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An experimental evolution study confirms that discontinuous gas exchange does not contribute to body water conservation in locusts

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The adaptive nature of discontinuous gas exchange (DGE) in insects is contentious. The classic 'hygric hypothesis', which posits that DGE serves to reduce respiratory water loss (RWL), is still the best supported. We thus focused on the hygric hypothesis in this first-ever experimental evolution study of any of the competing adaptive hypotheses. We compared populations of the migratory locust (*Locusta migratoria*) that underwent 10 consecutive generations of selection for desiccation resistance with control populations. Selected locusts survived 36% longer under desiccation stress but DGE prevalence did not differ between these and control populations (approx. 75%). Evolved changes in DGE properties in the selected locusts included longer cycle and interburst durations. However, in contrast with predictions of the hygric hypothesis, these changes were not associated with reduced RWL rates. Other responses observed in the selected locusts were higher body water content when hydrated and lower total evaporative water loss rates. Hence, our data suggest that DGE cycle properties in selected locusts are a consequence of an evolved increased ability to store water, and thus an improved capacity to buffer accumulated CO_2, rather than an adaptive response to desiccation. We conclude that DGE is unlikely to be an evolutionary response to dehydration challenge in locusts.

1. Introduction

Insects exchange respiratory gases through the tracheal system, which opens to the environment via segmental spiracles. Most insects can control spiracle closure, leading to a variety of gas exchange patterns, categorized as continuous, cyclic and discontinuous gas exchange (DGE) [1]. Even though, and perhaps because, it has been described only in five insect orders thus far [2], DGE has attracted substantial research focus for more than half a century. DGE cycles comprise three phases, typically characterized by spiracle state as assumed by monitoring CO_2 emission: the closed phase (C), in which all spiracles are closed; followed by the flutter phase (F), in which the spiracles open and close rapidly; and the subsequent open phase (O), during which the spiracles are opened [1,3].

Schneiderman [4] suggested that DGE serves for restricting respiratory water loss (RWL), based on the prolonged C-phase and largely inward convective gas transport during the F-phase (resulting from a drop in endotracheal air pressure during the C-phase). However, the general applicability of this hypothesis, later termed the hygric hypothesis [5], has since been questioned on both experimental and theoretical grounds (e.g. [6–8]). The controversy over the adaptive value of DGE has led to several alternative hypotheses (reviewed in [1]). Nevertheless, the classic hygric hypothesis is still well

supported by both single species studies (e.g. [9]) and large-scale comparative studies [10].

It has been shown that gas exchange patterns may change under desiccation selection [11]. However, to the best of our knowledge, this is the first study using an experimental evolution approach to directly test the adaptive significance of DGE. We selected migratory locusts (*Locusta migratoria*) for desiccation resistance for 10 consecutive generations, and compared their performance with that of control populations originating from the same laboratory stock. Measurements included desiccation tolerance, body water content, water loss rates and their components, and DGE prevalence and its characteristics. We predicted that desiccation-selected locusts would demonstrate a higher DGE prevalence as well as longer DGE cycles, owing to longer spiracle closure. Moreover, we expected desiccation-selected locusts to show lower RWL rates compared with controls, as predicted by the hygric hypothesis.

2. Methods

(a) Selection protocol
Four parallel populations ($N \approx 500$ each) of *L. migratoria* were established from the University of Haifa-Oranim stock population. Locusts were fed daily with fresh wheat shoots and dry oats ad libitum and were kept at $33.0 \pm 3.0^\circ$C, under a 14 L : 10 D photoperiod. In two of the populations, those undergoing desiccation selection, locusts were sexed at one-week post adult-eclosion and placed in separate cages (as females are more desiccation resistant), with access to dry oats only, until approximately 60–80% mortality was reached. During the selection process, the cages were flushed with dry air passed through silica gel columns in order to maintain approximately 15% relative humidity (RH). The two control populations were provided with fresh food and exposed to ambient humidity (approx. 50% RH) throughout. After selection, locusts were reintroduced into combined-sex cages and provided with fresh food and sand cups for egg laying. Eggs were incubated at 37°C, and hatchlings forming the next generation were placed in empty cages. Selection was repeated for 10 successive generations after which a sub-sample of locusts was reared for one generation off-selection to rule out parental effects on experimental results. Only males, one to two weeks post adult-eclosion, were used for all experiments.

