

The hyperoxic switch: assessing respiratory water loss rates in tracheate arthropods with continuous gas exchange

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Summary

Partitioning the relative contributions of cuticular and respiratory water loss in a tracheate arthropod is relatively easy if it undergoes discontinuous gas exchange cycles or DGCs, leaving its rate of cuticular water loss in primary evidence while its spiracles are closed. Many arthropods are not so obliging and emit CO₂ continuously, making cuticular and respiratory water losses difficult or impossible to partition. We report here that by switching ambient air from 21 to 100% O₂, marked spiracular constriction takes place, causing a transient but substantial – up to 90% – reduction in CO₂ output. A reduction in water loss rate occurs at the same time. Using this approach, we investigated respiratory water loss in *Drosophila melanogaster* and in two ant species, *Forelius*

mccooki and *Pogonomyrmex californicus*. Our results – respiratory water loss estimates of 23%, 7.6% and 5.6% of total water loss rates, respectively – are reasonable in light of literature estimates, and suggest that the ‘hyperoxic switch’ may allow straightforward estimation of respiratory water loss rates in arthropods lacking discontinuous gas exchange. In *P. californicus*, which we were able to measure with and without a DGC, presence or absence of a DGC did not affect respiratory vs total water loss rates.

Key words: *Drosophila melanogaster*, gas exchange, water loss, cuticular permeability, *Pogonomyrmex californicus*, *Forelius mccooki*.

Introduction

Insects are small but their ratio of surface area to volume is large. Water, contained in body volume, diffuses through the cuticle and the spiracles to the variably dry outside world. Unless this process is counterbalanced it will lead to death in a matter of hours or days. Insects are by far the most numerically successful and speciose animals on our planet, so the mechanisms by which they maximize their fitness in the face of this challenge are not without interest. The reader is referred to the excellent reviews of Edney (1977), Hadley (1994) and Chown (2002) for more details. Of the controversies that rage or smoulder in this scientific field, one that periodically ignites (but often yields more smoke than light) concerns the relative contributions of cuticular and respiratory water losses to overall water loss rates.

Water loss rate (WLR), and thus other parameters such as cuticular permeability that depend on the accurate measurement of WLR, are usually measured gravimetrically or via radioactive isotopes. This makes distinguishing respiratory from cuticular water loss rates difficult or impossible. It is possible to calculate – although with a wide margin of error – how much overall gas exchange is required to sustain a given catabolic flux rate, and then calculate the amount of water lost through this avenue by making assumptions about exchange

volumes (e.g. Lighton and Feener, 1989). Some insects make the process easier by limiting most of their respiratory water loss to discrete ‘bursts’ that can be separated from intervening periods of cuticular water loss by sensitive real-time gravimetric techniques (Machin et al., 1991; Lighton, 1992 and references therein) or by water vapor analysis (Hadley and Quinlan, 1982; Lighton et al., 1993; Gibbs et al., 2003; Gibbs and Matzkin, 2001), but such insects may be the exception rather than the rule (review by Lighton, 1996; but see Chown, 2002). And in the case of very small insects, this approach, even where possible in theory, is impractical because the resolution of current instrumentation is finite. This means, for example, that the relation between respiratory and cuticular water loss rate in such a widely studied organism as *Drosophila melanogaster* is currently uncertain because its gas exchange is generally continuous and its water vapor signal is barely detectable (Gibbs et al., 2003).

In a recent paper, Lehmann (2001) examined respiratory water loss rates in flying *Drosophila* by using water vapor as a tracer gas for tracheal ventilation, as was earlier shown in the far larger honeybee by Joos et al. (1997). Lehmann demonstrated for the first time that the degree of spiracular opening during flight is finely modulated to provide just-

sufficient oxygen delivery and carbon dioxide release, without imposing a needless respiratory water loss penalty. This approach worked well because of the huge respiratory gas exchange rates during flight, coupled with the ability to modulate flight intensity at will using a virtual-reality scenario (see also Dickinson and Lighton, 1995). However this approach is not practical in resting *Drosophila*, which exchange respiratory gases not only at a far lower rate than in flight, but also at a constant rate, although some partial exceptions may occur (Gibbs et al., 2003; Williams and Bradley, 1998).

In this paper we propose a novel approach to estimating minimal rates of respiratory water loss (RWL) even in small insects that continuously exchange respiratory gases. Our approach exploits the fact that the degree of spiracular opening in insects at rest is modulated by the partial pressures of both oxygen and carbon dioxide (Wigglesworth, 1935; Levy and Schneiderman, 1966a,b; and also see reviews by Kestler, 1985; Lighton, 1996). In steady state, a low and relatively constant intra-tracheal partial pressure of oxygen is maintained to facilitate inward diffusion of oxygen from the atmosphere. If the intra-tracheal P_{O_2} falls, the degree of spiracular opening rises; if intra-tracheal P_{O_2} rises, the degree of spiracular opening falls. Our approach replaces the air surrounding the insect under study with pure oxygen, briefly elevating inward oxygen diffusion rates by fivefold. The result was a transient period of increased intra-tracheal P_{O_2} and thus reduced the degree of spiracular opening accompanied by a depression in CO_2 and respiratory water vapor output. By using CO_2 as the tracer gas, and by using a sensitive flow-through water vapor analyzer to detect any accompanying change in water vapor flux, we can then estimate rates of respiratory water loss. As an added benefit the water vapor analysis (in conjunction with accurately known flow rates) allows real-time water loss rates to be measured with an accuracy approaching that of gravimetric methods, especially in small insects, without the disruption introduced by periodic weighing.

