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Intra-individual variation allows an explicit test of the hygric hypothesis for discontinuous gas exchange in insects

Caroline M. Williams[1], **Shannon L. Pelini**[2,†], **Jessica J. Hellmann**[2] **and Brent J. Sinclair**[1,*]

[1]*Department of Biology, University of Western Ontario, London, ON N6A 5B7, Canada*
[2]*Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556, USA*
**Author for correspondence (bsincla7@uwo.ca).*
†*Present address: Harvard Forest, Harvard University, Petersham, MA 01366, USA*

The hygric hypothesis postulates that insect discontinuous gas exchange cycles (DGCs) are an adaptation that reduces respiratory water loss (RWL), but evidence is lacking for reduction of water loss by insects expressing DGCs under normal ecological conditions. Larvae of *Erynnis propertius* (Lepidoptera: Hesperiidae) naturally switch between DGCs and continuous gas exchange (CGE), allowing flow-through respirometry comparisons of water loss between the two modes. Water loss was lower during DGCs than CGE, both between individuals using different patterns and within individuals using both patterns. The hygric cost of gas exchange (water loss associated with carbon dioxide release) and the contribution of respiratory to total water loss were lower during DGCs. Metabolic rate did not differ between DGCs and CGE. Thus, DGCs reduce RWL in *E. propertius*, which is consistent with the suggestion that water loss reduction could account for the evolutionary origin and/or maintenance of DGCs in insects.

Keywords: discontinuous gas exchange; respiratory water loss; Lepidoptera

1. INTRODUCTION

Discontinuous gas exchange cycles (DGCs) have evolved independently at least five times in insects (Marais *et al.* 2005). The evolutionary pressures that lead to DGCs are debated (Chown *et al.* 2006). DGCs consist of three phases: closed phase during which spiracles are closed and there is no external gas exchange; flutter phase where spiracles rapidly open and close, allowing bulk inflow of air, and open phase where spiracles are open to allow unrestricted gas exchange (Chown *et al.* 2006).

Three main adaptive hypotheses have been proposed to explain the origin and maintenance of DGCs (Chown *et al.* 2006). The hygric hypothesis contends that DGCs have evolved to limit respiratory water loss (RWL) by maximizing the time that the spiracles are closed, and minimizing water efflux due to bulk inward convection in the F-phase (Chown *et al.* 2006). The chthonic–hygric hypothesis (Lighton & Berrigan 1995) states that DGCs originated in insects inhabiting hypoxic and hypercapnic (primarily underground) environments to increase O_2 and CO_2 diffusion gradients, with coincidental water savings. The oxidative damage hypothesis (Hetz & Bradley 2005) suggests that DGCs minimize oxidative damage during periods of low metabolic demand, by maintaining low tracheal PO_2 while retaining delivery capacity during periods of high metabolic demand (e.g. flight).

Here, we focus on the water retention benefits of DGCs, primarily addressing the hygric hypothesis. We note the difficulty in distinguishing the hygric and chthonic–hygric hypotheses based on water loss, but the hygric hypothesis may be rejected independently of the chthonic and oxidative damage hypotheses since CO_2 and O_2 partial pressures are central to the latter (Chown *et al.* 2006). The hygric hypothesis predicts that (i) water lost per CO_2 released will be lower for insects using DGCs (see also Lighton & Turner 2008) and (ii) DGCs will decrease RWL.

Measurement of water loss within DGCs shows that RWL is greater when the spiracles are open (see Chown 2002). DGCs are longer in species from xeric environments (White *et al.* 2007), while cyclic and continuous patterns are more prevalent in mesic habitats (Marais *et al.* 2005). RWL was lower in individual ants that did not express DGCs; however, those individuals also had lower metabolic rates (Gibbs & Johnson 2004). Manipulation of environmental variables can force insects to abandon DGCs (e.g. Lighton & Turner 2008; Terblanche *et al.* 2008), but to our knowledge there have been no comparisons of RWL in individuals that use both DGCs and continuous gas exchange (CGE) under ecologically relevant conditions.

Erynnis propertius (Lepidoptera: Hesperiidae) overwinter as quiescent late-instar larvae in rolls of dry oak leaves (Prior *et al.* 2009). Quiescent larvae probably experience desiccation during the overwintering period as no feeding occurs. Under benign conditions, individuals use both DGCs and CGE, allowing a direct comparison of water loss rates both between and within individuals during DGCs and CGE. From the hygric hypothesis, we expect the ratio of water loss to CO_2 emission to be lower during DGCs than during CGE, and that the contribution of RWL to total water loss will be lower during DGCs. Support for these predictions under benign environmental conditions is a prerequisite for comprehending the role of water loss in the evolution of DGCs.

