RESEARCH ARTICLE

The effect of discontinuous gas exchange on respiratory water loss in grasshoppers (Orthoptera: Acrididae) varies across an aridity gradient

Shu-Ping Huang[1], Stav Talal[1,2], Amir Ayali[2] and Eran Gefen[1,*]

ABSTRACT

The significance of discontinuous gas-exchange cycles (DGC) in reducing respiratory water loss (RWL) in insects is contentious. Results from single-species studies are equivocal in their support of the classic 'hygric hypothesis' for the evolution of DGC, whereas comparative analyses generally support a link between DGC and water balance. In this study, we investigated DGC prevalence and characteristics and RWL in three grasshopper species (Acrididae, subfamily Pamphaginae) across an aridity gradient in Israel. In order to determine whether DGC contributes to a reduction in RWL, we compared the DGC characteristics and RWL associated with CO_2 release (transpiration ratio, i.e. the molar ratio of RWL to CO_2 emission rates) among these species. Transpiration ratios of DGC and continuous breathers were also compared intraspecifically. Our data show that DGC characteristics, DGC prevalence and the transpiration ratios correlate well with habitat aridity. The xeric-adapted *Tmethis pulchripennis* exhibited a significantly shorter burst period and lower transpiration ratio compared with the other two mesic species, *Ocneropsis bethlemita* and *Ocneropsis lividipes.* However, DGC resulted in significant water savings compared with continuous exchange in *T. pulchripennis* only. These unique DGC characteristics for *T. pulchripennis* were correlated with its significantly higher mass-specific tracheal volume. Our data suggest that the origin of DGC may not be adaptive, but rather that evolved modulation of cycle characteristics confers a fitness advantage under stressful conditions. This modulation may result from morphological and/or physiological modifications.

KEY WORDS: Hygric hypothesis, DGC, Insect, Respiration, Tracheal volume

INTRODUCTION

Insects exchange respiratory gases with their external environment through their gas-filled tracheal system, which opens to the external environment through segmental pairs of spiracles. Most insect species control spiracle opening and closure, leading to a variety of gas exchange patterns. These are often categorized as continuous, cyclic and discontinuous gas exchange (Chown, 2011).

Discontinuous gas exchange cycles (DGC) are characteristic of quiescent insects (Lighton, 1996), and have been reported in insects of at least five different Orders (Marais et al., 2005). They typically consist of three phases, characterized by spiracular behaviour:

closed (C), flutter (F) and open (O). During the C phase, the spiracles are shut, with minimal gas exchange with the external environment. The oxygen in the tracheal system is gradually consumed and CO_2 generated from the tissues is dissolved into the haemolymph and diffuses to the tracheae. As a result of the higher solubility of CO_2 compared with that of oxygen, a negative endotracheal pressure between the ambient environment and the tracheal system builds up during the C phase. Oxygen decline triggers the F phase, during which the spiracles flutter rapidly and allow a mostly inward gas transfer. The continued increase in P_{CO_2} during the F phase triggers the O phase when P_{CO_2} reaches a threshold (Levy and Schneiderman, 1966; Lighton, 1996; Förster and Hetz, 2010). In the O phase spiracles open wide, leading to a rapid exchange of respiratory gases with the insect's surroundings. Tracheal oxygen replenishment and CO_2 washout lead to spiracle closure, and repeated gas exchange cycles. The O phase is often referred to as a burst phase, while the F and C phases are collectively referred to as an interburst phase (Chown et al., 2006).

Although the adaptive value of DGC has attracted research attention for decades, a common explanation remains elusive (Chown, 2011). Three main hypotheses have been proposed for the adaptive significance of DGC: the hygric hypothesis (Buck and Keister, 1955), which suggests that DGC reduces respiratory water loss (RWL); the chthonic hypothesis (Lighton and Berrigan, 1995), which suggests that DGC maximizes respiratory gas partial pressure gradients between insect tracheae and the surrounding atmosphere, thus facilitating gas exchange (and reducing RWL) in hypercapnic and/or hypoxic subterranean environments; and the oxidative damage hypothesis, which suggests that DGC reduces oxidative damage to the tissues (Hetz and Bradley, 2005).

To date, none of the existing adaptive hypotheses has been backed by unequivocal experimental support (Chown et al., 2006; Quinlan and Gibbs, 2006; Chown, 2011). The hygric hypothesis has received considerable experimental support. For example, chronic exposure of cockroaches to low humidity results in shorter bursts of gas exchange, correlated with reduced rates of body mass loss (Schimpf et al., 2009). Employing DGC is also associated with increased desiccation and starvation resistance (Schimpf et al., 2012). Lower rates of RWL are associated with DGC compared with continuous exchange in lepidopteran larvae (Williams et al., 2010), but findings from similar studies are contradictory to predictions of the hygric hypothesis in ants (Gibbs and Johnson, 2004; Lighton et al., 2004; Lighton and Turner, 2008) and cockroaches (Groenewald et al., 2013). Moreover, earlier studies provided the first experimental challenge to the hygric hypothesis, showing that DGC is abolished in dehydrated insects when body water conservation is most essential (Hadley and Quinlan, 1993; Quinlan and Hadley, 1993). A more recent study showed that neither acclimation to dehydrating conditions nor acute exposure to

[1]Department of Biology, University of Haifa-Oranim, Tivon 600600, Israel. [2]Department of Zoology, Tel Aviv University, Tel Aviv 6997801, Israel.