(b) Desiccation resistance
Selected locusts were weighed (initial wet mass) (CPA224S, Sartorius, Göttingen, Germany) and placed in a custom-built metal-mesh cage divided into individual enclosures, allowing contact between neighbouring insects. The locusts were provided with dry oats only and kept at 30°C, RH ≈ 50%, under a 14 L : 10 D photoperiod. Each individual was checked twice a day and its time of death noted. It was then weighed (final wet mass) and dried at 60°C for 48 h for dry mass determination. Body water content was calculated by subtracting the locust's dry mass from its initial wet mass. In addition, water content at death was calculated by subtracting the locust's dry mass from its final wet mass.

(c) Respirometry
Flow-through respirometry was performed at 25°C using a dual water vapour and CO_2 analyser (LI-7000, LiCor, Lincoln, NE, USA). Following 12–24 h food deprivation, locusts were acclimated for 1 h in a 23 ml glass metabolic chamber. Atmospheric air, scrubbed of CO_2 and water vapour by passing it through silica gel/Ascarite® columns, was supplied at a flow rate of 200 ml min^{-1} using a mass flow controller (MC-500SCCM-D;

Alicat Scientific, Tuscon, AZ, USA). Gas exchange was monitored and analysed over the subsequent 1–2 h, using a UI-2 data acquisition interface and EXPEDATA software (Sable Systems International, Las Vegas, NV, USA). Partitioning of evaporative water loss (EWL) into its cuticular and respiratory components was based on the assumption that during the interburst (combined closed and flutter phases; figure 1a) water vapour is lost exclusively through the integument.

(d) Statistics
Statistical analysis was performed using SPSS 19.0 (IBM). Because there was no significant difference between replicate populations within-treatment in any measured variable ($p > 0.5$), replicate population data were pooled for analyses of selection treatment effects. Treatment-dependent effects were tested by analysis of covariance (ANCOVA; dry body mass as a covariate). Desiccation survival, body mass and DGE cycle durations were tested by *t*-test. DGE prevalence was compared using χ^2-test. When sphericity could not be assumed, we used the Huynh–Feldt corrected *F*-value and d.f. values.

3. Results

Desiccation-selected locusts survived approximately 36% longer than the control, when provided with dry food only ($t_{95.026} = 4.943$, $p < 0.001$). As can be seen in table 1, body mass of the selected locusts was higher compared with control ($t_{114} = 2.958$, $p = 0.004$), while there was no difference in body dry mass ($t_{114} = 1.094$, $p = 0.276$), indicating an evolved increase in body water content in response to selection ($F_{1,114} = 13.656$, $p < 0.001$). By contrast, dehydration tolerance, as defined by body water content when succumbing to dehydration, did not change in response to selection ($F_{1,114} = 0.870$, $p = 0.353$) (table 1).

Respirometry measurements revealed no significant difference in DGE prevalence ($\chi^2_1 = 0.124$; $p = 0.997$) between treatments. There was no significant treatment effect on metabolic rates (expressed as CO_2 emission rates) ($F_{1,68} = 0.985$, $p = 0.324$). By contrast, EWL rates were lower in desiccation-selected locusts ($F_{1,68} = 4.194$, $p = 0.044$). There was no significant treatment effect on RWL ($F_{1,68} = 0.167$, $p = 0.684$), indicating an evolved increase in cuticular resistance to water loss ($F_{1,68} = 4.189$, $p = 0.045$) (table 2).

Despite the similar DGE prevalence among treatments, their DGE characteristics differed significantly (figure 1b). Selected locusts exhibited 40% longer cycle durations than the control ($t_{68} = 3.642$, $p = 0.001$). A breakdown of the cycle into its burst and interburst phases (figure 1a) revealed that differences in cycle duration resulted from changes in the interburst ($t_{68} = 3.790$, $p < 0.001$) but not the burst duration ($t_{68} = 1.119$, $p = 0.267$). Calculation of CO_2 accumulation during the interburst (see [12]), assuming constant cellular metabolic rate despite intermittent gas exchange with the environment, revealed that the selected locusts had evolved a significantly higher capacity to accumulate CO_2 ($F_{1,68} = 9.763$, $p = 0.003$) (table 2).

