasses can be exchanged between the tracheal system and the atmosphere. Apart from elevated respiratory water loss, keeping the spiracles open for longer can also result in respiratory alkalosis via an excess dumping of CO_2. We therefore propose that the increased O-phase observed under dehydrated conditions is likely to be a consequence of respiratory acidosis and the need to excrete high levels of CO_2 buffered in the reduced volume of haemolymph.

A similar argument has been made by Woods and Smith, who found that insects have higher transpiration ratios than predicted by their universal model for water costs of gas exchange (Woods and Smith, 2010). They argue that the reason for this is that most of the insects included in their model use cyclic or DGE patterns. These insects therefore need to increase their O-phase duration above that which would be necessary solely for sufficient O_2 uptake, as during the C-phase, CO_2 is buffered in the body fluids and needs to be expelled during the O-phase to maintain acid–base homeostasis (Woods and Smith, 2010). Consequently, more respiratory water is lost to the atmosphere owing to the spiracles being kept open for longer periods to achieve pH regulation. Harrison (Harrison, 1989) found that in the American locust the acid–base status of the insect's haemolymph is regulated by ventilation, with a lower haemolymph pH (as would be present in our dehydrated individuals, see below) leading to increased ventilation frequencies. Snyder et al. (Snyder et al., 1980) also suggested that pH controls ventilation frequency in the cockroach (*Nauphoeta cinerea*) (see also Matthews and White, 2011a). An increase in ventilation frequency will lead to a decrease in the duration of the different phases of the DGE cycle, and could ultimately lead to the abolition of DGE altogether. Our finding that

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Table 3. Summary of statistics for generalized linear models testing for effects of acclimation and experimental treatment, as well as interactions between acclimation and experimental treatment, on mean phase durations, $\dot{V}_{CO_2}$, O-phase emission volume and water loss rate

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

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

dehydrated individuals of *P. tricolor* abandon DGE means we cannot reject the predictions of haemolymph pH regulation acting as a factor controlling DGE (Table 1). Nonetheless, other factors associated with haemolymph volume reduction may be involved. In addition, a *post hoc* assessment of sample size that might affect our conclusions suggested a sample size of ~34 individuals per treatment (see Materials and methods, '*Post hoc* power test'). Bearing this caveat in mind for the weaker effects, the major significant effects detected in this study nevertheless provide substantial insight into insect respiratory and DGE control.

With haemolymph pH regulation being the only factor for which the *a priori* predictions were not rejected, the next step is to examine whether dehydrated individuals do indeed have lower haemolymph pH values when compared with hydrated individuals. We found a significant reduction in haemolymph pH in dehydrated versus control individuals (6.6±0.1 and 7.0±0.3, respectively; $P=0.018$). These values fall well within the range of haemolymph pH values found for other grasshopper and locust species [*Melanoplus bivittatus*, pH 7.12 (Harrison, 1988); *Taeniopoda eques*, pH 6.98 (Harrison and Kennedy, 1994); *Schistocerca gregaria*, pH 7.31 (Harrison et al., 1990)]. These results confirm our predictions and suggest that in *P. tricolor* the DGE cycle is being used to regulate haemolymph pH in dehydrated individuals, and that this is a higher priority than the potential importance of reducing respiratory water loss or protecting tissues against oxidative damage. We therefore propose a scheme (outlined in Fig. 4) for a hierarchy of abiotic stressors influencing DGE and its ability to reduce respiratory water loss or oxidative damage. The following stressors may be prioritized by *P. tricolor* in decreasing order of importance: (1) maintain oxygen supply to match cellular demand; (2) excrete CO_2 and avoid

Table 4. Mean effect sizes and their signs for all recorded variables for each of the different acclimations (5% O_2, 40% O_2, hydrated, dehydrated) at each of the different treatments (5% RH, 90% RH, 5% O_2, 40% O_2, control), calculated as the mean deviation from the grand mean

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

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

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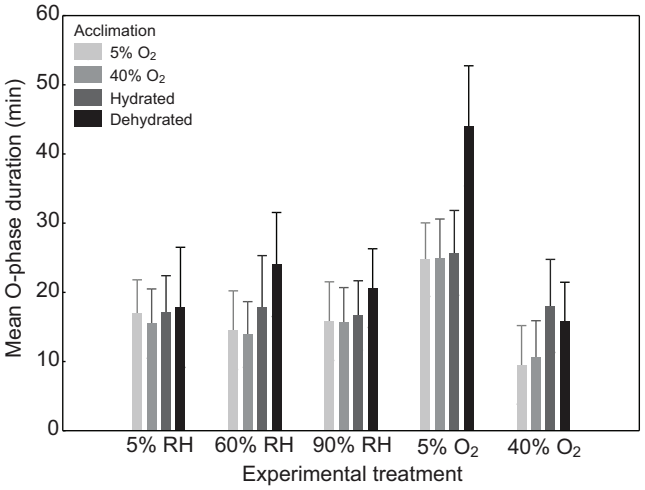

