

The role of discontinuous gas exchange in insects: the chthonic hypothesis does not hold water

Allen G. Gibbs^{1,*} and Robert A. Johnson²

¹*Department of Ecology and Evolutionary Biology, 1041 E. Lowell Street, University of Arizona, Tucson, AZ 85721 USA* and ²*School of Life Sciences, Arizona State University, Tempe, AZ 85287-4501, USA*

*Author for correspondence (e-mail: agibbs@arl.arizona.edu)

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Summary

Insects breathe through valved openings (spiracles) in their cuticle. Many insects open and close their spiracles in a cyclic pattern (discontinuous gas-exchange cycles, or DGC). These cycles were observed over half a century ago, their hypothesized function being to minimize loss of water from the tracheal system. However, numerous recent studies have found that respiration accounts for a small fraction of total water loss, and that insects stop performing DGC at times when this pattern would be most useful. Thus, the importance of cyclic gas exchange for water conservation has been challenged. The leading alternative is the chthonic hypothesis, which proposes that DGC originated in insects from hypercapnic (high CO₂) environments (e.g. burrows) to aid in release of carbon dioxide. By keeping the spiracles closed, insects would concentrate CO₂ and increase the gradient for outward diffusion of CO₂. CO₂ would be released rapidly when the spiracles opened, and respiratory water loss would be reduced. The chthonic hypothesis therefore predicts that DGC minimizes the ratio of respiratory water loss to CO₂ release relative to other modes of gas exchange. We tested

the chthonic hypothesis by simultaneously measuring water loss and CO₂ release in reproductive females (queens) of the seed-harvester ant *Pogonomyrmex barbatus*, a burrowing species from North American deserts. Queens used one of three patterns of gas exchange, discontinuous, cyclic and continuous. We resolved the problem of separating cuticular transpiration and respiratory water loss for individuals that used continuous gas exchange by developing a regression method that can be used across all patterns of gas exchange. The ratio of respiratory water loss to CO₂ release did not differ among ants using different patterns of gas exchange, in contrast to the expectation of the chthonic hypothesis. Metabolic rate, however, varied with gas-exchange pattern, and was lowest for individuals that used discontinuous gas exchange, intermediate for individuals using cyclic gas exchange, and highest for individuals using continuous gas exchange.

Key words: discontinuous gas-exchange cycle, queen mating stage, *Pogonomyrmex barbatus*, respiratory water loss, seed-harvester ant.

Introduction

Gas exchange in terrestrial environments comes at the cost of evaporative water loss from respiratory surfaces. Comparative physiologists have therefore long been interested in whether different ventilatory patterns affect respiratory water loss. Insects in particular vary tremendously in their modes of gas exchange, both within and among species (Lighton, 1998; Shelton and Appel, 2001; Chown, 2002). The classical pattern is that of discontinuous gas exchange, or discontinuous gas-exchange cycles (DGC; Lighton, 1996, 1998). These cycles consist of three phases: the closed (C) phase, when the spiracles are sealed and no gas exchange occurs; the flutter (F) phase, when the spiracular valves open and close rapidly; and the open (O) phase, when the spiracles open wide and allow maximum diffusion of gases. The F phase has been thought to be particularly critical for water conservation, because convective influx of oxygen-rich air into the tracheal system allows O₂ uptake with minimal water loss.

However, the F phase may be absent, or CO₂ can be released continuously without the spiracles closing in some insects, including those from arid habitats (Lighton and Berrigan, 1995; Quinlan and Lighton, 1999).

The paradigm that DGC are an adaptation to reduce respiratory water loss has been challenged in recent years. Several studies reveal that respiratory water loss comprises <15% of total water loss, even when the spiracles are open (e.g. Lighton, 1992; Quinlan and Hadley, 1993; Quinlan and Lighton, 1999; Bosch et al., 2000; Chappell and Rogowitz, 2000; Rourke, 2000). Thus, respiratory water loss is a relatively minor component of the overall water budget, so that the pattern of gas exchange may not significantly affect overall water balance. In addition, many insects cease performing DGC at high temperatures or when dehydrated (Quinlan and Hadley, 1993; Chappell and Rogowitz, 2000; Rourke, 2000), conditions under which water conservation should be most

important. These considerations have led many authors to conclude that the standard explanation for discontinuous gas exchange is inadequate (Lighton, 1996, 1998; Shelton and Appel, 2001; Chown, 2002).