Measurement of RWL during steady-state CO_2 output is of particular interest in species that are also capable of engaging in a discontinuous gas exchange cycle or DGC. The selective advantage of the stringent spiracular control required for the DGC is usually claimed to be a reduction in RWL relative to continuous, usually primarily diffusion-based, gas exchange, but this argument has recently been challenged (Lighton and Berrigan, 1995; Lighton, 1998 and references therein). Direct comparisons of RWL between members of the same species in the presence or absence of the DGC have not, to our knowledge, been made to date and offer a means to evaluate the classic 'hygric' or RWL-reduction hypothesis (Lighton, 1998) for the evolution of the DGC in insects and certain other tracheate arthropods.

Respiratory water loss at rest is an important parameter. Perhaps less important, but still interesting, is the maximal rate of respiratory water loss. This is a function of total tracheal system area and maximal degree of spiracular opening, and it is reasonable to assume that it will scale with maximal aerobic

capacity. By replacing the oxygen, in turn, with nitrogen, the maximal rate of respiratory water loss through diffusion can also be measured. (It is possible that during activities, such as locomotion, that the maximal rate of respiratory water loss may be enhanced by active mechanisms, such as convection.) Measurement of this parameter is facilitated because the response to anoxia of an insect continuously exchanging respiratory gases is a rapid and maximal increase in spiracular opening (see Lighton and Fielden, 1996; Klok et al., 2002). The resulting efflux of water vapor can then be directly measured and compared to resting respiratory and cuticular water loss rates.

Materials and methods

Animals

To investigate the feasibility of the 'hyperoxic switch' for measuring respiratory water loss rates in insects without a marked discontinuous gas exchange cycle or DGC, we employed three diverse insects: *Drosophila melanogaster* Meigen (mass ~1 mg), the tiny ant *Forelius mccooci* McCook (mass ~0.25 mg), and the harvester ant *Pogonomyrmex californicus* Buckley (mass ~7 mg). We used small groups of *Drosophila* and *Forelius* to improve resolution; this is not problematic because these insects did not engage in the DGC in our experiments. *P. californicus* does exchange respiratory gases discontinuously (Quinlan and Lighton, 1999), so we elevated its temperature to 40°C at which point our sample of ants switched to continuous gas exchange. We also recorded a sample of ants at 20°C to evaluate RWL during the DGC.

Foragers of *Forelius mccooci* and *Pogonomyrmex californicus* were collected from the University of California Elliot Chaparral Reserve and the Cuyacama Mountains, San Diego County, California, respectively. They were maintained in round polyethylene containers containing test tubes with water reservoirs backed by cotton wool 1–3 weeks before testing. The test tubes were wrapped in aluminum foil to block ambient light. Workers were maintained at ambient indoor temperatures (23±3°C) and ambient photoperiod (Northern Hemisphere fall). They were fed on crickets and sugar water *ad libitum*. *Drosophila melanogaster* were obtained from wild-type stocks in the laboratory of Stephen Roberts at the University of Nevada at Las Vegas. On arrival, the fly vials were opened and the flies were liberated. After 24 h the newly eclosed flies were decanted into a new vial, stored as above, and used for measurement on days 3–4 after eclosion. Flies were not sexed prior to measurement to minimize handling stress. The fly groups were approximately 50% of each sex (the ants, of course, were all female). Sex does not appear to significantly influence mass- or area-specific water loss rates in *D. melanogaster* (Gibbs and Matzkin, 2001 and references therein). Flies were reared with media until experiments on water loss were conducted.

Respirometry

Most metabolic and water-loss studies are conducted

separately by stop-flow (constant volume) and gravimetric techniques, respectively. Both techniques have poor temporal resolution. Stop-flow techniques yield integrated averages of catabolic flux rates over periods of an hour or more, during which bursts of activity may lead to serious measurement overestimates (Lighton et al., 2001 and references therein). Likewise, gravimetric studies cannot isolate mass-loss events unrelated to cuticular or respiratory water loss, such as excretion or salivation during grooming, which is a particular problem with *Drosophila*. Regular weighing also disturbs animals. By contrast, we used flow-through respirometry for all measurements. Although more demanding of instrumentation stability and resolution, flow-through respirometry minimizes these errors (Lighton, 1991), and also allows the evaluation of hypotheses that require high temporal resolution for experimental testing.

We used a Sable Systems International (www.sablesystems.com) TR-2 system (SSI; Las Vegas, Nevada, USA) for flow-through respirometry with a Li-Cor CO₂ analyzer (Lincoln, NE, USA; resolution <0.1 p.p.m. CO₂), supplemented by an SSI RH-100 water vapor analyzer with direct readout in Pa water vapor pressure (1 Pa of resolution or better and 1% accuracy), SSI's Datacan V data acquisition software with UI-2 16-bit measurement interface (basic accuracy 0.03%), and SSI ExpeData data analysis software. A Tylan FC-260 mass flow control valve attached to SSI's two-channel mass flow controller (TR-MFC1) controlled flow rates. Specimen temperatures were controlled to $\pm 0.1^\circ\text{C}$ by an SSI Pelt-4 temperature controller coupled to a SSI PTC-1 Peltier Effect cabinet with an internal volume of 8 l. The CO₂ analyzer was zeroed with CO₂-scrubbed outside air and spanned at 976 p.p.m. with a certified span gas. The water vapor analyzer was zeroed with nitrogen and spanned by bubbling air through pure water at an accurately known temperature $\sim 5^\circ\text{C}$ lower than ambient, setting the RH-100 to its dewpoint mode, and adjusting it to read the correct water temperature.