Fig. 2. Summary results of the effects of experimental treatment and acclimation on the mean duration of the open (O)-phase. Both experimental and acclimation treatment had a significant effect on O-phase duration (Wald χ^2=57.023, d.f.=4, P<0.001; Wald χ^2=21.631, d.f.=3, P<0.001, respectively). Mass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

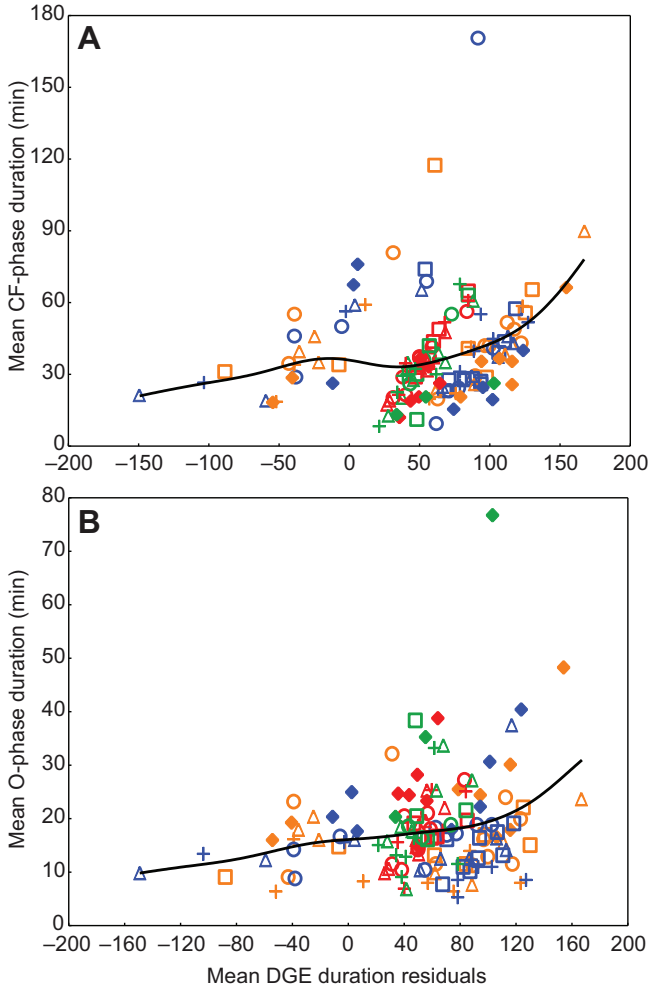

Fig. 3. Scatterplot of the relationships between DGE phase durations (O-, CF-phase) and the mean DGE duration in the different experimental treatments and acclimations for *P. tricolor*. Relationship between (A) the mean duration of a closed/flutter (CF)-phase with the residuals of the mean discontinuous gas exchange (DGE) cycle duration (i.e. DGE cycle–body mass relationship) (y=1988.786+0.091x, r=0.227, P=0.008); and (B) the mean duration of an O-phase with the residuals of the mean duration of a DGE cycle (y=933.820+0.041, r=0.2325, P=0.006). All individuals were included in the analysis (N=138) and each point on the graphs represents the mean value of 2–5 DGE cycles per individual. Residual data were used for mean DGE cycle duration, as mass is a significant covariate for this variable (Wald χ^2=5.661, d.f.=1, P=0.017). Fitted lines are distance-weighted least squares. The different acclimation treatments are represented by different colours (blue, hyperoxia; orange, hypoxia; green, dehydrated; red, hydrated), while the different experimental treatment groups are represented by different symbols [circles, 21% O_2, 5% relative humidity (RH); squares, 21% O_2, 60% RH; triangles, 21% O_2, 90% RH; diamonds, 5% O_2, 60% RH; crosses, 40% O_2, 60% RH]. For example, an orange triangle indicates that an individual received an acclimation treatment of 5% O_2, and its CO_2 release was recorded at the experimental treatment of 21% O_2 and 90% RH.

