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RESEARCH ARTICLE

Discontinuous gas-exchange cycle characteristics are differentially affected by hydration state and energy metabolism in gregarious and solitary desert locusts

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ABSTRACT

The termination of discontinuous gas exchange cycles (DGCs) in severely dehydrated insects casts doubt on the generality of the hygric hypothesis, which posits that DGCs evolved as a water conservation mechanism. We followed DGC characteristics in the two density-dependent phases of the desert locust *Schistocerca gregaria* throughout exposure to an experimental treatment of combined dehydration and starvation stress, and subsequent rehydration. We hypothesized that, under stressful conditions, the more stress-resistant gregarious locusts would maintain DGCs longer than solitary locusts. However, we found no phase-specific variations in body water content, water loss rates (total and respiratory) or timing of stress-induced abolishment of DGCs. Likewise, locusts of both phases re-employed DGCs after ingesting comparable volumes of water when rehydrated. Despite comparable water management performances, the effect of exposure to stressful experimental conditions on DGC characteristics varied significantly between gregarious and solitary locusts. Interburst duration, which is affected by the ability to buffer CO_2, was significantly reduced in dehydrated solitary locusts compared with gregarious locusts. Moreover, despite similar rehydration levels, only gregarious locusts recovered their initial CO_2 accumulation capacity, indicating that cycle characteristics are affected by factors other than haemolymph volume. Haemolymph protein measurements and calculated respiratory exchange ratios suggest that catabolism of haemolymph proteins may contribute to a reduced haemolymph buffering capacity, and thus a compromised ability for CO_2 accumulation, in solitary locusts. Nevertheless, DGC was lost at similar hydration states in the two phases, suggesting that DGCs are terminated as a result of inadequate oxygen supply to the tissues.

KEY WORDS: Buffering capacity, Desiccation, DGCs, Grasshoppers, Haemolymph, Oxygen, Starvation

INTRODUCTION

Insects breathe through an elaborate network of gas-filled tracheal tubes that ramify into fine tracheoles, which penetrate tissues and reach individual cells. Gas exchange with the environment occurs through paired, segmental, cuticular openings called spiracles (Nation, 2008). Most insect species have the ability to control the opening and closing of spiracles, which results in a variety of gas exchange patterns, often categorized as continuous, cyclic and discontinuous gas exchange cycles (DGCs) (e.g. Chown, 2011). The continuous pattern is characterized by a steady CO_2 emission rate because the spiracles are constantly open. The cyclic pattern is characterized by periodic bursts of CO_2 emission, changing from high to low, following the rhythmic closing and opening of the spiracles. DGCs have three phases characterized by spiracular status: the closed phase (C); the flutter phase (F); and the open phase (O) (Levy and Schneiderman, 1966).

While gas exchange with the environment is not continuous during DGCs, cellular respiration is. Hence, during the C phase, closing the spiracles results in a gradual decrease in the tracheal oxygen partial pressure (P_{O_2}). Although CO_2 accumulates in the body, and its partial pressure increases in the trachea during the C phase, endotracheal pressure drops as CO_2 is more water soluble than O_2. The developing hypoxic tracheal environment, upon reaching a threshold value, triggers the flutter phase (F), which is characterized by rapid opening and closing of the spiracles (Levy and Schneiderman, 1966). Sub-atmospheric tracheal pressure results in predominantly inward gas transport during the F phase and P_{CO_2} continues to increase until it reaches a critical value, which triggers prolonged opening of the spiracles (O phase), during which most of the gas exchange with the external environment occurs (e.g. Levy and Schneiderman, 1966; Lighton, 1996; Förster and Hetz, 2010).

Despite the extensive research attention that DGCs have attracted, they are by no means found in all insects. Indeed, they have only been reported, to date, in seven insect orders: Lepidoptera, Coleoptera, Hymenoptera, Blattodea, Orthoptera, Hemiptera and Diptera, (Marais et al., 2005; Gray and Bradley, 2006; Contreras and Bradley, 2009), in addition to other tracheated arthropods, such as centipedes, ticks and solifuges (Chown, 2011). Moreover, in insects that do exhibit DGCs, the pattern is reported to be limited to periods of quiescence (e.g. Lepidopteran pupae, or resting adult insects; see Matthews and White, 2011). It has also been suggested that insect gas-exchange patterns form a continuum, in which DGCs are limited to periods of low metabolic rates (Contreras and Bradley, 2009).

Several adaptive hypotheses have been proposed for the evolution and maintenance of DGCs (Chown et al., 2006; Quinlan and Gibbs, 2006; Chown, 2011). Initially, it was proposed that DGCs serve to restrict respiratory water loss (RWL) rates (the 'hygric hypothesis'), a suggestion that is based on the prolonged C-phase and a largely inward convective gas transport during the F-phase (Levy and Schneiderman, 1966; Kestler, 1985). However, the general applicability of the classic hygric hypothesis has since been questioned on both experimental and theoretical grounds. Mesic ants exhibited DGCs, whereas a closely related xeric species did not (Lighton and Berrigan, 1995). In addition, grasshoppers and beetles abandoned DGCs following severe dehydration stress, when stricter management of body water stores

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would be expected (Hadley and Quinlan, 1993; Quinlan and Hadley, 1993; Chown and Holter, 2000; Groenewald et al., 2014). Abolishment of DGCs when body water (and haemolymph in particular) is lost can be explained by a reduced CO_2 buffering capacity during spiracle closure, before the spiracles open again and allow CO_2 washout (Lighton, 1996).

The desert locust *Schistocerca gregaria* Forsskål 1775 (Orthoptera: Acrididae) exhibits density-dependent polyphenism, consisting of the two morphologically, behaviourally and physiologically distinct gregarious and solitary phases (Pener and Simpson, 2009). Among the multitude of reported phase-related differences, the two density-dependent phases vary in environmental stress resistance (Wang et al., 2007), as well as in the neural control of respiratory gas-exchange patterns (Berman et al., 2013). In this study, we used the locust phases for an intraspecific comparative investigation of the effect of experimental stress on DGC characteristics. Our goal was to use the phase-specific variation in stress resistance to elucidate the mechanistic constraints on DGCs, through a comparison of cycle properties in different hydration states, during a combined desiccation and starvation stress leading to a halt in the DGC pattern. We hypothesized that the reportedly more stress-resistant gregarious locusts would maintain DGCs longer following prolonged exposure to the experimental dehydrating conditions. Additionally, we expected that DGC expression would be restored in both phases following rehydration, in accordance with the role of body water in accumulation of CO_2 during spiracle closure.