*Author for correspondence (gefene@research.haifa.ac.il)

List of abbreviations

C phase	closed phase
CWL	cuticular water loss
DGC	discontinuous gas exchange cycle
EWL	evaporative water loss
F phase	flutter phase
O phase	open phase
RWL	respiratory water loss
$\dot{V}_{CO_2}$	CO_2 emission rate

low humidity resulted in shorter burst or longer interburst duration in grasshoppers (Orthoptera, Acrididae) (Groenewald et al., 2014).

However, comparative studies generally support a role for DGC in water conservation. A meta-analysis of DGC characteristics, habitat aridity and temperature provided support for the hygric hypothesis (White et al., 2007). Comparative studies also showed that xeric species modulate DGC characteristics to restrict RWL (Duncan and Byrne, 2000; Chown and Davis, 2003). To the best of our knowledge, work by Chown and Davis (2003) provides the only comparative study examining RWL associated with DGC, demonstrating that xeric species have reduced RWL rates correlated with DGC characteristics, e.g. longer interburst phase, shorter burst phase and/or shorter cycle length. To date, comparative DGC studies have largely focused on Coleoptera (Duncan and Byrne, 2000; Chown and Davis, 2003) and Hymenoptera species (Schilman et al., 2005).

One of the early major criticisms of the hygric hypothesis for the evolution of DGC stems from the findings of Hadley and Quinlan (1993) that dehydrated grasshoppers abolish DGC. Phylogenetic analysis of the distribution of gas exchange patterns in insects suggested that DGC has evolved at least five times independently (Marais et al., 2005). We therefore aimed to test whether the predictions of the hygric hypothesis would be supported using a comparative approach in Orthopterans. Our study animals were three closely related grasshopper species (Orthoptera: Acrididae, subfamily Pamphaginae): the xeric-adapted *Tmethis pulchripennis* (Serville 1838) and the mesic *Ocneropsis bethlemita* (Bolivar 1893) and *Ocneropsis lividipes* Fishelson 1985, distributed across an aridity gradient in Israel (Fig. 1).

In order to investigate whether DGC is associated with body water conservation, we examined the RWL associated with CO_2 release ('transpiration ratio') (Gibbs and Johnson, 2004) in both continuous and DGC breathers within species. We also examined the prevalence of DGC in each species by calculating the proportion of individuals exhibiting DGC. We hypothesized that DGC prevalence and other characteristics would correlate with the habitat aridity of these species. We predicted that: (1) DGC prevalence would be positively correlated with habitat aridity; (2) *T. pulchripennis* would have a longer interburst phase and/or a shorter burst phase compared with the two mesic species; and (3) DGC and variation in its characteristics would be translated to water savings. Tissue oxygen demand and tracheal conductance limiting its supply are major factors affecting gas exchange pattern characteristics in grasshoppers (Groenewald et al., 2014; Huang et al., 2014). Therefore, we measured grasshopper tracheal volume in an attempt to correlate DGC characteristics with potential variation in available oxygen storage volume during the interburst phase.

RESULTS
DGC prevalence and characteristics

A significantly higher DGC prevalence was detected in *T. pulchripennis* and *O. bethlemita* (60.6% and 51.6%, respectively) compared with *O. lividipes* (19.1%) (both $P<0.017$, Fisher's exact test; Table 1). Fig. 2 presents typical species-specific
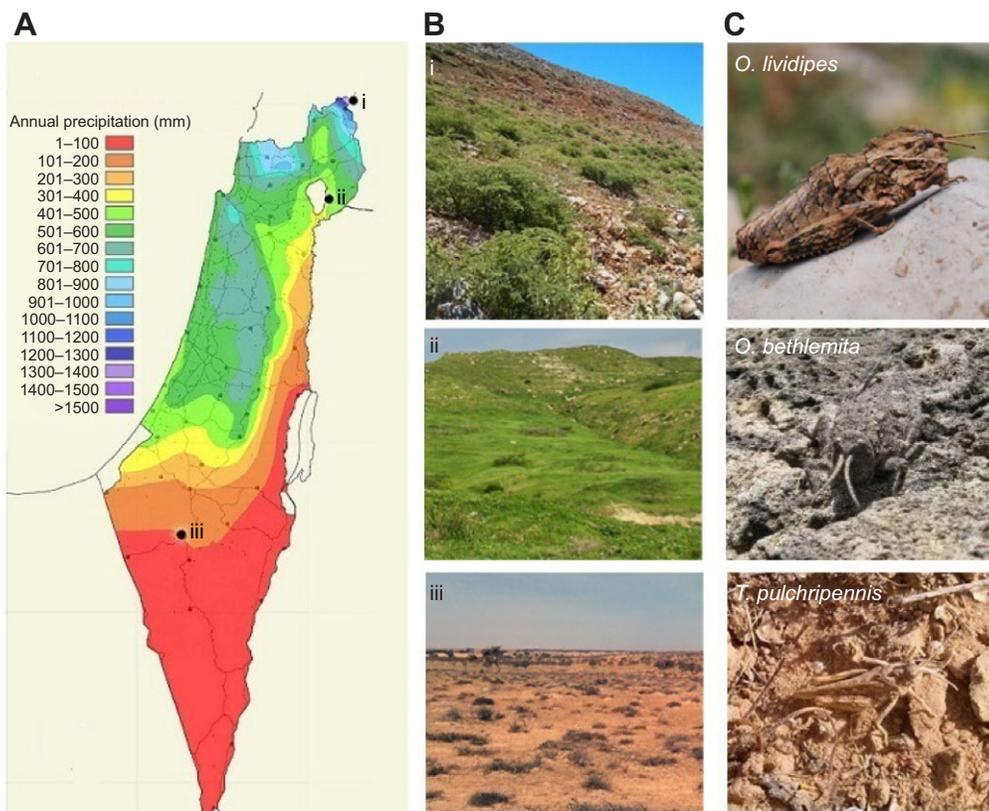