excess pH variation; and finally, (3) protect against oxidative damage and save water. The one overarching factor that influences all of the stressors and their relative importance is the morphology of the organism, which will affect, for example, the organism's spiracular/tracheal conductance, as well as modes of gas exchange [diffusion versus convection (e.g. Duncan et al., 2010; Wobschall and Hetz, 2004; Groenewald et al., 2012)]. Only once the two main priorities of taking up enough oxygen for aerobic metabolism and releasing CO_2 that accumulated due to cellular respiration have been satisfied can the DGE cycle be employed to attempt to regulate any of the subsequent stressors (e.g. energetic cost minimization, oxidative damage, water conservation). For example, we found that for dehydrated individuals, at the treatments where O_2 levels remained at 21% O_2 and only RH levels were altered, an increase in O-phase duration was observed, although not significantly. Because oxygen levels remain normoxic, priority 1 (maintaining adequate O_2 supply; Fig. 4) can easily be satisfied and the animal can therefore move on to priority 2 (avoid excess pH variation; Fig. 4). The increase in O-phase duration could thus be due to the animal trying to regulate its haemolymph pH, as its oxygen demand (the top priority) has already been satisfied. However, dehydrated individuals at the treatment of 5% O_2 need to regulate their spiracular activity to both maintain an adequate O_2 supply to tissues and regulate haemolymph pH levels (priority 1 and 2; see Fig. 4). Interactions occur between these two stressors (Fig. 4), and the effects of hypoxia and decreased haemolymph pH could therefore be synergistic, as these two factors together lead to a greater increase in O-phase duration than either did alone. Such a line of reasoning is supported by the findings of Matthews and White (Matthews and White, 2011a), who showed that in the cockroach *Nauphoeta cinerea*, conditions of hypoxia and hypercapnia are synergistic and lead to an increase in ventilation frequency.

MATERIALS AND METHODS

Study species and maintenance

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

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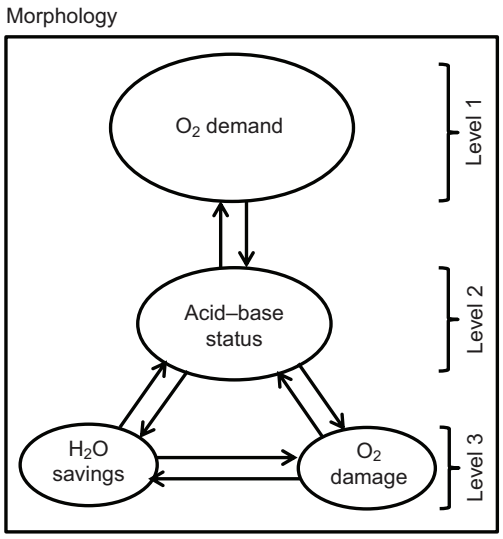

Fig. 4. Schematic hierarchy of abiotic stressors influencing DGE responses. See Discussion for a detailed explanation. Different levels indicate stressors prioritized by the insect in decreasing order of importance, with level 1 indicating the stressor that is of highest priority to the animal; level 3 indicates stressors that can only be regulated once priorities from level 1 and 2 have been satisfied. Arrows represent the interactions between the various stressors.

association with mesic habitats could mean that they are sensitive to dehydration stress, and that they are thereby more likely to be influenced by hydration levels.

A total of 300 individuals was collected and divided into two major experimental blocks. The first experimental block investigated the effects of oxygen acclimation, and the second experimental block tested the effects of hydration status on DGE (see Fig. 5).

Acclimation to variation in ambient oxygen

The acclimation period commenced immediately after collection of sufficient individuals. Individuals (males and females) were randomly divided into two groups: hypoxia (5% O_2) and hyperoxia (40% O_2). The acclimation period lasted for more than 7 days and took place under the same temperature, RH and photoperiod at which the initial collected population was kept. For the two different treatment gasses (5% or 40% O_2, balance nitrogen), a pressurized gas cylinder (Air Products South Africa Pty, Cape Town) was used to feed the gas into the acclimation containers at a constant flow rate of 150 ml min^{-1}, which was verified with a hand-held electronic flow meter (Model ADM1000, Agilent Technologies, Wilmington, DE, USA). An oxygen analyser (Pacific CA Systems, Union Gap, WA, USA) was used to confirm that the oxygen concentrations were at the desired levels within the acclimation containers. Temperature and humidity conditions within the containers were recorded with temperature/humidity iButtons.