We carried out daily respirometry measurements on adult male locusts in the two phases prior to and during exposure to a combined desiccation and starvation stress, until they abolished DGCs. The insects were then provided with water and respirometry was repeated within 24 h. Analysis of DGC characteristics suggested that factors other than water availability may have been responsible for the stress-induced variation in DGC performance, and we therefore examined the effect of exposure to experimental stress on the concentration of haemolymph proteins, an important non-bicarbonate buffer in grasshoppers (Harrison et al., 1990). We also monitored changes in haemolymph osmolality as a measure of haemolymph volume change in the two phases, assuming similar osmoregulatory capacities. Phase-specific effects of stress exposure on haemolymph protein concentrations prompted calculation of the respiratory exchange ratio (RER), for which we measured oxygen consumption and CO_2 emission rates using closed-system respirometry. Finally, we examined whether phase-specific variations in DGC characteristics were correlated with carbonic anhydrase (CA) activity levels in the muscle and fat body of gregarious and solitary locusts.

MATERIALS AND METHODS

Locusts

We used *S. gregaria* from our stock populations at the University of Haifa-Oranim. Locusts were fed daily with fresh grass, wheat shoots and dry oats *ad libitum* and were kept at 33.0±3.0°C, supplemented with light bulbs for thermoregulation, and a 14 h light:10 h dark photoperiod. Hatchlings of the same egg pods were randomly assigned to either crowded cages or individual solitary chambers within 3 h of hatching, in order to achieve gregarious and solitary adults, respectively (see Berman et al., 2013). Locusts of the two density-dependent phases were reared in separate rooms under similar conditions. All experiments and measurements were carried out on male locusts, 1–2 weeks after adult eclosion.

Dehydration and rehydration

Locusts were allowed at least 3 days of acclimation to the experimental conditions (30.0±0.5°C, RH ∼50% and 14 h light:10 h dark) (MIR-554 incubator, Panasonic, Japan), and were denied access to food for 12–24 h before the initial respirometry measurement (day 1). Following this measurement, locusts were returned to the incubator and kept in 2 litre containers for gregarious locusts and in 0.5 litre containers for each individual solitary locust, without access to food or water. Measurements of the two locust phases were not carried out at the same time, and therefore solitary and gregarious locusts were not kept in the same incubator simultaneously. Respirometry was then repeated daily (see below) for each individual locust until DGC was relinquished. The last day on which locusts still exhibited DGCs during the prolonged stress was termed day L. The animals were then returned from the metabolic chamber to their respective containers and offered drinking water prior to one additional respirometry measurement on the following day (day R). All individuals were weighed daily to the nearest ±0.1 mg (CPA224S, Sartorius, Goettingen, Germany) following respirometry. Body mass (M_b) loss (% of initial) during dehydration and mass gain following rehydration were calculated while accounting for dry mass of the faeces. Faeces mass for gregarious locusts was calculated by averaging the total faeces mass collected daily from their container.

Respirometry

Analysis of DGC characteristics was based on flow-through respirometry, which was carried out at 20°C and in the dark in order to reduce activity and thus increase the likelihood of DGCs. Individual locusts were placed in a 23 ml glass metabolic chamber covered with a black cardboard sleeve. Atmospheric air was scrubbed of CO_2 and water vapour by passing it through silica gel/Ascarite® columns and into the metabolic chamber at a flow rate of 200 ml min^{-1} using a mass flow controller (MC-500SCCM-D; Alicat Scientific, Tuscon, AZ, USA). Locusts were acclimated to the metabolic chamber, the measurement temperature (20°C), and the flow rate for 1 h prior to initiation of respirometry. Water vapour and CO_2 emission rates ($\dot{V}_{CO_2}$) were measured with a LI-7000 dual analyser (LiCor, Lincoln, NE, USA). Data collection and analysis were carried out using a UI-2 data acquisition interface and Expedata software (Sable Systems International, Las Vegas, NV, USA). Baselines were recorded at the beginning and end of each individual recording by passing dry CO_2-free air directly through the analyser. Cycle phases were characterized as 'burst' (O phase) and 'interburst' (C+F phases) because the F phase could not be reliably distinguished from the C phase by using the CO_2 trace. The gas exchange pattern was categorized as DGC when three consecutive cycles consisted of a clear >2 min interburst. Cycle characteristics were calculated by averaging values for these consecutive cycles. Occasionally, when cycle duration was exceptionally long (>30 min) or when DGCs were interrupted by locust activity before being restored, calculation of cycle properties was based on two cycles only.

Cuticular water loss rate was defined as the water emission rate during the interburst. Respiratory water loss was calculated by subtracting the cuticular water loss rate from the water emission rate during the burst.

RER calculation

Closed-system respirometry was carried out in order to measure respiratory gas exchange rates for RER calculation. Individual locusts were placed in 60 ml syringes, after washing the syringes with dry CO_2-free air. The syringes were then sealed and placed at 20°C for 25 min, after which 30 ml of the syringe gaseous atmosphere were injected into a stream of dry, CO_2-free, air at a flow rate of 200 ml min^{-1}, which was passed through a magnesium perchlorate column, a LI-7000 CO_2 analyser, an Ascarite® column and then an Oxzilla II oxygen analyser (Sable Systems International, Las Vegas, NV, USA). Closed-system respirometry and M_b measurements were repeated daily during 6 days of dehydration (the calculated mean time prior to DGC abolishment; see Results). The locusts were then rehydrated on the next day of exposure to experimental stress, and respirometry was repeated 24 h later (day R). Baselining was repeated between individual measurements by passing dry CO_2-free air directly through the analysers.

Haemolymph chemistry

Additional locusts were used for determination of haemolymph osmolality and protein content at various hydration levels (during dehydration and after

rehydration). Haemolymph was drawn with a sharpened glass capillary and 8 µl were used for measuring osmolality with a vapour pressure osmometer (5100C; Wescor, Logan, UT, USA).