2. MATERIAL AND METHODS

Erynnis propertius larvae were reared from adults caught in spring 2007 from Oregon, USA, and British Columbia, Canada. Larvae were fed fresh Garry oak (*Quercus garryana*) leaves until the sixth instar when they became quiescent (Pelini *et al.* 2009). Then, larvae were housed in Sanyo MIR-153 incubators (Sanyo Scientific, Bensenville, IL) in 25 ml plastic containers on moist vermiculite without food, at 8 : 1°C (day : night) and a 13D : 11L photoperiod. Total water content was determined gravimetrically for nine individuals that were not used in respirometry.

Volume of water and CO_2 released per unit time ($\dot{V}H_2O$, $\dot{V}CO_2$) by *E. propertius* larvae ($n = 39$) were measured for 4 h at 8°C after 3 h

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acclimation using flow-through respirometry (Lighton 2008; details in electronic supplementary material). Each larva was measured once, at a randomly assigned time between 8.00 and 20.00. All comparisons were made between complete DGCs and 68 min blocks of CGE, the latter chosen to match the mean cycle time of the DGCs (see electronic supplementary material for details of data selection).

Mean ± s.e.m. is reported throughout. All statistical analyses were performed in R (R Project v. 2.8.1; www.r-project.org/). Where ratios or percentages are presented, statistical analyses were performed on raw data using analysis of covariance (ANCOVA). $\dot{V}CO_2$ and $\dot{V}H_2O$ ($\mu l\,h^{-1}$) were compared between modes using repeated measures ANCOVA (individuals using mixed patterns) or ANCOVA (effect of mode between individuals) with the covariates mass and time. $\dot{V}H_2O$ ($\mu l\,h^{-1}$) between individuals was also compared using an ANCOVA with the covariates mass and time, and $\dot{V}CO_2$ to determine whether the molar ratios were significantly different between groups. $\dot{V}CO_2$ and $\dot{V}H_2O$ were $\log_{10}$-transformed prior to this analysis. To determine the hygric cost of gas exchange (Gibbs & Johnson 2004), $\dot{V}H_2O$ was regressed against $\dot{V}CO_2$ and the resulting slope used to estimate the incremental increase in water loss associated with CO_2 release (electronic supplementary material, figure S1). $\dot{V}H_2O/\dot{V}CO_2$ slopes were compared between continuous and discontinuous gas exchange with a *t*-test (between individuals) or paired *t*-test (within individuals). Cuticular water loss for all individuals and modes was estimated as the intercept of the $\dot{V}H_2O/\dot{V}CO_2$ regression (Gibbs & Johnson 2004) and compared between CGE and DGCs using an ANCOVA with total water loss as a covariate. RWL was calculated by subtracting cuticular from total water loss and compared between CGE and DGCs using an ANCOVA with cuticular water loss as a covariate.

3. RESULTS

No movement was detected in any larvae during respirometry. Fifteen individuals used solely CGE, 18 individuals used solely DGCs and six individuals switched between patterns during the course of one measurement period (figure 1). In those that switched between patterns, four of six switched from CGE to DGCs, with one switching from DGCs to CGE, and a sixth switching from CGE to DGCs and back again. Water loss declined during a respirometry run for both modes of gas exchange ($F_{2,12} = 22.72$, $p < 0.001$), while $\dot{V}CO_2$ did not ($F_{2,12} = 1.17$, $p = 0.34$). Total water content of $n = 9$ caterpillars was 3.08 ± 0.4 g H_2O g(dry mass)$^{-1}$. $\dot{V}CO_2$ did not differ between gas exchange patterns either within or between individuals (within: $F_{1,3} = 2.64$, $p = 0.20$; between: $F_{1,29} = 2.61$, $p = 0.12$; table 1). Time and mass were not statistically significant covariates of $\dot{V}CO_2$ in either analysis ($p > 0.1$).