Acclimation to variation in moisture availability

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

Respirometry treatment conditions

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

Flow-through CO_2-based respirometry was undertaken to record $\dot{V}_{CO_2}$ (see Lighton, 2008). For the two different oxygen treatments (5% and 40% O_2, balance nitrogen), a pressurized gas cylinder (Air Products South Africa Pty,

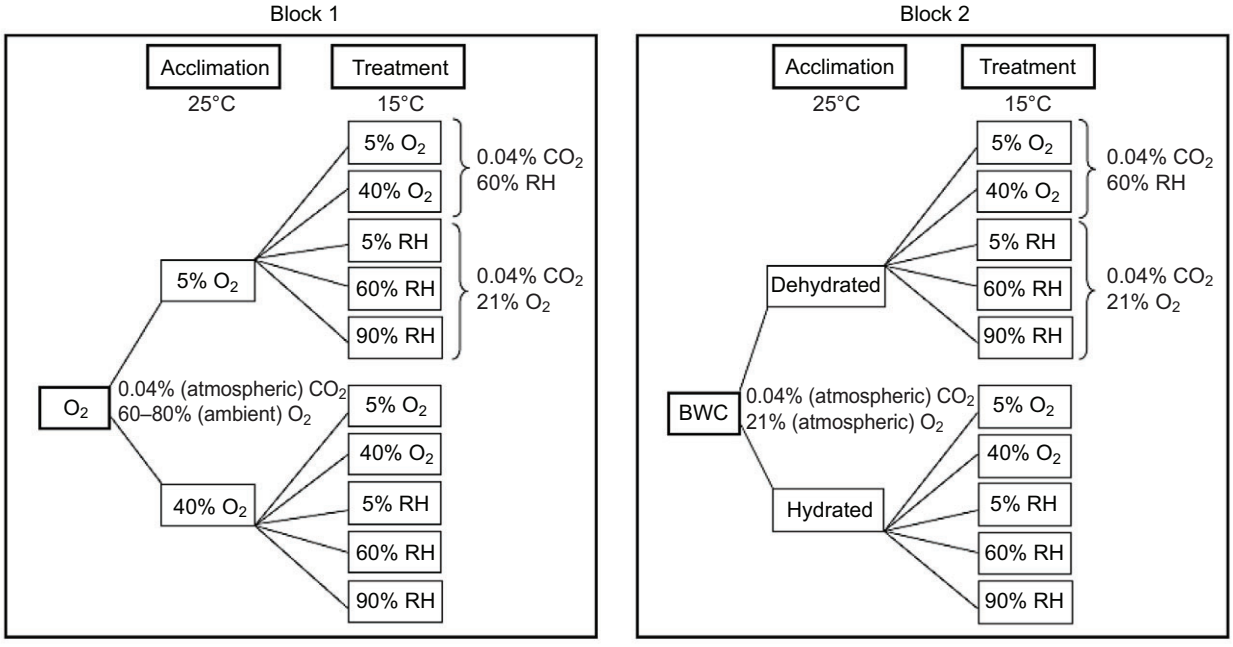

Fig. 5. Schematic diagram of the experimental design for the oxygen and hydration status exposures (acclimations) and the range of treatments employed to assess DGE modulation. BWC, body water content.

Cape Town, South Africa) fed one of the gas mixtures into soda lime (Merck, Gauteng, South Africa) and 50:50 silica gel/Drierite (silica gel, Merck; Drierite, Sigma-Aldrich, St Louis, MO, USA) scrubber columns. For the treatment at normoxia (21% O_2), an aquarium pump was used to feed atmospheric air through the scrubber columns. Air was regulated at a constant flow rate of 200 ml min^{-1} by a flow control valve (Sierra Side-Trak, Sierra Instruments Inc., Monterey, CA, USA) connected to a mass flow controller (MFC-2, Sable Systems, Las Vegas, NV, USA). Thereafter, air flowed through the zero channel of an infrared CO_2–H_2O analyser (Li-7000, Li-Cor, Lincoln, NE, USA).

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

Each individual was weighed to 0.1 mg prior to and after each trial by using a digital microbalance (Model MS104S, Mettler Toledo, Greifensee, Switzerland). In each respirometry system, a single grasshopper was placed in a darkened cuvette and given ~20 min to settle in the cuvette (at 15°C) before recording of CO_2 release commenced. Baselines (between 15 and 30 min) were recorded at the beginning and end of each trial by using an identical setup, but without the test animal. Individuals' $\dot{V}_{CO_2}$ levels were measured and recorded for 6–12 h per individual. Owing to sexual size dimorphism, female grasshoppers were measured in a 20 ml cuvette and males in a 10 ml cuvette. Individuals were fasted for at least 8 h before recording of CO_2 release commenced.