Our system is diagrammed in Fig. 1. Bev-A-Line low-permeability tubing was used throughout to minimize water vapor adsorbance errors (Thermoplastic Processes Inc., Georgetown, DE, USA). Briefly, air from outside the lab building was pulled by an SSI TR-SS1 sub-sampler through a Drierite/Ascarite/Drierite drying column (Ascarite; Thomas Scientific, Swedesboro, NJ, USA) to remove CO₂ and H₂O, then into the mass flow control valve, which was set to an STP-corrected flow rate appropriate to the organism (20 ml min⁻¹ for *Forelius* and *Pogonomyrmex*, or 40 ml min⁻¹ for *Drosophila*). After the mass flow control valve, the gas flow passed through a second column containing Ascarite and magnesium perchlorate, which dries air to a greater extent than Drierite and helped to stabilize the water vapor baseline. The prepared air then entered the temperature cabinet, which was set to 20 or 40°C, passed through a helical aluminum temperature equilibration coil, and then entered the respirometry chamber. Because of the small size of *Forelius mccooki*, we employed a 4 cm length of 3 mm I.D. low-permeability Bev-A-Line tubing as the chamber. For the other

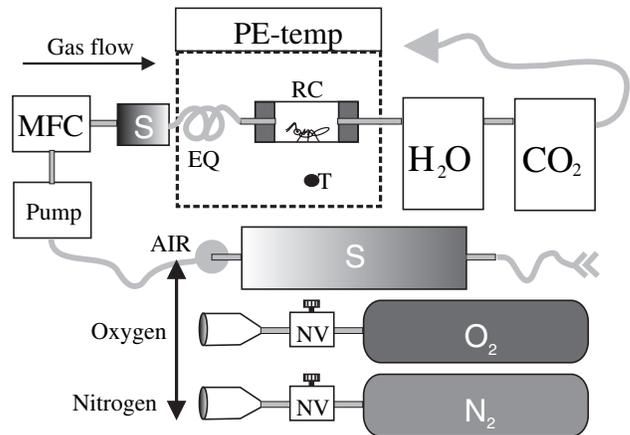


Fig. 1. Simplified diagram of the respirometry system. Not to scale. Data acquisition system not shown. Oxygen and nitrogen tanks are at the bottom; each opens into a manifold through which oxygen or nitrogen flow can be controlled with a needle valve (NV). MFC, mass flow control valve (associated controller not shown). Dashed rectangle is temperature-controlled cabinet (Peltier effect; PE-temp). S, scrubber (H₂O and CO₂); EQ, equilibration coil; RC, respirometry chamber; T, thermocouple; H₂O, water vapor analyzer; CO₂, carbon dioxide analyzer. See text for details.

animals we employed a sealed glass/metal chamber with an interior volume of about 3 ml (SSI TRRM). Finally, air left the respirometry chamber (having gathered CO₂ and H₂O from the insects on its way), entered the RH-100 water vapor analyzer, and then traveled to the CO₂ analyzer.

As outlined in the Introduction, we could at any time switch pure nitrogen or oxygen for the dry air that normally passed through the system. This was accomplished by disconnecting the normal drying column, and instead allowing the system's pump to pull gas from a manifold into which either dry oxygen or dry nitrogen flowed at a rate of approximately 100 ml min⁻¹. During a typical hyperoxic switch run, a recording was started that established the baselines for the carbon dioxide and water vapor analyzers. Meanwhile a group of 10 *Forelius* or a single *Pogonomyrmex* was gathered and weighed to 0.01 mg with a Mettler AG245 balance (Mettler-Toledo Inc., Columbus, OH, USA). After weighing the recording was paused and the animals were placed in the respirometry chamber. A built-in stainless steel filter disk prevented the smaller animals from creeping from the chamber into the system's tubing. The flow rate through the system was increased to 100–200 ml min⁻¹ for 3–5 min to speed equilibration, and then lowered to the appropriate flow rate (see above) for a further 5–10 min before re-starting the recording. The recording continued until a plateau in water loss rate was reached. At that point the gas flowing through the system was changed to pure oxygen. After a further ~ 20 min, the oxygen was changed to nitrogen, and the recording continued until the animals' carbon dioxide production fell to near baseline levels. Finally, the recording was paused, the animals were removed from the chamber, the chamber was replaced, and after equilibration another baseline taken.

We weighed *Drosophila* and recorded their water vapor and CO₂ output in the same manner as that used for the ants except for the following. First, we cooled flies for easier handling, and then placed groups of 4–11 individuals in the respirometric chamber to record CO₂ and water vapor emission. After that, flies were weighed and the mass of water lost during the recording (as determined from the respirometry data) was added to their post-recording weight.

Each recording consisted of a variable number of data points taken at 2 s intervals, using finite impulse response digital filtration to reduce short-term noise (Lighton, 1991). Recordings typically lasted for 1.5–2.0 h including initial and final baselines.

Analysis and statistics

Hyperoxic switch recordings were analyzed using Sable Systems ExpeData software (β release 3.1). For each hyperoxic switch recording:

- (1) The CO₂ and H₂O baselines were rotated and subtracted
- (2) CO₂ in p.p.m. was converted to μl h⁻¹ by multiplying by flow rate in ml h⁻¹ and dividing by 10 (for formulae see Lighton, 1991)
- (3) H₂O vapor pressure in Pa was converted to mg H₂O ml⁻¹ by dividing by (T×461.5) where T was the temperature of the water vapor sensor in Kelvin
- (4) H₂O concentration in mg ml⁻¹ was converted to mg h⁻¹ water loss rate by multiplying by flow rate in ml h⁻¹
- (5) Plateau CO₂ emission and H₂O loss rates were selected (pre-oxygen treatment)
- (6) Mean plateau CO₂ and H₂O loss rates were stored in an internal spreadsheet
- (7) Mean cabinet temperature over this interval was stored
- (8) The post-oxygen decline in CO₂ emission rate was selected and its magnitude stored
- (9) The post-oxygen decline in H₂O loss rate was selected and its mean value stored
- (10) The maximal rate of CO₂ emission, post-nitrogen, over 30 s, was selected and its mean value stored
- (11) The maximal rate of H₂O loss, post-nitrogen, over 30 s, was selected and its mean value stored
- (12) The secondary plateau rate of H₂O loss, post-nitrogen, was selected and its mean value stored.