Fig. 1. Collection sites and typical habitats of the three grasshopper species. (Ai) Mt Hermon; (Aii) Susita; (Aiii) Bor Mashash, Negev. Mean annual precipitation map (1981–2010) follows http://www.ims.gov.il/IMS/CLIMATE/ClimaticAtlas/RainMaps.htm. (B) Typical habitats of the locations identified in A. (C) The study species: *Ocneropsis lividipes*, *Ocneropsis bethlemita* and *Tmethis pulchripennis*.

Table 1. DGC prevalence and characteristics in the study grasshoppers

Species	T. pulchripennis	O. bethlemita	O. lividipes
DGC prevalence[1]	60.6%[a]	51.6%[a]	19.1%[b]
Sample size	33	31	58
DGC characteristics			
Sample size[2]	20	16	22
Body mass (g)	0.51±0.07[b]	0.96±0.12[a]	0.98±0.09[a]
Cycle length (min)	9.0±4.8	11.2±3.6	10.7±3.8
Burst phase (min)	2.0±0.6[b]	4.4±1.3[a]	4.5±1.6[a]
Interburst phase (min)	6.9±4.5	6.8±3.1	6.1±3.1
Interburst phase ratio[3]	72.2±10.4%[a]	58.7±11.7%[b]	56.1±11.8%[b]
Burst phase ratio[3]	27.7±10.4%[b]	41.2±11.7%[a]	43.8±11.8%[a]
$\dot{V}_{CO_2}$			
Total (μl h^{-1} g^{-1})	269.3±55.8	288.1±85.2	274.3±38.9
Interburst phase (μl h^{-1})	14.6±5.4[c]	44.1±24.0[b]	76.1±37.9[a]

Data for *Tmethis pulchripennis*, *Ocneropsis bethlemita* and *Ocneropsis lividipes* are means±s.d. (except for DGC prevalence and sample size). DGC, discontinuous gas exchange cycle; $\dot{V}_{CO_2}$, CO_2 emission rate (as a measure of metabolic rate).
Different letters indicate a significant difference between species.
[1]Based on the first measurement of each individual.
[2]Data for *O. lividipes* include individuals that did not exhibit DGC in the first measurement.
[3]Phase ratio=100%×phase length/DGC cycle length.

DGC patterns: DGC of *T. pulchripennis* is characterized by negligible CO_2 emission during the interburst followed by a sharp CO_2 burst phase. In comparison, *O. bethlemita* and *O. lividipes* display an interburst phase consisting of what appears as very short spiracle closure phase followed by a gradual increase in CO_2 emission rate leading to the burst phase. The CO_2 emission rate during the interburst was significantly lower in *T. pulchripennis* compared with that of the other two species (Table 1; $F_{2,24.8}$=37.6, $P<0.001$, Welch's ANOVA).

The interburst duration did not significantly differ among these three species (Table 1). However, *T. pulchripennis* had a shorter burst phase (2.0±0.6 min), resulting in a lower proportion of burst to cycle duration (27.7%) compared with that of the other two species (burst duration: 4.4–4.5 min, 41.2–43.8% of the cycle length). The mass-specific metabolic rate (expressed as CO_2 emission rate, $\dot{V}_{CO_2}$) during DGC did not vary significantly among the three species (Table 1; supplementary material Fig. S1).

RWL and transpiration ratio

The contribution of RWL to total water loss was significantly lower in *T. pulchripennis* (6.8%) than in the other two species (10.1–14.2%) ($P<0.017$, Kruskal–Wallis test; Table 2). In all three species, DGC was associated with significantly lower metabolic rates compared with continuous gas exchange (all $P<0.05$, Kruskal–Wallis test; Fig. 3A). However, only *T. pulchripennis* displayed a lower transpiration ratio (mol H_2O/mol CO_2) when breathing with DGC compared with continuous gas exchange (Fig. 3B).

Tracheal volume and body water content

Mass-specific tracheal volume was significantly higher in *T. pulchripennis* than in the other two species (Table 3). Moreover, the large tracheal volume of *T. pulchripennis* was significantly higher than the expected value based on the tracheal volume–body size relationship for 11 grasshopper species (Fig. 4). In contrast, body water content did not differ significantly among the three study species (66.2–67.3%).