4. Discussion

We report the first-ever experimental evolution approach, to our knowledge, explicitly taken to test any of the adaptive hypotheses posited for the evolution and maintenance of DGE in insects. We chose to test here predictions of the
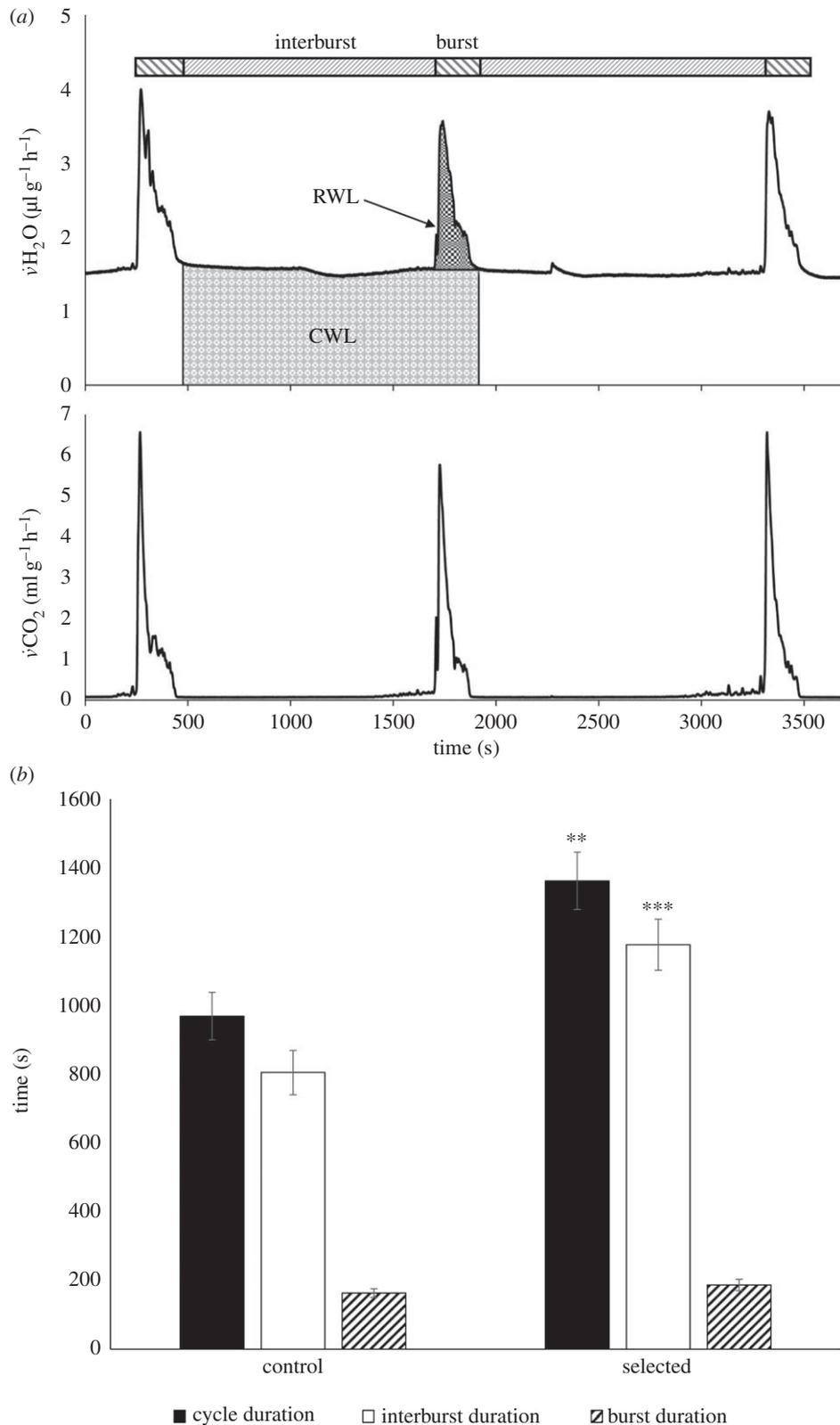
Figure 1. DGE cycle characteristics. (*a*) Typical CO_2 and water emission traces of *L. migratoria* (selected male; body mass = 1.341 g). Top horizontal bar divides the cycle into its interburst and burst components. Filled areas represent cuticular (CWL) and respiratory (RWL) water losses during one cycle. (*b*) Durations (mean $\pm$ s.e.) of DGE cycle and its components in control and selected populations. Asterisks indicate significant differences from control values: ** $p < 0.01$; *** $p < 0.001$.

hygric hypothesis. To do so, we selected replicate populations of *L. migratoria* for desiccation resistance and compared their gas exchange patterns and water relations traits with those of control populations. A significant response to 10 generations of selection was reflected in increased desiccation resistance, indicating that the ancestral populations contained substantial genetic variation upon which selection could act. The

evolved mechanisms for improved resistance included the ability to store more body water when hydrated and reduced EWL rates. However, there was no change in the locusts' desiccation tolerance: namely, the ability to withstand low body water content when dehydrated. This is in agreement with previous findings from *Drosophila* (reviewed in [13]), suggesting a general strategy in insect stress response.