Haemolymph protein concentrations were determined using a standard colorimetric assay (BCA1, Sigma-Aldrich). Haemolymph samples were collected over ice. The samples were centrifuged at 1400 ***g*** for 5 min to remove cells and prevent clotting. From each sample, 5 µl of the supernatant was diluted with 495 µl distilled water and then re-diluted ×3. The colorimetric assay was carried out by loading 8 µl triplicates from each sample onto a 96-well microplate and adding 200 µl of protein assay reagent (50 parts bicinchoninic acid solution to one part 4% $CuSO_4$). The plates were then incubated overnight at room temperature, before absorbance at 562 nm was measured (PowerWave XS2, BioTek Instruments, VT, USA). Protein concentrations were determined using standards of known concentrations of bovine serum albumin.

Body water content

Individual locusts (10 gregarious and 14 solitary) were weighed, dried at 60°C for 48 h and then re-weighed for water content determination. Preliminary experiments had shown that constant dry mass was reached after 48 h. Body water content was calculated by subtracting locust dry mass from their wet mass.

Tracheal volume

The tracheal system volume was estimated using the water displacement method (Bartholomew and Barnhart, 1984). Individual locusts (14 gregarious and 10 solitary) were weighed and then placed in a syringe containing 50 ml of soapy water and equipped with a 3-way valve. After ejecting air from the syringe, the valve was sealed and the plunger was pulled back and forth. The negative pressure inside the syringe drew air from the tracheal system through the spiracles, while the tracheal system became filled with water. When bubbles ceased to appear from the spiracles the locusts were removed from the syringes, blotted dry and re-weighed. Tracheal volume was estimated by subtracting initial M_b from final M_b, assuming water density of 1 g ml^{-1}.

Carbonic anhydrase activity

Flight muscle and fat body samples were dissected out from both solitary and gregarious locusts, washed with locust saline (Abrams and Pearson, 1982), weighed and transferred to a 1.5 ml vial containing 0.5 ml of homogenizing buffer [50 mmol l^{-1} Tris-HCl, 5 mmol l^{-1} 2-mercaptoethanol (both Sigma-Aldrich)]. Haemolymph samples were drawn with a sharpened glass capillary, and 30 µl aliquots were transferred to 1.5 ml vials containing 0.5 ml of homogenizing buffer. Tissue samples were sonicated (Microson XL 2005, Heat System Inc., Farmingdale, NY, USA) and then centrifuged at 12,400 ***g*** for 5 min at 5°C. Each measurement included 200 µl homogenizing buffer (without tissue sample) for the uncatalysed reaction followed by the use of 200 µl sample buffer solution supernatant for the catalysed reaction.

Carbonic anhydrase activity was measured by using a modified electrometric assay (Darlington et al., 1985). Briefly, 1 litre of distilled water was bubbled with CO_2 for at least 1 h to prepare CO_2-saturated water prior to the assay. Initially, 2.8 ml of assay buffer (25 mmol l^{-1} Tris-HCl at pH 8.4) were placed in the assay vessel, before adding 200 µl of sample buffer solution. The reaction, carried out at 2–3°C, was started by adding 3 ml of CO_2-saturated water into the assay vessel and measuring the time for a pH change from 8.3 to 6.3. Measurements of pH change were carried out using a pH electrode connected to a pH/blood-gas monitor (RHM73, Radiometer, Copenhagen, Denmark). Data were recorded on a desktop PC using a data acquisition card and software. The activity (A) was calculated according to the equation of Wilbur and Anderson (1948):

$$A = (t_0 + t_{cat})/t_{cat}, \quad (1)$$

where t_0 and t_{cat} are the time for the pH drop from 8.3 to 6.3 in the uncatalysed and catalysed reactions, respectively.

Statistics

Statistical analysis was performed using SPSS 19.0 statistical software (IBM). Phase-dependent effects were tested by ANCOVA (M_b as a covariate). Repeated measures analyses were used for determination of within-phase stress-exposure effects. When sphericity could not be assumed, we used the Huynh–Feildt corrected F value and d.f. values. Values are shown as means±s.e.m.

RESULTS

Daily respirometry measurements of gregarious and solitary locusts (wet M_b, 1.535±0.063 g; N=15, and 1.300±0.055 g; N=14, respectively) during experimental stress exposure revealed no significant phase-dependent effect on the latency to the termination of DGCs (∼7.2±0.5 days; t-test; t_{27}=0.281, P=0.78) (Table 1). Total water loss when DGCs were lost was similar (∼22.5% of initial M_b) in the two phases (Mann–Whitney U-test; U=104, P=0.98) (Table 1). There was also no significant phase effect on the amount of water ingested during subsequent rehydration (Mann–Whitney U-test; U=71, P=0.15) (Table 1). Further comparisons were made, with special emphasis on three time points or stages during exposure to the experimental conditions (Fig. 1): metabolic rates (expressed as CO_2 emission rates) measured on the last day prior to stress-induced abolishment of DGCs (day L) were significantly lower (by 35–40%) compared with initial values measured on the first day of the experiment (day 1; paired t-test; t_{14}=5.37 for gregarious and t_{13}=7.16 for solitary, P<0.001 for both phases). These lower metabolic rates remained unchanged following rehydration (day R, the first day after rehydration; Table 1). Both density-dependent locust phases exhibited similar metabolic rates and evaporative water loss rates (both cuticular and respiratory) during the experiment (ANCOVA;

Table 1. DGC maintenance, respiratory gases and water exchanges before and during dehydration, and following rehydration of gregarious and solitary locusts