Water loss was significantly lower during DGCs than during CGE both within and between individuals (within: $F_{1,3} = 34.75, p = 0.010$; between: $F_{1,28} = 5.59$, $p = 0.025$; figure 2, table 1). Time was not a statistically significant covariate ($p > 0.1$) for $\dot{V}H_2O$ either between or within individuals, nor was mass within individuals ($F_{1,3} = 2.64$, $p = 0.20$). However, mass was positively correlated with $\dot{V}H_2O$ between individuals ($F_{1,28} = 15.06$, $p < 0.001$). The ratio of $\dot{V}H_2O$ to $\dot{V}CO_2$ was higher during CGE between individuals ($F_{1,29} = 1.84$, $p = 0.02$; table 1). The slopes of the regression of $\dot{V}H_2O$ on $\dot{V}CO_2$ were higher during CGE than during DGCs between but not within individuals, although the trend was in the same direction (between: $t_{14} = 2.59$, $p = 0.020$; within: $t_5 = 1.11$, $p < 0.1$; table 1). RWL accounted for significantly more of the total water loss during CGE both between and within individuals (between: $F_{1,29} = 5.41$, $p = 0.027$; within: $F_{1,3} = 22.77$, $p = 0.017$; table 1). Cuticular water loss did not differ between DGCs and CGE either between ($F_{1,29} = 1.68$, $p = 0.206$) or within ($F_{1,3} = 1.69$, $p = 0.284$) individuals.

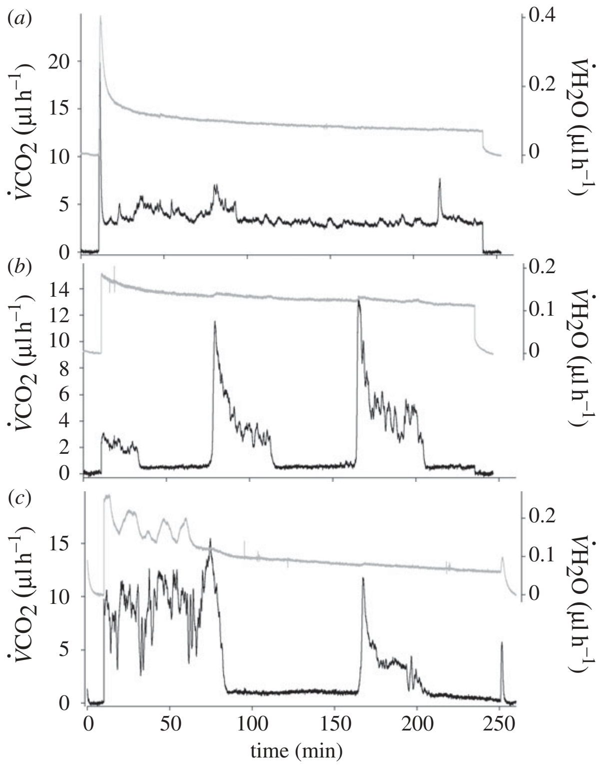

Figure 1. Example of CO_2 (grey lines) and H_2O (black lines) emission traces from larvae of *E. propertius*: (*a*) solely CGE; (*b*) solely DGCs and (*c*) a mixture of patterns.

4. DISCUSSION

To our knowledge, this is the first time the hygric hypothesis of DGCs has been tested in a species where individuals exhibit both modes of gas exchange with comparable metabolic rates and without differential water balance status (e.g. Hadley & Quinlan 1993). Water loss in *E. propertius* is higher during CGE, both within individuals that use both patterns and between individuals exhibiting one or other mode. Thus, in this species, a DGC appears to confer a significant water conservation benefit. This contrasts with experiments where the mode of gas exchange or metabolic rate is manipulated (e.g. Lighton & Turner 2008; Terblanche *et al.* 2008; Contreras & Bradley 2009; Schimpf *et al.* 2009) and suggests that water conservation is an advantage that could lead to the origin or maintenance of DGCs in insects.

Grasshoppers abandoned DGCs when stressed by desiccation (Hadley & Quinlan 1993); in contrast, only two of six individuals that switched went from DGCs to CGE as they lost water in our study. Only six of the 39 caterpillars we observed switched gas exchange modes. We hypothesize that this results from the short (4 h) observation period, and that longer recordings would reveal a greater incidence of switching.

Between individuals, the slope of a regression of $\dot{V}H_2O$ on $\dot{V}CO_2$ is higher during CGE, which indicates a reduced hygric cost of gas exchange during

Table 1. Gas exchange and water loss parameters in *E. propertius* larvae using both or either mode of gas exchange. Mean ± s.e.m. presented.