During these operations a master log file was recorded that spanned the analysis of all of the data files. If required (for example, to add an additional variable to the spreadsheet for each file), the master log file could be edited and then played back, allowing all of the data files to be re-analyzed very rapidly and without operator error. When complete, the data in the spreadsheet were saved as a delimited ASCII file with column headings. Once the data were in the spreadsheet, further data manipulations could be performed. These included the calculation of water vapor pressure saturation deficit from chamber temperature (formula in Lighton and Feener, 1989) and the calculation of ant surface area (*ibid*) and, thence, cuticular permeability.

Recordings of animals engaging in the DGC (i.e. *P.*

californicus at 20°C) were analyzed as described elsewhere (Lighton et al., 1993). Means are accompanied by *N* and s.e. unless otherwise noted.

Results

Water loss rates

Typical recordings for *Drosophila melanogaster*, *Forelius mccooki* and *Pogonomyrmex californicus* during continuous CO₂ emission are shown in Figs 2–4. Discontinuous gas exchange cycles of *P. californicus* are similar to those reported in its congeners (Quinlan and Lighton, 1999). All of the organisms we studied during hyperoxic switch runs were active for much or all of the duration of measurements, except in anoxia (Table 1). Six *P. californicus* at 20°C were inactive frequently enough to allow ~5 DGCs to be measured per individual. For this group of ants, DGC frequency was 3.75±1.73 s.e.m. mHz, and 0.13±0.05 s.e.m. μl CO₂ and 0.47±0.37 μg s.e.m. H₂O were emitted per open-spiracle phase. Table 1 contains the other relevant parameters for this group; note that RWL during the DGC as a percentage of total RWL is practically identical to that of the same species during continuous emission of CO₂ (see Table 1).

Calculation of total vs respiratory water loss rates

The hyperoxic switch technique we used in this study did, as predicted, caused a transient drop in carbon dioxide output (see Figs 2–4 and Table 1). The decline in carbon dioxide emission also caused a temporary decrease in water loss rate. Thus at steady-state, assuming diffusive gas exchange with fixed trans-spiracular partial pressure gradients:

$$\text{trans-spiracular } \dot{V}_{\text{H}_2\text{O}} = D_{\text{H}_2\text{O}} \times A/L \times \Delta P_{\text{H}_2\text{O}}, \quad (1)$$

and

$$\text{trans-spiracular } \dot{V}_{\text{CO}_2} = D_{\text{CO}_2} \times A/L \times \Delta P_{\text{CO}_2}, \quad (2)$$

where *A/L* are the summed areas and lengths of the diffusing paths for water and CO₂ (through the spiracles and down tracheae), and *D* is the diffusivity of the gas in question under the conditions of the experiment. Because water and CO₂ are transported via the same tracheae and spiracles, *A/L* are identical for CO₂ and water. From these equations, it can be seen that a given reduction in spiracular area will cause an identical proportional reduction in gas flux for both water and carbon dioxide, if the partial pressure gradients for water and carbon dioxide remain constant.

Exposure to pure oxygen produced short-term decreases in \dot{V}_{CO_2} of 46–88% (Table 1). If the partial pressure gradients for water and carbon dioxide did not change during these periods, then these decreases in \dot{V}_{CO_2} were caused by decreases in *A/L* of 46–88%. Thus, the simplest estimate of trans-spiracular $\dot{V}_{\text{H}_2\text{O}}$ would be

$$\text{minimal trans-spiracular } \dot{V}_{\text{H}_2\text{O}} = \Delta \dot{V}_{\text{H}_2\text{O}}, \quad (3)$$

where $\Delta \dot{V}_{\text{H}_2\text{O}}$ is the decrease in $\dot{V}_{\text{H}_2\text{O}}$ in response to hyperoxia. However, the low flow rate we used (20 ml min⁻¹ in the case of *F. mccooki* and *P. californicus*, and 40 ml min⁻¹ for *D. melanogaster*) limited the temporal resolution of the system,

Table 1. Water loss rate and cuticular permeability

Species	<i>Drosophila melanogaster</i>	<i>Forelius mccooki</i>	<i>Pogonomymex californicus</i>	<i>Pogonomymex californicus</i>
Temperature (°C)	20	20	40	20
Individuals measurement ⁻¹	4–11	10	1	1
<i>N</i>	9	6	7	6
Individual mass (µg)	1000±20	270±20	6760±530	7660±180
\dot{V}_{CO_2} (µl hr ⁻¹ insect ⁻¹)	1.98±0.14	0.26±0.01	8.98±0.60	1.69±0.25
MR (µW)	11.52±0.83	1.52±0.08	69.60±4.63	13.09±1.90
SMR (µW)	1.86±0.10	0.61±0.04	38.14±2.58	10.63±0.22
Total WLR (µg hr ⁻¹ insect ⁻¹)	45.57±3.21	15.33±1.15	547.05±78.86	106.62±12.42
Area-specific WLR (µg hr ⁻¹ cm ⁻²)	449.86±38.23	358.53±29.99	1461.14±156.71	265.46±29.79
Uncorrected CP (µg hr ⁻¹ cm ⁻² Torr ⁻¹)	25.84±2.19	20.57±1.70	26.51±2.84	15.25±1.71
Corrected CP (µg hr ⁻¹ cm ⁻² Torr ⁻¹)	19.74±1.33*	18.31±1.68*	25.22±2.96	14.39±1.61
Reduction CO ₂ (%)	46.12±4.08	71.77±2.55	88.35±5.56	NA
Reduction WLR (%)	9.43±1.13*	5.34±1.18*	4.79±1.24	NA
Respiratory WLR (%)	22.86±3.59*	7.59±2.01*	5.58±1.47	5.64±0.49
Peak CO ₂ (%)	109.73±8.65	208.61±8.60	327.66±36.07	NA
Peak WLR (%)	185.23±33.77	149.90±22.24	69.62±13.38	NA
Plateau WLR (%)	18.48±13.61	30.67±6.26	43.52±8.48	NA