	Gregarious	Solitary	P
Sample size	15	14	
M_b (g)	1.53±0.24[a]	1.30±0.20[b]	0.01
Mass loss to DGC loss (% of initial)	22.7±7.8	22.2±6.3	0.98
Time to DGC loss (days)	7.3±2.3	7.1±2.7	0.78
Water ingested (% of total loss)	33.6±20.0	44.4±10.6	0.15
sp$\dot{V}_{CO_2}$ (µl g^{-1} h^{-1})			
Day 1	172.5±40.8	181.2±18.7	0.32
Day L	104.5±9.9	118.9±20.9	0.09
Day R	104.2±13.0	117.2±16.5	0.12
sp$\dot{V}_{CO_2,max}$ (µl g^{-1} h^{-1})			
Day 1	2586.7±1067.9[a]	1587.5±456.5[b]	0.017
Day L	1216.6±634.1[a]	490.2±104.8[b]	0.004
Day R	1705.3±637.7[a]	742.8±286.1[b]	0.001
spEWL rate (µl g^{-1} h^{-1})			
Day 1	0.64±0.14	0.69±0.11	0.78
Day L	0.61±0.16	0.57±0.11	0.15
Day R	0.76±0.18	0.77±0.14	0.24
RWL ratio (%)			
Day 1	17.9±5.7	16.2±4.1	0.45
Day L	6.4±3.2	9.4±3.1	0.15
Day R	8.5±3.4	8.8±2.7	0.13

Values are means±s.d. (sp indicates mass-specific values).
Different superscript letters denote statistically significant differences (P<0.05).
Statistical tests included t-tests for body mass (M_b) and time to DGC loss, Mann–Whitney tests for percentage data and ANCOVA for rates, with M_b as a covariate. Respiratory water loss (RWL) data is shown as percentages of total evaporative water loss (EWL), but compared by ANCOVA on absolute values.

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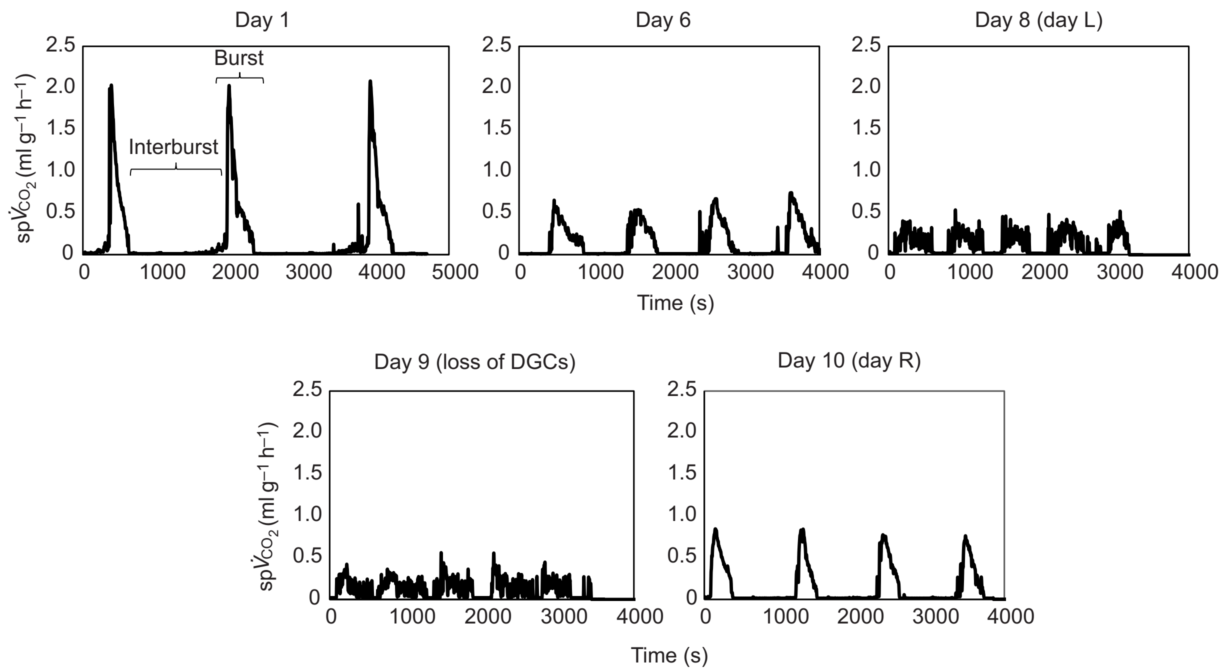

Fig. 1. Daily respirometry measurements show DGC properties in the desert locust *Schistocerca gregaria*. Typical CO_2 emission traces (solitary individual; M_b=1.546 g) before (day 1) and during exposure to prolonged stress (days 6, 8 and 9), and following rehydration (day R). Day L (day 8 for this specific individual) was the last day prior to abolishment of DGCs.

P>0.05 for days 1, L and R). The relative importance of respiratory water loss was also similar (Table 1) and within the range reported for other orthopterans (Quinlan and Hadley, 1993; Rourke, 2000).

Despite the above unexpected comparable desiccation-resistance performances, comparative analysis of the effect of the hydration state on DGC characteristics revealed a significant variation between the density-dependent phases. In gregarious locusts, cycle duration did not change during dehydration, and increased following rehydration ($F_{1.49,\ 20.83}$=4.19; P<0.05, but no Bonferroni *post hoc* support) (Fig. 2A). In contrast, cycle duration in the solitary locusts varied significantly with hydration state ($F_{1.43,\ 18.63}$=8.26; P<0.01), showing a significant decrease during dehydration and recovery to values not significantly lower than control following rehydration (Fig. 2A). A breakdown of the cycle into its burst and