Haemolymph pH measurement

Haemolymph pH of individual grasshoppers was measured for both a control (hydrated, N=12) and treatment (dehydrated, N=5) group. The small sample size of the treatment group when compared with the control group is due to the fact that haemolymph volume decreases in dehydrated individuals. This made it difficult to obtain a large enough volume of haemolymph to measure from individuals in the treatment group. To obtain different levels of dehydration within the individuals, individuals were treated as previously described (see Materials and methods, 'Acclimation to variation in moisture availability'). Once the desired level of dehydration was reached, haemolymph was extracted from the individual by removal of the hindlegs. Haemolymph was extracted from the wound with a 100 µl micropipette (Eppendorf Research, Merck Millipore) and transferred to a 200 µl Eppendorf tube. The average volume of haemolymph extracted per individual was 5–15 µl. After extraction, measurements were made as quickly as possible to minimize the amount of time that the haemolymph came into contact with atmospheric air. Needle-type pH microsensors (140 µm diameter), connected to a PreSens pH-1 micro fibre optic pH transmitter (PreSens GmbH, Regensburg, Germany), were used to measure haemolymph pH. The pH sensors were calibrated at 15°C using seven sodium phosphate buffers that ranged from pH 5 to 8 in 0.5 pH increments. Because the pH sensors are sensitive to the ionic strength of a sample, the sodium phosphate buffers were made up with an ionic strength the same as that of locust Ringer's solution (0.172) (Pearson and Robertson, 1981). The sodium phosphate buffers were checked with a pH meter (inoLab, pH 720, WTW GmbH, Weilheim, Germany) calibrated with standard pH 4 and 7 buffer solutions. To measure haemolymph pH, a hole was pierced through the Eppendorf lid containing the haemolymph, so that the pH probe could be inserted while minimizing contact of the haemolymph with atmospheric air. Haemolymph pH was measured every 1 s for a period of 3 min to allow pH readings to stabilize. Haemolymph pH readings were recorded with pH1-view software via a standard desktop computer.

Data analyses

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

Individual grasshoppers were assessed for the presence or absence of DGE based on their rate of CO_2 release ($\dot{V}_{CO_2}$). Contingency tables were then generated for the number of individuals that showed DGE under control and treatment conditions. Mean duration and mean $\dot{V}_{CO_2}$ for the different phases (CF- and O-phases) were compared between different treatments and acclimations. These data (presence/absence of DGE and different phase responses) were then used to test the *a priori* predictions regarding water, oxygen and pH regulation (Table 1).

Mass was a significant covariate for all parameters (P<0.05 in all cases), except for mean O-phase duration and WLR. Therefore, to adjust for the effects of size, the residuals of the dependent variable–body mass relationship were used when drawing correlations. Generalized linear models (GLZs, normal distribution, identity link function) were used for data analysis as several assumptions were not met, and GLZs are more robust to the violation of these assumptions. GLZs were used to test for effects of acclimation and treatment on mean DGE phase durations, $\dot{V}_{CO_2}$, O-phase volume and WLR. An interaction between acclimation and treatment was always tested for and any significant covariates were included in the analyses. For variables in which treatment or acclimation was found to have a significant effect, graphs including 95% confidence intervals were drawn to distinguish which of the treatments or acclimations differ significantly from the others. In addition, estimates of effect sizes were calculated for all the recorded variables for each of the different acclimation and treatment conditions (Table 4). Effect sizes were calculated as the mean deviation from the grand mean. Weighted least squares were used to test for correlations between the different variables, as this analysis is more robust to outliers and data that are not normally distributed. All statistical analyses were carried out in Statistica 11 (StatSoft Inc., Tulsa, OK, USA).

Post hoc power test

The recommended (*post hoc*) sample sizes for three different extreme cases in our results were calculated using Statistica software power calculator, assuming α=0.05 and β=0.8 (Crawley, 2007). This analysis suggested a sample size of approximately 34 individuals per treatment, meaning that for our experimental design a total sample size of 680 individuals would be required.

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Competing interests
The authors declare no competing financial interests.

Author contributions
B.G. carried out the experimental work, data extraction and analyses. All authors contributed to analyses, interpretation and writing.

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