DISCUSSION

The hygric hypothesis proposed that a reduction in RWL could provide an adaptive explanation for the evolution of DGC (Buck and Keister, 1955). Our data do not consistently support predictions of the hygric hypothesis, but still indicate that DGC may have an ecological significance for insects coping with environmental water scarcity. First, DGC prevalence among the three study species corresponded well to their respective habitat aridity. Second, DGC of xeric-adapted *T. pulchripennis* were characterized by very low CO_2 emissions during their interburst phase, and significantly shorter bursts compared with those of the two more mesic species (Table 1). Third, *T. pulchripennis* showed a significantly lower transpiration ratio than the other two species when expressing DGC, and is also the only species in which a lower transpiration ratio was calculated during DGC compared with continuous gas exchange (Fig. 3).

DGC prevalence is correlated with habitat aridity

A recent study (Schimpf et al., 2013) showed for the first time that DGC expression is a heritable trait, a prerequisite for natural selection regardless of the pattern's actual adaptive value. Surprisingly, DGC prevalence in the population is rarely addressed despite its suggested ecological relevance. In this study, we used relatively large sample sizes, which allowed us to calculate the DGC prevalence in each study species and to examine how these agree with the species' habitat water availability according to predictions of the hygric hypothesis. Our findings show that DGC prevalence indeed corresponds well with aridity levels in the respective species' natural habitats (Fig. 1, Table 1). In our preliminary study, a very low DGC prevalence (10.8%, N=37) was detected for another mesic species, *Prionosthenus galericulatus*, of the same subfamily (Pamphaginae) as our study species. This indicates that DGC prevalence in a population could be informative in examining its ecological and evolutionary significance. For example, DGC was reported to be predominant in xeric insects, while other breathing patterns (cyclic, continuous) were more predominant in mesic insects (Marais et al., 2005). Our study, however, shows that DGC prevalence is a quantitative attribute of a species that could relate to its ecological significance in the species' natural habitat.

The hygric hypothesis for the evolution of DGC has been challenged by the relatively low contribution of RWL to total water
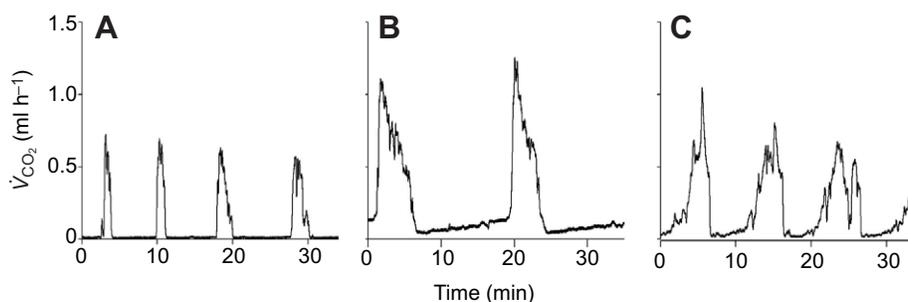

Fig. 2. Typical discontinuous gas-exchange cycle (DGC) traces for the three study species at 25°C. (A) *T. pulchripennis*, (B) *O. bethlemita* and (C) *O. lividipes*. $\dot{V}_{CO_2}$, CO_2 emission rate.

Table 2. Mass-specific rates of CO_2 and water vapour emission in DGC breathers in the study grasshoppers

Species	T. pulchripennis	O. bethlemita	O. lividipes
Body mass (g)	0.51±0.07[b]	1.10±0.12[a]	0.98±0.08[a]
Sample size	9	10	14
sp$\dot{V}_{CO_2}$ (ml h^{-1} g^{-1})	0.27±0.05	0.31±0.08	0.27±0.03
spEWL rate (µl h^{-1} g^{-1})	1.07±0.33	0.89±0.16	0.88±0.26
spCWL rate (µl h^{-1} g^{-1})	1.00±0.33	0.77±0.17	0.81±0.25
spRWL rate (µl h^{-1} g^{-1})	0.06±0.01	0.12±0.04	0.08±0.02
RWL (% of total)	6.8±2.3%[b]	14.2±6.2%[a]	10.1±3.0%[a,b]
Transpiration ratio	0.27±0.05[b]	0.75±0.22[a]	0.54±0.12[a]

Data are means±s.d. (except for sample size).
Data are mass-specific (sp) rates of: $\dot{V}_{CO_2}$, CO_2 emission; EWL, evaporative water loss; CWL, cuticular water loss; RWL, respiratory water loss.
Different letters indicate significant differences between species ($P<0.017$, Kruskal–Wallis test).