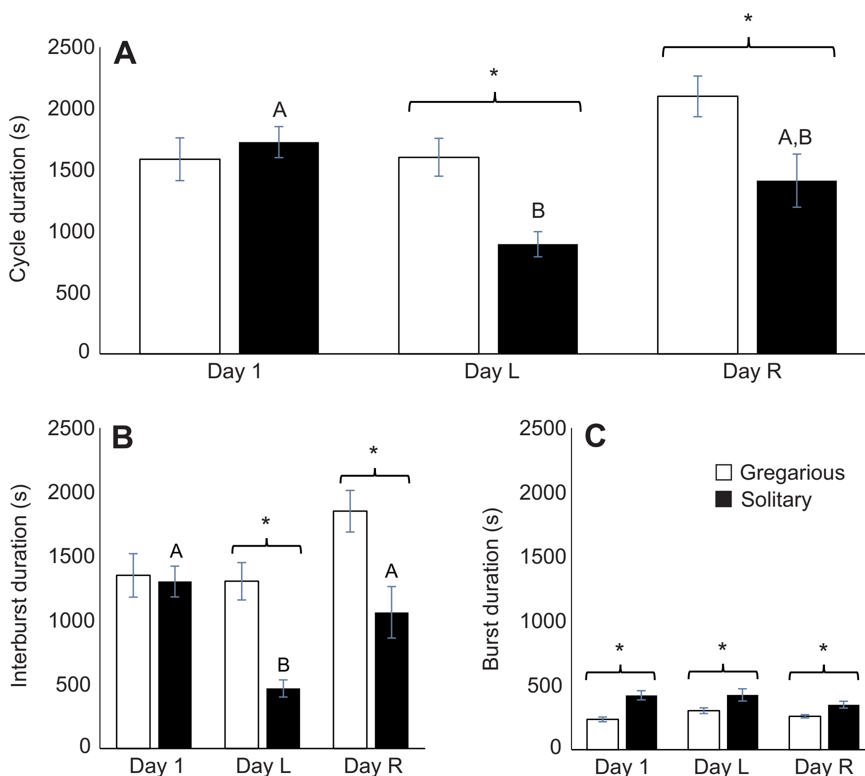

Fig. 2. Phase-specific variation in the effect of experimental treatment on DGC characteristics in locusts. Cycle (A), interburst (B) and burst (C) duration before exposure (day 1), on the last day of DGCs (day L) and following rehydration (day R) of gregarious and solitary locusts. Different letters indicate statistically significant treatment effects, whereas asterisks indicate significant phase variation (P<0.05).

interburst phases (Fig. 1) revealed that the differences in cycle characteristics between gregarious and solitary locusts resulted from changes in the duration of the interburst (gregarious, $F_{1.54,21.61}$=4.58; solitary, $F_{1.49,19.36}$=10.78; P<0.05) (Fig. 2B), whereas the burst duration did not change with hydration state in either gregarious or solitary locusts (gregarious, $F_{2,28}$=4.22, P=0.06; solitary, $F_{2,26}$=1.63, P=0.22) (Fig. 2C). Interestingly, burst duration was longer in solitary locusts compared with gregarious locusts in all the hydration states (day 1, $t_{19.88}$=4.64; day L, $t_{18.29}$=2.32; day R, $t_{19.05}$=3.01; P<0.05 for all hydration states). Accordingly, the similar respiratory gas-exchange rates, coupled with shorter burst duration, were reflected in mean maximum mass-specific $\dot{V}_{CO_2}$ values (peak burst sp$\dot{V}_{CO_2}$) twofold higher in the gregarious locusts (Table 1). Nevertheless, we found no phase-related difference in the mass-specific carbonic anhydrase (CA) activity in muscle (Mann–Whitney U-test; P=0.70, N=14 each) and fat body tissue (P=0.28, N=13 each). No CA activity was detected in the haemolymph of either gregarious or solitary locusts.

Assuming constant cellular metabolic rate despite cyclic gas exchanges with the environment, we calculated the locusts' ability to accumulate CO_2 during the interburst (multiplying interburst duration by mean $\dot{V}_{CO_2}$ and subtracting the volume of CO_2 that was emitted during the interburst for each individual) and how this was affected by hydration state in the two locust phases. Dehydration had a significant effect on the ability of both solitary and gregarious locusts to accumulate CO_2 during the interburst (21% and 57% of control values, respectively) ($F_{2,28}$=11.33, P<0.001 for gregarious; $F_{2,26}$=50.88, P<0.001 for solitary) (Fig. 3). In accordance with the effect of dehydration on haemolymph CO_2 accumulation capacities during the interburst in the two phases, gregarious locusts, but not solitary ones, demonstrated full recovery of CO_2 accumulation capacities following rehydration (Fig. 3).

The different effect of stress exposure on the ability of gregarious and solitary locusts to accumulate CO_2 during the interburst was not coupled with a significant difference in metabolic rates, water loss rates or ingested water volumes (during rehydration) (Table 1). There was also no phase-dependent variation in locust tracheal volume and body water content (Table 2), which represent the oxygen source and CO_2 sink during the interburst, respectively, and therefore could potentially affect differences in DGC characteristics

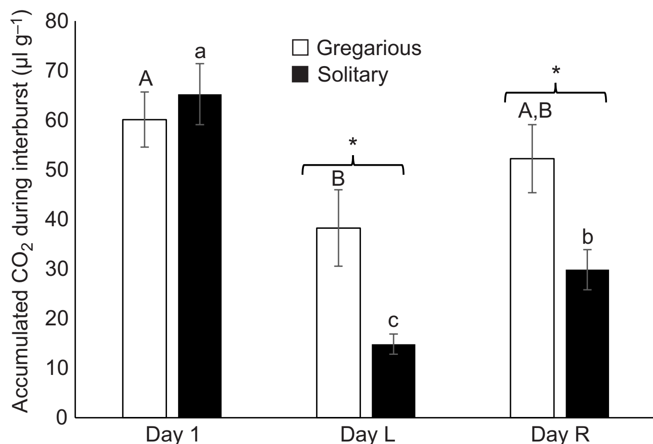

Fig. 3. Accumulation of CO_2 during the interburst at different experimental hydration states. Accumulation capacities in gregarious and solitary *S. gregaria* before and during dehydration and following rehydration were calculated as: (mean $\dot{V}_{CO_2}$×interburst duration) – interburst $\dot{V}_{CO_2}$. Different letters indicate statistically significant treatment effect, whereas asterisks indicate significant phase variation (P<0.05).

Table 2. Tracheal volume and total body water content in gregarious and solitary locusts

	Gregarious	Solitary	P
Tracheal volume			
Sample size	14	10	
M_b (g)	1.65±0.13	1.57±0.16	0.159
Tracheal volume (ml)	0.64±0.08	0.62±0.12	0.546
Total body water content			
Sample size	10	14	
M_b (g)	1.56±0.25[a]	1.27±0.17[b]	0.003
Water content (g)	0.92±0.09	0.79±0.10	0.193
Water content (% of M_b)	59.9±4.6	62.9±3.9	

Values are means±s.d.
Different superscript letters denote statistically significant differences (α=0.05). Statistical tests included t-tests for body mass (M_b) and ANCOVA for tracheal volume (M_b as a covariate) and body water content (dry M_b as a covariate).