Values are mean ± S.E. WLR, water loss rate. CP, cuticular permeability, either raw (cuticular + respiratory) or corrected by subtraction of respiratory water loss (see text). Peaks or reductions in \dot{V}_{CO_2} or $\dot{V}_{\text{H}_2\text{O}}$ are expressed as percent above or below the normoxic steady-state condition, respectively. Respiratory WLR is expressed as percent of total WLR. WLR plateau after N₂ is expressed as percent over the normoxic steady-state condition. SMR is standard metabolic rate predicted by the allometric equation for inactive tracheate arthropods (Lighton et al., 2001). NA, not applicable. *One value missing. See text, especially for RWL comparisons in *P. californicus* between 20°C (discontinuous gas exchange) and 40°C (continuous gas exchange). Torr=133.3 Pa.

blunting the measurement of the temporary spiracular constriction and its accompanying decreases in rates of CO₂ and H₂O emission. Moreover, the measurement of grouped of

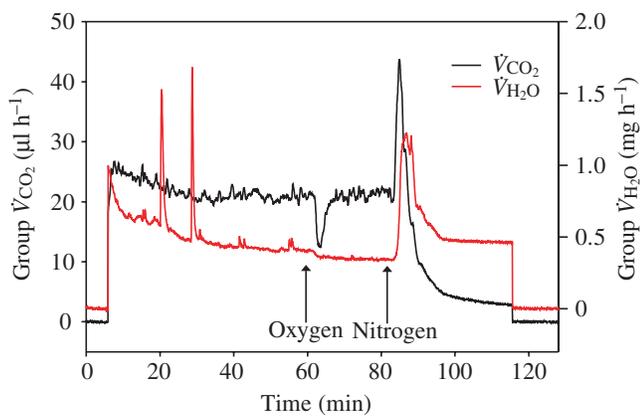


Fig. 2. Typical recording of a hyperoxic switch recording of *Drosophila melanogaster*. Eleven flies, total mass 9.41 mg, at 20°C and 40 ml min⁻¹ STP. The recording begins and ends with baselines. Two water excretion events can be seen at ~20–30 min into the recording. The large dip in \dot{V}_{CO_2} that follows hyperoxia is plainly visible at ~65 min. A modest drop in $\dot{V}_{\text{H}_2\text{O}}$ coincides with the drop in \dot{V}_{CO_2} . To minimize the influence of long-term drift on our measurements, we used only the magnitude of the $\dot{V}_{\text{H}_2\text{O}}$ drop immediately coinciding with the \dot{V}_{CO_2} drop. Following the introduction of nitrogen, a rapid rise in both $\dot{V}_{\text{H}_2\text{O}}$ and \dot{V}_{CO_2} are evident. \dot{V}_{CO_2} rapidly declines to near baseline levels, but $\dot{V}_{\text{H}_2\text{O}}$ plateaus at an intermediate level; see text.

insects might enhance this phenomenon owing to inter-individual variability in the modulation of the degree of spiracular opening. Using the ‘instantaneous correction’ of Bartholomew et al. (1981) is impractical here because the technique requires a clean enough signal to derive a usable first derivative from it, and we are operating near the noise floor of current instrumentation. Therefore, our hyperoxic $\dot{V}_{\text{H}_2\text{O}}$ decline data are very conservative measures on which to base estimates of respiratory water loss rates.

It is, however, possible to correct the $\dot{V}_{\text{H}_2\text{O}}$ data for the limited temporal resolution of the system and to assign an upper limit to respiratory water loss rates. This correction requires three assumptions. First, it is certainly reasonable to assume a ratiometric relation between the hyperoxic declines in \dot{V}_{CO_2} and $\dot{V}_{\text{H}_2\text{O}}$, as shown in Equations 1 and 2, bearing in mind that CO₂ comes only from the spiracles while H₂O comes from both the spiracles and the cuticle. The second assumption is that for a short time, the degree of spiracular opening is reduced to very low levels after exposure to hyperoxia – in fact *P. californicus* shows a decline of nearly 90%. We will assume a transient decline to zero. By this reasoning, we should increase the hyperoxic $\dot{V}_{\text{H}_2\text{O}}$ decline by the ratio of (100%:hyperoxic \dot{V}_{CO_2} decline%). If this assumption is reasonable, then we can assign an upper limit to respiratory water loss rates, as is shown in Table 1. So, assuming no changes in internal P_{CO_2} or $P_{\text{H}_2\text{O}}$:

$$\text{maximal trans-spiracular } \dot{V}_{\text{H}_2\text{O}} = \Delta \dot{V}_{\text{H}_2\text{O}} \times 1/x, \quad (4)$$