Table 1. Selection effects on desiccation resistance and body mass components. Asterisks indicate significant differences from control values: **$p < 0.01$; ***$p < 0.001$.

	mean $\pm$ s.e.	
properties	control ($N = 64$)	selected ($N = 52$)
desiccation survival (days)[a]	6.1 ± 0.3	8.3 ± 0.4***
body wet mass (g)	1.274 ± 0.015	1.340 ± 0.017**
body dry mass (g)	0.433 ± 0.008	0.445 ± 0.008
body water content (g)	0.841 ± 0.010	0.895 ± 0.011***
water content at death (g)	0.526 ± 0.009	0.520 ± 0.010

[a]At 30°C and RH $\approx$ 50%.

Table 2. DGE and gas exchange properties. Asterisks indicate significant differences from control values: *$p < 0.05$; **$p < 0.01$.

	mean $\pm$ s.e.	
properties	control ($N = 35$)	selected ($N = 35$)
DGE prevalence (%)	74 (35 of 47)	78 (35 of 45)
CO_2 emission rate (μl g^{-1} h^{-1})	979.8 ± 27.7	921.7 ± 19.6
evaporative water loss rate (μl g^{-1} h^{-1})	2.16 ± 0.06	1.94 ± 0.07*
cuticular water loss rate (μl g^{-1} h^{-1})	1.99 ± 0.06	1.77 ± 0.06*
RWL rate (μl g^{-1} h^{-1})	0.19 ± 0.01	0.18 ± 0.01
CO_2 accumulation during interburst (μl)	63.7 ± 5.0	85.6 ± 4.4**

One expected outcome of the selection procedure according to the hygric hypothesis was that of an increased use of DGE. However, an almost identical DGE prevalence in the selected and control populations lends support to the claim that evolution is unlikely to modulate RWL, which constitutes a relatively small fraction of the total EWL in insects [7]. In this study, we calculated RWL to be 9% of the total water loss, which is within the typical range of less than 15% previously reported for insects [8].

While it has been shown that DGE may not constitute a water conservation mechanism *per se*, modulation of its properties could result in water savings [14]. As expected, selection for desiccation resistance resulted in longer interbursts, during which gas exchange through the spiracles is minimal, whereas burst durations remained unchanged. Nevertheless, these evolved changes in DGE characteristics did not result in water saving. The observed significant changes in EWL rates could be attributed to increased cuticular resistance to water loss in the selected locusts, probably as a result of changes in the cuticular hydrocarbon quantity and/or composition. In fact, the ×1.12 higher cuticular resistance (table 2) and the ×1.19 higher water volume lost prior to death (table 1) largely explain the ×1.36 longer survival of the selected locusts (table 1).

The longer interbursts observed during DGE in the selected locusts, coupled with CO_2 emission rates similar to those of the controls, indicate that the former possess the ability to store more CO_2 in their tissues and haemolymph when the spiracles are closed and gas exchange with the environment does not occur. Rather than constituting an adaptive mechanism, allowing increased tolerance to harsh environmental conditions through tighter spiracle closure, this ability appears to be a by-product of the observed higher water content in the selected

locusts. During the interburst, CO_2 is largely dissolved in the body fluids, or hydrated to bicarbonate, and thus its storage capacity depends on body water content. Furthermore, we found a significantly higher haemolymph protein concentration in the selected locusts (S.T., 2016, unpublished data), and this important haemolymph buffer in locusts [15] may aid in alleviating acid–base balance alterations resulting from CO_2 accumulation. Interestingly, locust density-dependent phase-specific variations in haemolymph protein content were also associated with changes in DGE properties in the desert locust, *Schistocerca gregaria*, during desiccation [12].

This study adds to a growing body of evidence suggesting that DGE is unlikely to have evolved in orthopterans to restrict water loss to the environment.

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