(Matthews et al., 2012; Huang et al., 2015). However, as accumulation of CO_2 in body fluids incurs a challenge to acid–base balance, we compared haemolymph protein content, which is correlated with buffering capacity in *S. gregaria* (Harrison et al., 1990), in the two phases. Initially, haemolymph protein concentration was significantly higher in solitary (38.1±

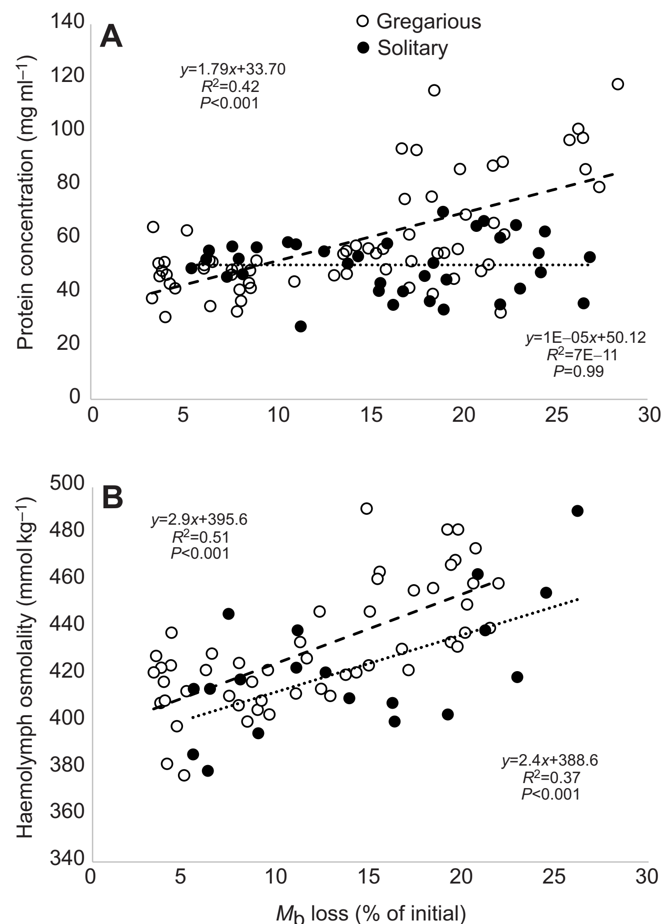

Fig. 4. Changes in haemolymph chemistry during exposure to experimental stress. Haemolymph protein concentration (A) and osmolality (B) as a function of loss of body mass in gregarious (open symbols, dashed lines) and solitary (filled symbols, dotted lines) *S. gregaria* during prolonged desiccation and starvation. Data for day 1 (0% mass loss) are given in the Results section.

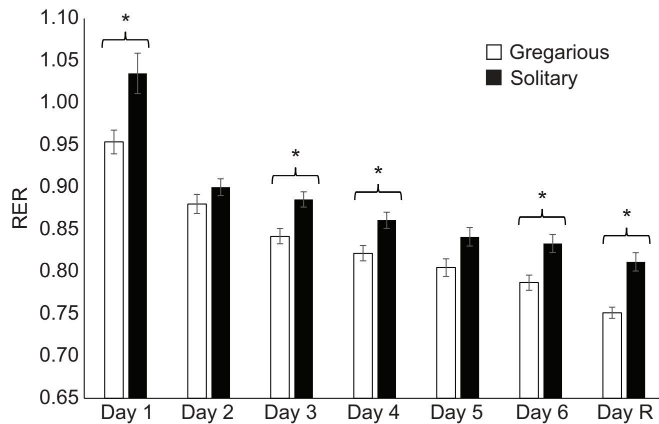

Fig. 5. Phase-specific variation in energy metabolism, as reflected in RER values, during exposure to experimental stress. Calculated respiratory exchange ratio (RER) values based on constant-volume respirometry of gregarious (*N*=18) and solitary (*N*=11) locusts before and during exposure to prolonged experimental stress, and following rehydration (day R). Asterisks indicate significant between-phase variation (*P*<0.05).

3.6 mg ml^{-1}) than in gregarious (25.0±1.9 mg ml^{-1}) locusts (*t*-test; t_{21}=3.55, *P*=0.002). During experimental stress and the ensuing decrease in haemolymph volume (Albaghdadi, 1987), haemolymph protein concentration increased in gregarious but not in solitary locusts (Fig. 4A). Haemolymph osmolality values (day 1 values were 397±3 and 387±6 mOsm kg^{-1} for gregarious and solitary locusts, respectively) measured during exposure to experimental stress suggest similar rates of haemolymph loss in gregarious and solitary locusts (test for equality of slopes; $F_{1,67}$=0.63, *P*=0.43) (Fig. 4B). Thus, while haemolymph protein concentration increased with haemolymph volume loss in gregarious locusts, unchanged concentration during dehydration and haemolymph volume loss suggest loss of haemolymph protein in solitary locusts. Data for day 1 (0% mass loss) haemolymph chemistry were not considered for the analysis of stress effects (Fig. 4) because of the potential postprandial effects, as well as that of elevated initial water loss rates during prolonged desiccation (Hadley, 1994).

Fig. 5 demonstrates a continuous drop in calculated RER values during 6 days of dehydration and following rehydration. Significantly lower RER values were recorded for the gregarious compared with the solitary locusts on all but days 2 and 5 of measurements, suggesting a higher use of lipid stores to meet energetic needs during the combined desiccation and starvation stress in the gregarious locusts.