loss (Chown, 2002). However, habitat aridity could provide extra selective pressure, whichwould result in an increased frequency of DGC-performing individuals in the population. Groenewald et al. (2014) reported 100% prevalence of DGC for the mesic grasshopper *Paracinema tricolor* (Orthoptera: Acrididae) at 15°C. Importantly, insect metabolic rate and DGC are temperature dependent (Contreras and Bradley, 2010), and therefore DGC prevalence is expected to change with experimental temperature. The employed experimental temperature in this study (25°C) was within the annual temperature range typical of the habitats of all three study grasshopper species. It is possible that interspecific variation in the ability to maintain spiracle closure and DGC with increasing temperature (and metabolic rate) would be unveiled only at higher experimental temperatures within the ecologically relevant range.
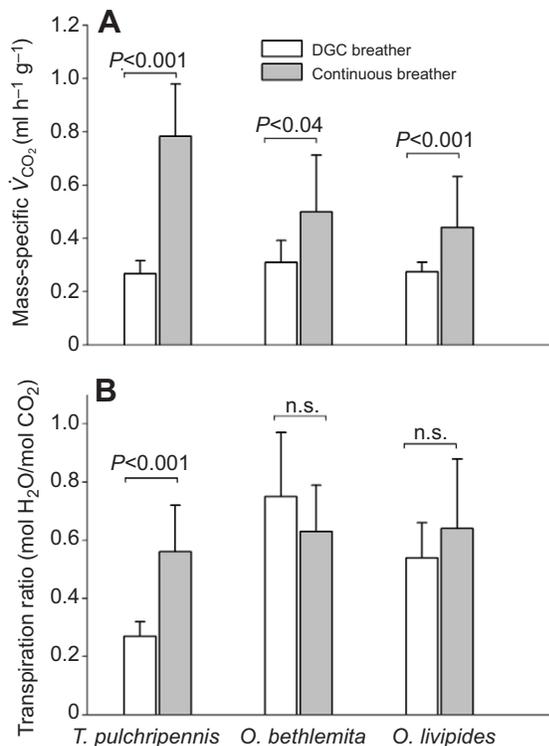

Fig. 3. Mass-specific $\dot{V}_{CO_2}$ and transpiration ratio in the three study species. (A) Metabolic rate expressed as $\dot{V}_{CO_2}$ and (B) transpiration ratio are shown for DGC and continuous breathers. Values are means+s.d.; N=8–9 for *T. pulchripennis* and *O. bethlemita*, N=14 for *O. lividipes*. n.s., not significant.

Table 3. Tracheal volume and body water content in the study grasshoppers

Species	T. pulchripennis	O. bethlemita	O. lividipes
Body mass (g)	0.45±0.06[b]	0.92±0.15[a]	1.01±0.11[a]
Sample size	26	20	36
Tracheal volume (µl)	178.9±31.0[b]	232.3±55.7[a]	270.8±68.1[a]
Mass-specific tracheal volume (µl g^{-1})	404.1±75.4[a]	255.0±77.6[b]	264.6±74.5[b]
Body water content (%)	67.3±2.1	66.2±3.6	66.9±2.3

Data are means±s.d. (except for sample size).
Different letters indicate significant differences between species.

Nevertheless, we note that DGC prevalence under field conditions is subject to the thermal biology and ecology of the respective species, for which data are currently not available.

Variation in DGC characteristics and RWL rates
Modulation of DGC characteristics has been reported to have possible implications for water balance in various insect species. For example, a shorter burst phase and cycle length have been reported in cockroaches in response to low humidity (Schimpf et al., 2009); xeric species exhibited a shorter burst phase, longer interburst phase or longer DGC, with or without an associated reduction in metabolic rate, compared with their mesic counterparts (Duncan et al., 2002; Chown and Davis, 2003; White et al., 2007). In this study, we show that variation in DGC characteristics is correlated with RWL. Interburst duration did not differ significantly among the study species, while the burst phase was shorter in the xeric-adapted *T. pulchripennis* than in the other two species. The lack of interspecific variation in interburst duration is in agreement with the finding of similar metabolic rates and body water content for the three species (Tables 1, 3), resulting in comparable rates of CO_2 buildup. In contrast, by percentage, the length of the burst
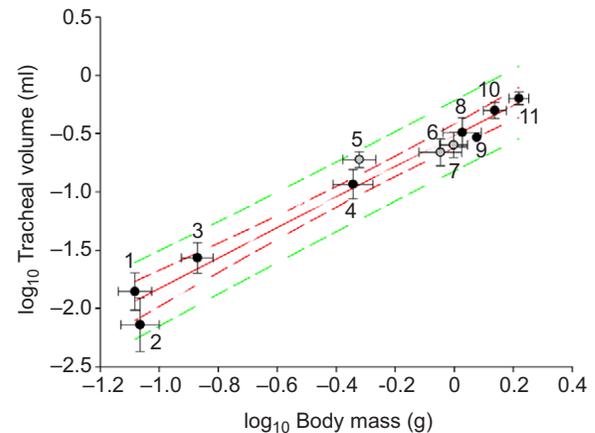

Fig. 4. Tracheal volume as a function of body mass in adult male grasshoppers. The log-transformed means (±s.d.) for the three study species (grey) and eight others (black; E.G., unpublished; Lease et al., 2006) were used to build the regression line. Regression equation: $\log_{10}$ tracheal volume=1.307×$\log_{10}$ body mass–0.519 (indicated by the solid red line, N=11, r^2=0.96; red dashed lines indicate 95% confidence intervals; green dashed lines indicate 95% prediction intervals). Species are numbered as follows: 1, *Pyrgomorpha conica* (N=31; w); 2, *Pyrgomorphella granosa* (N=22; nw); 3, *Notostaurus anatolicus* (N=5; w); 4, *Prionosthenus galericulatus* (N=29; nw); 5, *T. pulchripennis* (N=26; w); 6, *O. bethlemita* (N=20; nw); 7, *O. lividipes* (N=38; nw); 8, *Poekilocerus bufonius* (N=32; w); 9, *Schistocerca americana* (Lease et al., 2006; w); 10, *Locusta migratoria* (N=19; w); and 11, *Schistocerca gregaria* (N=14; w). w, winged; nw, non-winged.

phase accounted for about 27% of the DGC cycle for *T. pulchripennis*, which was shorter than that of the other two mesic species (>40% of the cycle length) (Table 1). Moreover, *T. pulchripennis* showed significantly lower CO_2 emission rates during the interburst than did the other two species, suggesting tighter spiracle closure with possible consequences for water vapour emission during the interburst. Together, a shorter burst phase and tighter spiracle closure in the interburst phase explain the lower RWL in *T. pulchripennis* compared with the *Ocneropsis* sp.