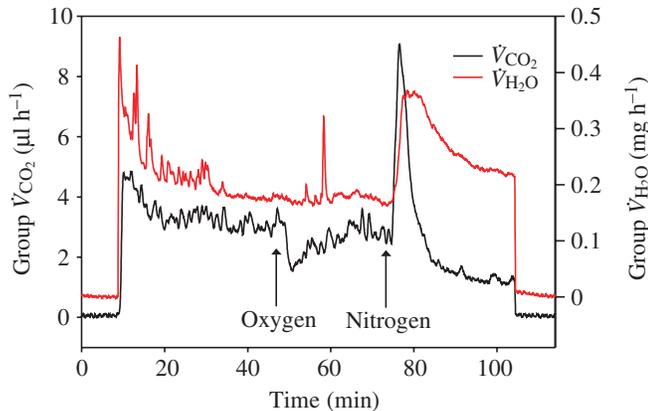


Fig. 3. Typical recording of a hyperoxic switch recording of *Forelius mccooki*. Ten ants, total mass 3.28 mg, at 20°C and 20 ml min⁻¹ STP. The recording begins and ends with baselines. Intermittent water excretion events can be seen near the start of the recording and at ~60 min. The large dip in \dot{V}_{CO_2} that follows hyperoxia is plainly visible at ~50 min. The drop in $\dot{V}_{\text{H}_2\text{O}}$ coincides with the drop in \dot{V}_{CO_2} and immediately precedes a small excretion event. We noticed that water excretion events often occurred ~5–20 min after initiation of hyperoxia in all species. Because we used only the magnitude of the $\dot{V}_{\text{H}_2\text{O}}$ drop immediately coinciding with the \dot{V}_{CO_2} drop, these events, though annoying, usually did not interfere with our measurements. Following the introduction of nitrogen, a rapid rise in both $\dot{V}_{\text{H}_2\text{O}}$ and \dot{V}_{CO_2} are evident. \dot{V}_{CO_2} rapidly declines to near baseline levels, but $\dot{V}_{\text{H}_2\text{O}}$ starts to plateau at an intermediate level; see text.

where $\Delta\dot{V}_{\text{H}_2\text{O}}$ is the decrease in $\dot{V}_{\text{H}_2\text{O}}$ in response to hyperoxia, and x is the factorial decrease in \dot{V}_{CO_2} in response to hyperoxia.

However, as noted above, this estimate of maximal transpiracular $\dot{V}_{\text{H}_2\text{O}}$ assumes no changes in the partial pressure gradients for water or carbon dioxide. This is the third assumption of this model. While it seems likely that tracheal air stays saturated with water within the tracheae during hyperoxia, it is possible that in these insects, as in grasshoppers (Gulinson and Harrison, 1996), that hyperoxia induces a rise in tracheal P_{CO_2} . As can be seen from Equation 2, if ΔP_{CO_2} doubled in response to hyperoxia, a 50% drop in \dot{V}_{CO_2} would be associated with a 100% reduction in A/L. We attempted to minimize this problem by using measurements of $\dot{V}_{\text{H}_2\text{O}}$ and \dot{V}_{CO_2} from only the initial minutes after exposure to hyperoxia.

It is trivial to modify Equation 4 to allow for a less than complete reduction in the degree of spiracular opening across the hyperoxic switch; this assumption simply allows us to set an upper limit to the contribution of respiratory water loss to total water loss.

Discussion

Catabolic flux rates

Catabolic flux rates for *Drosophila* in this study are very similar to those determined by Gibbs et al. (2003; their Fig. 4): \dot{V}_{CO_2} approximately 2 $\mu\text{l h}^{-1} \text{fly}^{-1}$ (measurement temperature not specified but presumably close to 24°C; our measurements were made at 20°C). Lehmann et al. (2000) found rather higher

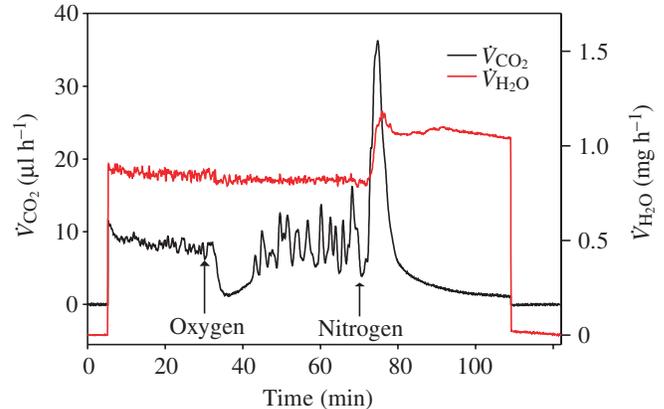


Fig. 4. Typical recording of a hyperoxic switch recording of *Pogonomyrmex californicus*. One ant, mass 8.25 mg, at 40°C and 20 ml min⁻¹ STP. The recording begins and ends with baselines. The large dip in \dot{V}_{CO_2} that follows hyperoxia is plainly visible. The drop in $\dot{V}_{\text{H}_2\text{O}}$ coincides with the drop in \dot{V}_{CO_2} . Following the introduction of nitrogen, a rapid rise in both $\dot{V}_{\text{H}_2\text{O}}$ and \dot{V}_{CO_2} are evident. \dot{V}_{CO_2} rapidly declines to near baseline levels, but $\dot{V}_{\text{H}_2\text{O}}$ plateaus at an intermediate level; see text.

rates: >4 $\mu\text{l h}^{-1} \text{fly}^{-1}$ at 24°C. Williams and Bradley (1998), by contrast, found a remarkably low \dot{V}_{CO_2} of 0.7 $\mu\text{l h}^{-1} \text{fly}^{-1}$ at an unspecified temperature, and ~2–4 $\mu\text{l h}^{-1} \text{fly}^{-1}$ at 25°C (Williams and Bradley, 1998). It would appear that *Drosophila* \dot{V}_{CO_2} is a moving target, and activity levels will certainly influence readings by a large percentage, as will temperature. In any event our measurements fall in the middle of the range of reported values after allowing for temperature, suggesting that our results are typical. The ants, except for *P. californicus* at 20°C, showed \dot{V}_{CO_2} substantially (2×) higher than predicted on the basis of current consensus equations at 20 or 40°C (Lighton et al., 2001), as expected for intermittently active animals.