DISCUSSION

The finding that dehydrated grasshoppers abandon DGCs (Hadley and Quinlan, 1993; Quinlan and Hadley, 1993) provided early experimental evidence contrasting predictions of the hygric hypothesis for the evolution and maintenance of DGCs in insects. The phase-dependent differences in locust DGC characteristics reported here were not correlated with variation in respiratory water losses, and thus also do not support the hygric hypothesis. In addition to contributing to a still ongoing debate regarding the adaptive value of DGCs, the observation that dehydrated insects relinquish spiracular control raises questions relating to the mechanistic basis of DGC maintenance under stressful conditions. Despite the overall enhanced stress resistance attributed to the gregarious phase in the migratory locust *Locusta migratoria* (Wang et al., 2007), we did not find significant differences between the desert locust density-dependent phases in water loss rates and the duration of exposure to stress before DGCs were abandoned. Initial body water content of hydrated locusts of both phases was similar, as was body water content when DGC was abolished. Nevertheless, the significantly different effects of the combined desiccation and starvation experimental stress on DGC characteristics in gregarious and solitary locusts highlight the links between DGC characteristics, water balance and energy metabolism.

As a result of continuous cellular respiration, the ability to maintain spiracle closure during the interburst is limited by accumulation of CO_2, which leads to a threshold P_{CO_2} that triggers the beginning of the O-phase (Förster and Hetz, 2010). In the present study, this ability was affected during exposure of locusts to the experimental treatment both by decreasing the volume of body fluids and by changing $\dot{V}_{CO_2}$. We did not measure haemolymph volume, but dehydration of adult *S. gregaria* to ~20% mass loss has been reported to result in a 40–50% loss of haemolymph volume (Albaghdadi, 1987), which would result in a considerably compromised ability to accumulate soluble CO_2 during the interburst. The finding that dehydrated gregarious locusts maintained control of interburst duration values (Fig. 2B) appears to contradict the expected effect of reduced haemolymph volume on CO_2 accumulation capacity. However, the 35–40% decrease in $\dot{V}_{CO_2}$ values between day 1 and day L means that the interburst duration was not proportionally shortened with haemolymph volume depletion. Calculation of CO_2 accumulation during the interburst yielded several interesting observations. First, while analysis of cycle characteristics appeared to suggest no link between hydration state and DGC performance in gregarious locusts, the decrease in $\dot{V}_{CO_2}$ with exposure to experimental stress, coupled with unchanged interburst duration, translate into a compromised CO_2 accumulation capacity. Second, the decreased ability to accumulate CO_2 during the interburst as a result of dehydration was more pronounced in solitary than in gregarious locusts (Fig. 3), despite their comparable hydration states. Third, rehydration in gregarious locusts resulted in recovery of initial levels of interburst CO_2 accumulation, whereas recovery in solitary locusts was only partial (Fig. 3). Together, these observations indicate that DGCs were affected by factors other than body water availability during the exposure to experimental stress.

Accumulation of CO_2 in the haemolymph during the interburst incurs disturbance to the acid–base balance, the magnitude of which is determined by the haemolymph buffering capacity. Haemolymph proteins provide an important non-bicarbonate buffer in *S. gregaria* (Harrison et al., 1990) and, therefore, we compared haemolymph protein concentration in gregarious and solitary locusts as a measure of their haemolymph buffering capacity. In gregarious locusts, a significant increase in haemolymph protein concentration was observed with increasing dehydration level (Fig. 4A). Assuming a loss of 40–50% haemolymph volume at mass loss levels of ~20% (Albaghdadi, 1987), an increase of similar proportions in protein concentration (Fig. 4A) suggests that haemolymph protein content remained intact in dehydrated gregarious locusts. In contrast, haemolymph protein concentration remained unchanged across the same range of experimental dehydration levels in the solitary individuals. Similar water loss rates for solitary and gregarious locusts (Table 1), and assumed similar rates of haemolymph depletion (supported by similar rates of increase in haemolymph osmolality; Fig. 4B), suggest that haemolymph protein content decreased during exposure to experimental stress in the solitary locusts, potentially compromising their haemolymph buffering capacity. RER calculation (Fig. 5) indicated that both solitary and gregarious locusts increasingly relied on lipid stores for their

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metabolic needs as exposure to stressful conditions progressed. This observation is in agreement with previous findings for gregarious *L. migratoria* (Loveridge and Bursell, 1975) and for *Romalea guttata* and *Taeniopoda eques* (Quinlan and Hadley, 1993). Interestingly, RER values were significantly higher in solitary compared with gregarious locusts. This suggests a higher reliance on non-lipid metabolic fuels, and perhaps the catabolism of haemolymph proteins in the former. This phase-specific difference in energy metabolism is correlated with significantly higher lipid content in gregarious locusts, for which lipid provides an exclusive metabolic fuel supporting migratory flight (Schneider and Dorn, 1994; Ayali and Pener, 1995; Ayali et al., 1996; Pener et al., 1997). It may also correlate to the function of adipokinetic hormone (AKH) during stress (e.g. Perić-Mataruga et al., 2006), as well as to the well-established phase-dependent differences in AKH response (primarily, the increased AKH-induced mobilization of lipids in gregarious compared with solitary locusts; Ayali and Pener, 1992; Ayali et al., 1994).

Despite the correlation between changes in haemolymph protein content and interburst CO_2 accumulation capacities that result from experimental stress, it is likely that other factors are also at play. Significantly higher initial protein concentrations in the haemolymph of solitary (compared with gregarious) locusts are not reflected in greater CO_2 accumulation during the interburst. In addition, the difference in haemolymph protein content at about 20% loss of initial mass (Fig. 4A), typical of day L, cannot explain the greater than twofold difference in CO_2 accumulation between locust phases (Fig. 3). Nevertheless, the contribution of haemolymph proteins to the capacity for CO_2 accumulation is expected to be lower in the hydrated state, when haemolymph volume is twofold higher and its ionic strength is lower (Fig. 4B). The impact of proteins on haemolymph buffering depends on their concentration and composition, both of which could vary with insect age, sex, diet and nutritional status. In addition, given the wide range of phase-specific physiological variation, we cannot rule out the possibility that haemolymph proteins in solitary and gregarious locusts vary in composition too. Literature data on the specific buffer value of haemolymph proteins is based on haemolymph of female *S. gregaria*, 3–4 weeks after adult eclosion (Harrison et al., 1990), whereas males were used in the present study. Sex-specific variation in haemolymph amino acid composition has been reported for young adult *S. gregaria* (Benassi et al., 1961). Interestingly, the haemolymph of males was reported to exhibit a considerably higher concentration of histidine, the imidazole groups of which have pKa values of 7–8 and thus provide most of the buffering capacity at physiologically relevant pH values (Truchot, 1987). We therefore refrained from attempting to quantify the extent of the link between haemolymph protein content and CO_2 accumulation during the interburst. Nevertheless, it would be interesting to investigate whether sex-specific variation in haemolymph amino acid composition is reflected in CO_2 accumulation capacities in *S. gregaria*.