DGC is correlated with reduced RWL in the xeric-adapted *T. pulchripennis* only

The contribution of DGC to water savings in insects is controversial. Larvae of *Erynnis propertius* (Lepidoptera: Hesperiidae) conserve body water better when employing DGC (Williams et al., 2010), whereas RWL in ants is not affected by gas exchange patterns (Gibbs and Johnson, 2004; Lighton et al., 2004; Schilman et al., 2005). To the best of our knowledge, this is the first single-study, interspecific analysis of closely related species comparing the effect of gas exchange pattern (DGC versus continuous) on RWL. In all three study species, DGC was associated with low metabolic rate (Fig. 3A). However, among the three species, only the xeric-adapted *T. pulchripennis* enhanced body water conservation (i.e. a lower transpiration ratio) by employing DGC in comparison with continuous respiratory gas exchange (Fig. 3B).

Both metabolic rate and water loss rate vary with body size in insects (Zachariassen et al., 1987; Chown, 2002), and therefore an interspecific comparison of RWL rates would depend on mass dependence of both these variables across the study species. Moreover, the overlap in adult body size across the study species is only partial (Table 1), which further complicates comparison of absolute water loss rates. The relative importance of RWL across the study species was minor, ranging from 6.8% to 14.2% of total losses (Table 2). These values are in agreement with previously reported values for *Melanoplus sanguinipes* (15%; Rourke, 2000) and for *Romalea guttata* and *Taeniopoda eques* (<10%; Quinlan and Hadley, 1993). Significantly lower proportions of RWL in *T. pulchripennis* may support a role for modified DGC characteristics in reducing water loss in this xeric-adapted species (Tables 1, 2) (see Groenewald et al., 2013, and references therein). Moreover, calculation of RWL based on water vapour emission during the burst phase only (see Materials and methods) underestimated RWL for the *Ocneropsis* sp. but not for *T. pulchripennis* (see Fig. 2 for CO_2 emission traces). Nevertheless, the relative importance of RWL as an index for adaptive response to habitat water scarcity is questionable because the other main component of water loss, cuticular water loss (CWL), often varies with environmental aridity (Chown, 2002).

We therefore used the transpiration ratio to evaluate the RWL associated with CO_2 emission during different gas exchange patterns employed by our study species. This index is independent of the possible varying dependence of metabolic rate and water loss rate on body size within and across species. It is also of considerable ecological significance because it estimates the cost in water loss per unit of energy expended in each species (Woods and Smith, 2010). Analysis of RWL rates and oxygen uptake rates available from the literature suggested that transpiration ratios in insects exhibiting DGC were in fact higher than predicted by their metabolic rates (Woods and Smith, 2010). Adding data from this study to the model (Fig. 5) suggests a possible taxon effect, with most grasshopper RWL rates lower than predicted by oxygen uptake rates. The significantly low transpiration ratio in *T. pulchripennis*
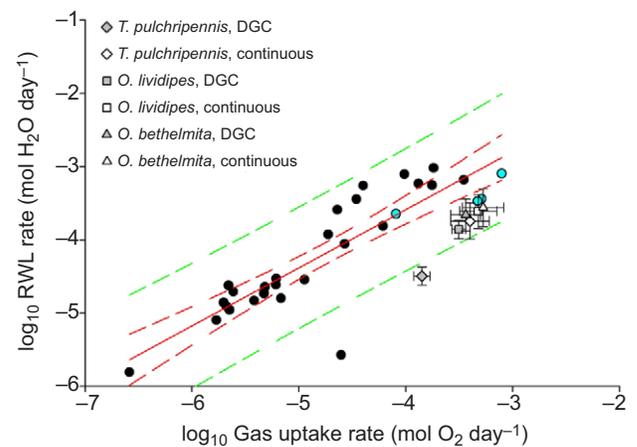
Fig. 5. Respiratory water loss (RWL) rate as a function of oxygen consumption rate. Data are means (±s.d.) for the three study species exhibiting DGC (grey symbols) and continuous (open symbols) patterns superimposed on available insect data (black circles) from the literature as plotted by Woods and Smith (2010). Light blue circles represent literature data for three grasshopper species exhibiting various gas exchange patterns. Regression equation for literature data: $\log_{10}$ RWL rate=$0.792 \times \log_{10}$ gas uptake rate-0.421 (indicated by the solid red line, $r^2=0.77$; red dashed lines indicate 95% confidence intervals; green dashed lines indicate 95% prediction intervals) (following Groenewald et al., 2013). Oxygen consumption rates were calculated for the three study grasshopper species by assuming a respiratory exchange ratio of 0.84 (Lighton, 2008).

employing DGC is highlighted as it falls below and outside the model prediction limits altogether (Fig. 5), and thus supports the role of this gas exchange pattern in the water budget of this species. Moreover, the independence of RWL from the gas exchange pattern in the two other study species suggests that DGC may not have evolved as a water-saving mechanism. Instead, an evolved variation in DGC characteristics may confer a fitness advantage in stressful environments (Chown, 2011). Mechanistically, the species-specific effect of DGC on transpiration ratios reported here could result from varying reliance on convective and diffusive exchanges during the O-phase, with the former expected to contribute to lower RWL rates (Kestler, 1985; Chown et al., 2006). Shorter burst duration (Table 1) and higher tracheal volume (Fig. 4) in *T. pulchripennis* suggest enhanced tracheal convection in this species.