The hyperoxic switch

We are not aware of any previously published report of a reduction in \dot{V}_{CO_2} following exposure to hyperoxia in any insect. However, a senior study by Wigglesworth (1935) in the flea, showed the modulation of spiracular opening by gas composition. Particularly, in his Fig. 4H (Wigglesworth, 1935), it is clear that the closed phase of the last abdominal spiracle lasts for a longer time in pure oxygen; however, the transient effects of hyperoxia on \dot{V}_{CO_2} have not previously been reported. Levy and Schneiderman (1966a) did not cite the Wigglesworth's 1935 study, but they demonstrated a similar phenomenon. With silkworm pupae, they observed an increase in the period of spiracular constriction with increasing ambient O_2 ($P_{\text{O}_2}=60\%$), and hypothesized a theoretical spiracular response in pure O_2 . We suggest that the hyperoxic switch effect is widespread and could be a useful tool for arthropod respiratory physiologists. This method may be especially applicable for small arthropods with continuous gas exchange and for which gas exchange occurs primarily by diffusion,

which obviously appears to be the case in our study from the kinetics of gas exchange shown in our graphs.

As alluded to in the Results, our raw measurements may underestimate the effects of the hyperoxic switch on the degree of spiracular opening. This is because of the limited temporal resolution of our respirometry system, chiefly in the analyzers rather than in the flow characteristics of our respirometer chambers at the flow rates we used. We propose (see Results) a simple correction to mitigate this effect and to allow calculation of an upper limit to respiratory water loss rates. Direct visual observation of spiracular activity during the hyperoxic switch would provide valuable information regarding the validity of our correction technique.

Total water loss rates

Our respirometrically measured rates of total water loss in *D. melanogaster* are similar to those of Gibbs and Matzkin (2001; $\sim 42 \mu\text{g h}^{-1} \text{fly}^{-1}$, their Fig. 3), and the gravimetric measurements of Lehmann et al. (2000; $30 \mu\text{g h}^{-1} \text{fly}^{-1}$, their Table 2). The overall water loss rate of *D. melanogaster* divided by estimated surface area and water vapor pressure deficit yields its gross cuticular permeability (i.e. including the respiratory component). This value, again, is similar to the value that can be calculated from published data (*ibid.*).

The WLR of *F. mccoeki* has not been previously described but is typical for a xeric ant (Duncan and Lighton, 1994). That of *P. californicus* is far higher on an area-specific basis than that of the other two insects, but this is to be expected in view of its higher measurement temperature (40°C vs 20°C). When corrected for water vapor pressure saturation deficit, its gross cuticular permeability is lower than, but not dissimilar to, that of two congeners, *P. rugosus* and *P. occidentalis* (mean $28.4 \mu\text{g h}^{-1} \text{cm}^{-2} \text{Torr}^{-1}$; where $\text{Torr} \equiv 133.3 \text{ Pa}$; Quinlan and Lighton, 1999).

Respiratory water loss rates during continuous CO_2 emission

Among the ants we measured, respiratory water loss rates varied from $\sim 5\text{--}7\%$ of total water loss rates, which is similar to values measured in ants that express a DGC; 2% (*Camponotus vicinus*; Lighton, 1992); 8% (*Cataglyphis bicolor*; Lighton, 1992); 4% (*Pogonomyrmex occidentalis*, which is very similar to *P. californicus* in body size and morphology; Quinlan and Lighton, 1999) and 2% (*Pogonomyrmex rugosus*; Quinlan and Lighton, 1999). They are well within the range for insects in a variety of other orders for which estimates exist in the literature (see especially Table 1 in Chown, 2002).

Where *Drosophila melanogaster* is concerned, there are very few published estimates of respiratory water loss in animals at rest. As Gibbs et al. (2003) bemoan, 'Unfortunately, water loss from individual flies in our experiments was so low that we could not measure it reliably... Carbon dioxide readings never reached zero, indicating that at least one spiracle remained open at all times, which would also reduce our ability to distinguish respiratory losses. Thus, we were unable to reliably detect increases in water loss caused by spiracular

opening...' Williams and Bradley (1998) were fortunate enough, however, to find – of the 50 flies they examined – two that exhibited sufficient discontinuity in CO_2 emission to allow them to assess respiratory water loss, which they estimated at $\sim 25\%$ of total water loss rate. This is very similar to our figure, which can therefore be provisionally accepted as a reasonable figure for non-flying *D. melanogaster*.

We were somewhat struck by the fact that the $\dot{V}_{\text{H}_2\text{O}}$ trace often appeared to stay below pre-hyperoxic levels, whereas the \dot{V}_{CO_2} trace invariably returned to pre-hyperoxic levels. It is possible that this may reflect a lasting reduction in the degree of spiracular opening caused by hyperoxia, in conjunction with a higher internal P_{CO_2} that establishes itself at a level sufficient to maintain normal rates of CO_2 emission through the constricted spiracles. Then again, most of the $\dot{V}_{\text{H}_2\text{O}}$ traces showed a long-term downward trend, making firm statements about long-term changes in $\dot{V}_{\text{H}_2\text{O}}$ problematic. This was another reason why we restricted our measurements to the section of the $\dot{V}_{\text{H}_2\text{O}}$ trace immediately coincident with the \dot{V}_{CO_2} hyperoxic decline (time difference only 5 min or less on average). For *D. melanogaster*, which showed the strongest long-term downward trend in $\dot{V}_{\text{H}_2\text{O}}$, we restricted our measurements to an interval of only 3.19 ± 1.05 min (mean \pm s.e.; $N=9$) immediately following the hyperoxic switch. This was because water excretion events often took place shortly after the effects of hyperoxia manifested themselves (see Fig. 3). We are at a loss to explain this apparent behavioral effect either of hyperoxia or, conceivably, of moderate endotracheal hypercapnia caused by spiracular constriction.