Considering their similar initial body water contents, water loss rates, and the latency to abolishment of DGCs, locusts of the two density-dependent phases are likely to have similar haemolymph volumes on day L. Solubility of CO_2 in the haemolymph is also not expected to vary among the two phases, given the observed effect of dehydration on their haemolymph osmolality (Fig. 4B). Therefore, the similar measured $\dot{V}_{CO_2}$ values (Table 1) should be reflected in comparable rates of interburst CO_2 build-up in body fluids. Nevertheless, the interburst duration was significantly shorter in dehydrated solitary compared with gregarious locusts (Fig. 2A), suggesting that loss of haemolymph proteins and compromised buffering capacity resulted in a sharper increase in haemolymph P_{CO_2}, which reached the critical values for triggering spiracle opening more rapidly. The effect of variation in haemolymph protein management was also highlighted by DGC characteristics following rehydration, which replenished some of the water but not the proteins lost during desiccation and starvation. All of the rehydrated insects recovered DGCs after having ingested the same volume of water, but only the gregarious locusts recovered control values of CO_2-accumulating capacity (Fig. 3B). Although the rehydrated solitary locusts re-established control interburst durations (Fig. 2), this was achieved at significantly lower CO_2 production rates (Table 1). This indicates that factors other than water budget are associated with changing the DGC characteristics during exposure to stressful conditions.

However, why did solitary and gregarious locusts abolish DGCs at the same hydration state, following similar periods of exposure to desiccation and starvation stress? Maintenance of haemolymph buffering capacity is correlated with longer interburst periods in the gregarious compared with the solitary locusts under stressful conditions. Hence, unless the effect of dehydration on threshold P_{CO_2} values for spiracular opening (Bursell, 1957; Miller, 1964) varies in the two phases, gregarious locusts should be expected to maintain DGCs for longer. A possible explanation lies in the hierarchy of factors affecting DGC expression and its characteristics (Groenewald et al., 2014). According to the model suggested by those authors, DGCs will be expressed and their characteristics will be determined by acid–base balance considerations, as long as the pattern allows adequate oxygen supply to the tissues. Decreasing metabolic rates under stressful conditions prolong the ability to express DGCs, but their abolishment at similar hydration states yet varied haemolymph buffering capacities, in the two locust phases, suggests that it is triggered by a failure to meet tissue oxygen demand. Similar tracheal volumes (Table 2) mean that gregarious and solitary locusts do not vary in the magnitude of their oxygen resources once the spiracles are closed. It has recently been shown that *S. gregaria* complement diffusive oxygen transport to their tissues with active ventilation during the interburst (Huang et al., 2014). A decrease in haemolymph volume and pressure may impair the compression of air sacs and thus compromise ventilatory gas transport in the tracheal system (Harrison et al., 2013), as was also reflected in reduced maximal $\dot{V}_{CO_2}$ values in dehydrated locusts during the burst (Fig. 1, Table 1). Likewise, the accumulation of more CO_2 during the interburst following rehydration (Fig. 3) was not matched by an increased duration of O-phase (Fig. 2C), which is in line with higher maximal $\dot{V}_{CO_2}$ values during the burst (Fig. 1, Table 1) and a role for the increased haemolymph volume in active ventilation. Interestingly, this contradicts the suggestion that CO_2 accumulation during DGC results in longer spiracle opening than is necessary for oxygen uptake, and thus that exhibiting the pattern incurs a water vapour loss penalty (Woods and Smith, 2010). An additional effect of haemolymph water loss on oxygen supply could be that of elevated fluid viscosity, which would further limit what is already the main barrier for oxygen supply: namely, diffusion through the fluid-phase from the tracheole terminals to the tissues (Timmins et al., 2000). This would also contribute to correlation between haemolymph loss rate and timing of DGC loss in both gregarious and solitary *S. gregaria*, independent of variation in their haemolymph buffering capacity.

Finally, burst duration was not affected by hydration state, and was consistently longer in solitary than in gregarious locusts (Fig. 2C). The burst phase is triggered by elevated tissue (and

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tracheal) CO_2 levels (Levy and Schneiderman, 1966), but it is less clear what dictates the timing of spiracle closure, which initiates the subsequent interburst (Heinrich and Bradley, 2014). The consistently shorter burst phase in gregarious locusts, irrespective of hydration state, provides additional support for a fundamental difference in the regulation of respiratory gas exchange in the two phases of *S. gregaria* (Berman et al., 2013). Rapid elimination of CO_2 during the burst is expected to require a high tissue carbonic anhydrase (CA) concentration/activity (Quinlan and Gibbs, 2006). Although we found no difference in the mass-specific fat body tissue CA activity between the density-dependent phases, the considerably higher fat content in gregarious locusts (Schneider and Dorn, 1994; Ayali and Pener, 1995) may result in an overall higher CA activity, and thus a more rapid release of CO_2. In addition, a shorter burst (and higher peak burst $\dot{V}_{CO_2}$) in the gregarious locusts may result from more vigorous active ventilation during the burst.

In conclusion, we did not find phase-specific differences in resistance to desiccation in *S. gregaria*, and variation in DGC characteristics was not reflected in water savings. However, the phase-specific effects of stress exposure on DGC characteristics, despite the comparable water budgets, revealed an important role of energy metabolism in the maintenance of DGCs. Stress response in the form of changing metabolic rates and the use of various metabolic fuels could explain intra- and potentially also interspecific variation in DGC characteristics. Our results indicate that the choice of metabolic fuel could affect cycle characteristics, not only according to typical RER values, but also as a result of its effect on the buffering capacity of body fluids.

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Author contributions
E.G. and A.A. attracted funds. All authors conceived and designed the experiments. S.T. performed the experiments and analysed the data. All authors contributed to manuscript preparation.

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