Underlying mechanism for interspecific differences in DGC-associated RWL

The results of this study highlight the need for further research leading towards a better understanding of the mechanistic basis for species-specific DGC characteristics and their ecological relevance. We recorded a significantly longer burst phase for the two mesic species compared with the xeric-adapted species. It has been suggested that some insects may need to keep their spiracles open longer than others during the burst phase in order to eliminate the CO_2 dissolved in the haemolymph during the interburst – perhaps longer than is necessary for obtaining sufficient O_2 (Woods and Smith, 2010). It is possible that a shorter burst duration, associated with a reduced water loss (Chown and Davis, 2003), may result from more extensive active ventilation. Alternatively, the elimination rate of CO_2 accumulated in the haemolymph during the interburst may depend on carbonic anhydrase activity during the burst. Therefore, variation in carbonic anhydrase activity could be responsible for interspecific variation in burst duration if the rate of bicarbonate conversion back to CO_2 is a limiting step in CO_2

washout. Surprisingly, tissue buffering capacity and the role of carbonic anhydrase in insect gas exchange have received little research attention to date (Quinlan and Gibbs, 2006).

Measurement of tracheal volume (Table 3) provides an interesting possible link between this gas exchange system's morphology and its effect on DGC characteristics and RWL. The mass-specific tracheal volume of *T. pulchripennis* is ca. 1.5-fold higher than that of either of the two mesic species. Moreover, the tracheal volume of *T. pulchripennis* is also significantly higher than predicted by the relationship between tracheal volume and body mass for 11 grasshopper species (Fig. 4). A considerably higher oxygen storage volume, coupled with similar metabolic rates to those of the other study species (Table 1), suggests a slower decline in endotracheal P_{O_2} during the interburst in *T. pulchripennis*. Reaching critical P_{O_2} and P_{CO_2} values is known to trigger the F and O phases, respectively (Förster and Hetz, 2010). This could explain the DGC trace typical of *T. pulchripennis* (Fig. 2A), where an F phase was not detectable in any individual, perhaps as a result of a considerably delayed triggering by low P_{O_2}. In contrast, the other two species exhibited what appeared to be a short C phase and an obvious F phase (Fig. 2B,C) with $\dot{V}_{CO_2}$ gradually increasing during the interburst in all individuals (Table 1). This suggestion is in agreement with a dominant role for tracheal oxygen transport capacity in shaping DGC characteristics in grasshoppers (Groenewald et al., 2014; see also Huang et al., 2014). Importantly, CO_2 emission during the interburst would be accompanied by emission of water vapour, further increasing losses resulting from longer burst duration.

It should be noted that *T. pulchripennis* was the only winged (flying) species among our study animals. A larger mass-specific tracheal volume for *T. pulchripennis* compared with the other two study species could have evolved in order to meet the high tissue oxygen demand during flight. Our experimental setup cannot tease apart the potential benefits of a larger tracheal volume for meeting tissue oxygen demands or for body water conservation through reduced respiratory water loss. In addition, correlation with habitat type in this study lacks phylogenetic rigor as the two *Ocneropsis* sp. are more likely to share traits because of their presumed shared evolutionary history. The interspecific relationship between tracheal volume and body size based on data for 11 winged and wingless grasshopper species (Fig. 4) indicates that tracheal volume scales with mass$^{1.307}$ (Fig. 4), meaning a higher mass-specific investment in the tracheal system in larger species. This relationship is similar to the mass-scaling coefficient of 1.294 reported for four species of non-flying and rarely flying tenebrionid beetles spanning three orders of magnitude in body mass (Kaiser et al., 2007). The similar coefficients, despite considerably larger mass-specific tracheal volumes in grasshoppers (Lease et al., 2006; this study) compared with tenebrionid beetles, reaffirms the suggestion that structural limitations on tracheal growth provide a barrier to insect size in normoxic conditions (Kaiser et al., 2007). Nevertheless, the tracheal volume of *T. pulchripennis* is significantly higher than predicted by allometry (Fig. 4). Although our dataset cannot rule out a possible link between mass-specific tracheal volume and wing status, a tracheal volume of 420 µl g^{-1} for the winged desert locust *Schistocerca gregaria* (S.T. et al., unpublished data) compared with 246 µl g^{-1} for its winged mesic congener *Schistocerca americana* (Lease et al., 2006) mirror values reported here for the xeric-adapted *T. pulchripennis* and the mesic *Ocneropsis* sp., respectively (Table 3). This could support an evolved, morphologically derived modification of DGC characteristics, which may result in water savings in xeric species. A large tracheal system would also enhance the

thermoregulatory performance of *T. pulchripennis*, shown to be capable of respiratory evaporative cooling (Prange, 1990).