In any event we conclude that the hyperoxic switch technique has potential utility to students of the respiratory physiology of tracheate arthropods.

Respiratory water loss rates during the DGC

The RWL of *P. californicus*, expressed as a percentage of total WLR, did not change whether or not it expressed a DGC (Table 1). This is an interesting result, and serves to caution that simplistic evolutionary hypotheses regarding the evolution of the DGC, e.g. the long-held 'hygric hypothesis', may not hold water (Lighton, 1998 and references therein; with respectful apologies to Allen Gibbs). It should be mentioned here that hypotheses regarding the RWL-reduction benefits of the DGC should be exclusively fielded under the banner of the hygric hypothesis; the chthonic hypothesis (Lighton, 1998) is exclusively concerned with gas exchange under severe hypoxic or hypercapnic conditions, not with what may (or may not) occur in conditions where partial pressures of respiratory gases are more quotidian, as in CO_2 -scrubbed air.

We have no reason to believe that our sample of ants was expressing the DGC abnormally. The DGCs and catabolic flux rates of our sample of *P. californicus* were almost identical to those reported by Quinlan and Lighton (1999), compensating where necessary for the 5°C higher temperature of the ants in that study. In addition their catabolic flux rates are very similar to those predicted for motionless ants of that mass and

temperature by the allometric equation of Lighton et al. (2001); $P=0.25$, see Table 1.

However, the WLRs of *P. californicus* during continuous and discontinuous CO₂ emission are not as directly comparable as one might wish, calling for a note of interpretative caution. This is because the temperatures of the ants expressing the two gas exchange strategies differed by 20°C. This temperature disparity should not affect the ratio of respiratory to total water loss rates, especially because WLR (as expressed in Table 1) is also standardized by the water vapor pressure saturation deficit at each temperature. WLR per unit water vapor pressure saturation deficit is generally independent of temperature (Edney, 1977; Lighton and Feener, 1989 and references therein). Catabolic flux rate is strongly temperature dependent, increasing some fourfold from 20 to 40°C, and should elevate RWL at the higher temperature. However, this effect (of uncertain magnitude with regard to RWL, accompanied by unknown changes in the degree of spiracular opening) would increase RWL to total WLR ratios at the higher temperature, so it would make the present comparison more conservative. This is because the RWL to total WLR ratio would be expected to be higher at 40°C, exaggerating any putative reductions in RWL caused by the DGC at 20°C, a temperature at which RWL should decrease anyway because of decreased catabolism. This makes the parity reported here of RWL as a percentage of total WLR, during continuous and discontinuous CO₂ emission, all the more interesting, although it would be preferable to turn the DGC on or off at a single temperature and within a single individual while monitoring WLR. Such studies could go some way towards resolving the controversies surrounding the selective correlates of the DGC (Lighton, 1998; Chown, 2002; and references in both).

Response to anoxia

A large increase in CO₂ emission followed exposure to nitrogen (Figs 2–4; Table 1), which was caused by the opening of the insects' spiracles after detection of anoxia. However, the biological meaning of this peak is difficult to interpret in this context because it consists of material (CO₂) that requires O₂ for its production, but O₂ was something that the insects, in their case, no longer had. (For this reason, convective gas exchange caused, for example, by abdominal pumping, would rapidly cease during anoxia.) The CO₂ peak therefore consisted largely of carbon dioxide escaping from the tracheal system and hemolymph, steadily decreasing after the initial peak with classic first-order-decay kinetics. This contrasts with water vapor output, which in the short term is independent of oxygen availability and is, therefore, a better indicator, for present purposes, of total respiratory throughput capacity. Not surprisingly, *D. melanogaster*, as an insect capable of flight, showed the largest anoxic factorial increase in $\dot{V}_{\text{H}_2\text{O}}$, but *F. mccooki* was not far behind. To anyone who has watched *F. mccooki* in the field this comes as no surprise, because this tiny ant forages at high speed at high substrate temperatures (Hölldobler and Wilson, 1990; J.R.B.L., personal observations) and is

therefore presumably capable of high aerobic throughput. Ants have elsewhere been shown to be capable of attaining factorial aerobic scopes close to those required for flight (Roces and Lighton, 1995).

The episode of peak water loss following exposure to nitrogen is brief, because, we hypothesize, the terminal tracheoles rapidly lose their small reservoirs of free water in the absence of metabolic activity (Wigglesworth, 1983). After the reservoirs evaporate, water diffuses into the tracheoles from the hemolymph and then diffuses through the tracheal system and out through the spiracles at a much lower, and more constant, rate. Other possible explanation for the fall in water vapor rates after a period of time in anoxia could be the cessation of some active process that is facilitating the high rates of water loss.

It is reasonable to infer from the far higher maximal rate of water vapor loss through the spiracles immediately after exposure to nitrogen (see above), that the rate-limiting step in this phase of the anoxia response is the diffusion of water across the tracheolar membranes, rather than spiracular and tracheal resistance. Thus, following the initial brief peak of water loss, there follows a lower plateau at an approximate steady state composed of cuticular plus tracheal water loss (not strictly respiratory water loss because respiration has ceased). We assume this steady state to represent the steady flux of water vapor from the tracheoles, following the wholesale loss of water-vapor-saturated air from the endotracheal space during the initial stages of anoxia.

Measuring $\dot{V}_{\text{H}_2\text{O}}$ and \dot{V}_{CO_2} kinetics after exposure of tracheate arthropods to anoxia may therefore yield non-invasive and, if of short duration, non-lethal comparative information on maximal degree of spiracular opening, tracheal volume, tracheal conductance and summed tracheolar surface areas.

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