			mixture of patterns	
	CGE	DGCs	CGE	DGCs
N	15	18	6	
fresh mass (mg)	252.9 ± 19.2	230.5 ± 10.1	273.64 ± 22.3	
$\dot{V}CO_2$ ($\mu l\,h^{-1}$)	6.84 ± 1.48	3.67 ± 0.36	6.40 ± 1.24	4.81 ± 1.42
$\dot{V}H_2O$ ($\mu l\,h^{-1}$)	0.216 ± 0.048	0.121 ± 0.022^a	0.184 ± 0.049	0.116 ± 0.026^b
$\dot{V}H_2O$ (moles) : $\dot{V}CO_2$ (moles)	0.105 ± 0.071	0.037 ± 0.007^a	0.030 ± 0.005	0.028 ± 0.005
RWL ($\mu l\,h^{-1}$)	0.0209 ± 0.0076	0.0048 ± 0.0013^a	0.0233 ± 0.0101	0.0134 ± 0.0070^b
cuticular water loss ($\mu l\,h^{-1}$)	0.1951 ± 0.0492	0.1170 ± 0.0217	0.1698 ± 0.0471	0.1030 ± 0.0224
RWL as percentage total water loss (%)	12.8 ± 4.9	4.4 ± 2.0^a	13.2 ± 1.4	9.9 ± 3.9^b
hygric cost of gas exchange (slope of $\dot{V}H_2O$ on $\dot{V}CO_2$)	$2.015 \times 10^{-3} \pm 6.14 \times 10^{-4}$	$4.140 \times 10^{-4} \pm 6.50 \times 10^{-5a}$	$2.416 \times 10^{-3} \pm 1.50 \times 10^{-3}$	$8.678 \times 10^{-4} \pm 2.93 \times 10^{-4}$

[a] Significant difference ($p < 0.05$) between individuals using either DGCs or CGE.
[b] Significant difference ($p < 0.05$) between modes of gas exchange within individuals that use both modes.

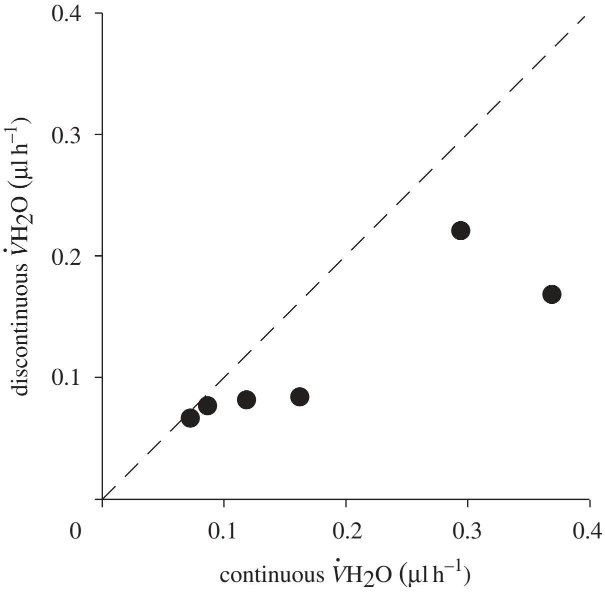

Figure 2. Water loss during continuous compared with discontinuous gas exchange in *E. propertius* individuals that used both modes. The line indicates equal water loss in both modes.

DGCs in *E. propertius*. In contrast, the $\dot{V}H_2O/\dot{V}CO_2$ slope did not differ among queens of *Pogonomyrmex barbatus* under different modes of gas exchange, but a DGC was abandoned at higher metabolic rates (Gibbs & Johnson 2004). We found no difference in $\dot{V}CO_2$ between *E. propertius* individuals using DGCs and CGE. This challenges the oxidative damage hypothesis, which predicts that metabolic rate should determine the mode of gas exchange used by an individual through its influence on tracheal PO_2 (Contreras & Bradley 2009).

Clearly, DGCs decrease RWL compared with CGE, while cuticular water loss does not differ. The relative contribution of RWL during DGCs (4.4%) in *E. propertius* is at the low end for tracheate arthropods, but RWL during CGE (12.8%) is consistent with other species (Chown 2002; Lighton *et al.* 2004). $\dot{V}H_2O$ declined throughout the experiment for both patterns of gas exchange which may indicate a steady decline in cuticular water loss, but is more likely due to water adhering to the inside of the plastic tubing. Baseline correction accounts for this effect (Lighton 2008). The fitness benefit of modulating RWL has been questioned (Chown 2002). However, caterpillars would take 19 days at 0% RH to reach an injurious 30 per cent reduction in water content if using DGCs, compared with only 10.6 days using CGE (see electronic supplementary material). Furthermore, insects adapted to xeric environments typically have reduced cuticular water loss (Chown 2002), increasing the benefit of reduced RWL. However, whether the RWL reduction during DGCs confers a fitness advantage requires further investigation.

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