In conclusion, our data suggest that DGC does not result in water savings per se but rather provides a platform for modulation of cycle characteristics that could result in significant water savings in xeric species. Modification of this kind may be possible through a range of morphological and physiological routes, encompassing variation in tracheal dimensions, ventilatory muscle activity, and tissue and haemolymph buffering capacity. A better understanding of the role of DGC and its characteristics in stress resistance will require further comparative studies, which would ideally include data on the respective species' life history, behaviour, thermal biology and habitat microclimates.

MATERIALS AND METHODS

Grasshoppers

Adult male grasshoppers were collected from three locations in Israel, varying considerably in mean annual precipitation, during 2013–2014 (Bor Mashash, Negev for *T. pulchripennis*: ~100 mm; Susita for *O. bethlemita*: 533 mm; Mt Hermon for *O. lividipes*: >1000 mm) (Fig. 1). The grasshoppers were kept in the laboratory at 25°C, on a 14 h light:10 h dark photoperiod cycle (MIR-554 incubator, Panasonic, Japan), and were provided with fresh grass, natural vegetation and dry oats. Respirometry was carried out 2–4 days following insect collection.

Respirometry

Animals were provided with food until 1 h prior to flow-through respirometry at 25°C. Each grasshopper was introduced into a 9 ml metabolic chamber 1 h before measurements began. The incurrent air stream was scrubbed of H_2O and CO_2 by a Drierite/Ascarite column and passed through the metabolic chamber at a constant flow of 200 ml min^{-1} using factory-calibrated mass flow controllers (Alicat, Tucson, AZ, USA). Excurrent air was then passed through a CO_2/H_2O analyser (LI-7000, Li-Cor Biosciences, Lincoln, NE, USA). Data acquisition and analyses were carried out using ExpeData software (Sable Systems International, Las Vegas, NV, USA). Readings of CO_2 and H_2O were taken every 0.1 s. Cycle phases were classified as burst (O) and interburst (C+F) phases because the F phase could not always be objectively distinguished from the C phase using the CO_2 emission trace.

Respirometry traces were inspected by eye, and only recordings that showed apparent negligible CO_2 emission rates during the interburst period were classified as DGC, and used for analyses of cycle characteristics. The data for the last three cycles of each CO_2 and water vapour trace were extracted and analysed. Water vapour emission during the interburst period was assumed to represent CWL only, and the RWL was calculated by subtracting CWL from the total evaporative water loss (EWL) during a cycle.

Transpiration ratios were calculated by regressing H_2O emission rates on CO_2 emission rates for each animal (Gibbs and Johnson, 2004). Mean r^2 values for the three species ranged from 0.80 to 0.86 and 0.70 to 0.74 for DGC and continuous exchange, respectively (sample size was 8–14 in each of the species×exchange pattern combinations). Regression line intercepts allowed calculation of CWL of continuous breathers (Gibbs and Johnson, 2004). DGC traces were used for comparing CWL estimations based on the regression method and the traditional method described above, with mean difference <2% in all three species.

Because of a gas analyser technical failure, water vapour emission values from the first year of the study were discarded, resulting in different sample sizes for DGC and EWL values in Tables 1 and 2.

Tracheal volume and body water content

The tracheal volume was estimated using a water displacement method (Bartholomew and Barnhart, 1984). Grasshoppers were CO_2-anaesthetized, weighed and then placed into a plastic syringe (60 ml) filled with soapy water. Air was drawn out of the tracheal system and replaced with fluid by moving the plunger back and forth. The grasshopper was then blotted dry

and weighed again. Tracheal volume was estimated as the increase in body mass, assuming a water density of 1 g cm^{-3}. Mass-specific tracheal volume was calculated as tracheal volume divided by dry mass. The animals' dry mass was measured following drying in a 60°C oven for 48 h. The body water content was calculated as follows: body water content (%) =100%×(wet body mass−dry body mass)/(wet body mass).

Statistics

Shapiro–Wilk's test and Levene's test were used to examine the normality of the data distribution and to assess homoscedasticity. Percentage data were arcsine transformed before statistical analyses. We used one-way ANOVA to compare body mass and species effect on DGC characteristics, tracheal volume and body water content, using Tukey's HSD procedure for *post hoc* comparisons. When data did not meet the homogeneity assumption after data transformation, Welch's ANOVA was used instead, with the Games–Howell procedure used for *post hoc* comparisons. Fisher's exact test was used to test the species difference in DGC prevalence. Interspecific variation in RWL (as % total EWL) and transpiration ratio was tested using the non-parametric Kruskal–Wallis test because of the small sample size. The significance level (α) was set at 0.05. In non-parametric tests, α was adjusted to 0.017 (=0.05/3) using Bonferroni correction to account for multiple comparisons.

Limited overlap in body size among the study species prevented comparisons of mass-corrected RWL rates, and their respiratory and cuticular components. However, we combined data from the literature with unpublished data from our laboratory to construct allometric relationships of metabolic rate (supplementary material Fig. S1) and tracheal volume (Fig. 4) with body mass in grasshoppers. We then carried out Kruskal–Wallis tests (with Bonferroni corrections) on residuals in order to compare our study species.

Values throughout the text are presented as means±s.d., unless otherwise mentioned. We used JMP (SAS Institute) and SPSS (SPSS Inc., Chicago, IL, USA) for statistical analysis.

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