Discontinuous gas-exchange cycles are taxonomically widespread, but not universal, and their evolutionary origin is unknown. Lighton (1996, 1998; see also Lighton and Berrigan, 1995) noted the prevalence of DGC in fossorial insects, which inhabit microclimates where CO₂ levels may be relatively high. Consequently, Lighton proposed the chthonic hypothesis, which suggests that DGC originated as a mechanism to improve gas exchange while at the same time minimizing respiratory water loss. Under the chthonic hypothesis, insects build up CO₂ when the spiracles are closed, creating an increased gradient for outward diffusion when they open. The same amount of CO₂ can then be excreted during shorter periods of spiracular opening, thus reducing the associated respiratory water loss. The chthonic hypothesis, therefore, makes the specific prediction that the ratio of respiratory water loss to CO₂ release will be lower in insects performing DGC than in those using other modes of gas exchange. The null hypothesis is that this ratio will not be affected by the pattern of gas exchange.

Alternatively, DGC could reduce tracheal oxygen levels, thereby increasing the gradient for O₂ uptake in hypoxic habitats (which are often hypercapnic). In either case, the chthonic model emphasizes minimizing respiratory water loss while allowing effective gas exchange (Lighton and Berrigan, 1995; Lighton, 1998). For example, Lighton (1998) notes that an alternative mode of gas exchange would be to open the spiracles continuously. Even in hypercapnic or hypoxic habitats, sufficient gradients for diffusive gas exchange would eventually be established. However, he concludes that this "... is not a viable strategy except in water-saturated air." Thus, the chthonic hypothesis can be viewed as an extension of the traditional water conservation hypothesis, that attempts to explain the adaptive significance of DGC under the specific environmental conditions in which these cycles may have evolved.

We tested the chthonic model by measuring the respiratory water loss:CO₂ release ratio in reproductive females (queens) of the seed harvester ant *Pogonomyrmex barbatus* F. Smith (Formicidae: Myrmicinae). Our central hypothesis was that this ratio would be lowest in ants performing DGC, relative to those using other modes of gas exchange. *Pogonomyrmex barbatus* is a soil-dwelling species that occurs in arid regions of southwestern North America (Johnson, 2000a,b). Summer rains trigger the mating flights, and the sexuals fly to mating aggregations that contain many thousands of individuals (Hölldobler, 1976). Each female mates with several males, then tears off her wings and digs a burrow in which she starts her colony. Water-loss rates more than double from the time when the queen flies from her nest to several days after mating (Johnson, 2000a; Johnson and Gibbs, 2004). The queen supports herself and rears her first brood of workers, which emerge after 4–5 weeks at 30°C, solely using her body reserves (Johnson, 2002). Water conservation during this period

underground is critical to success of the new colony (Johnson, 2000c), so *P. barbatus* provides an ideal species to test the chthonic hypothesis.

Materials and methods

Collections

Ants *Pogonomyrmex barbatus* F. Smith were collected in July and August near Rodeo, New Mexico, USA. We excavated mature alate (winged) females from their nest prior to mating flights, and newly mated, dealate queens were collected as they walked from the mating aggregation, but before beginning to excavate their new nests. 2 days later, we excavated mated dealate queens from their incipient nests. Each ant was placed in a 1.5 ml microcentrifuge tube that had been punctured for ventilation, along with a small piece of moist paper towel to maintain hydration. Ants were then shipped *via* overnight express to the laboratory; respirometry measurements were performed within 1 week.

Respirometry

We measured water-loss rates and metabolic rates for each ant using flow-through respirometry. Ants were placed in 5 ml glass-aluminum chambers in a darkened incubator at 30°C. Dry, CO₂-free air was pumped through the chambers at 100 ml min⁻¹ to a Li-Cor (Lincoln, Nebraska, USA) LI-6262 infrared CO₂ and water vapor sensor. Chambers containing the ants were placed in the respirometer for an acclimation interval of approximately 3 h, then data were recorded for 30 min. Time-averaged data were recorded every 5 s and analyzed using Datacan V software (Sable Systems; Las Vegas, Nevada USA). Ants were weighed to the nearest 0.1 mg before and after each run. Estimates of water loss during the run, as calculated from mass loss, were consistent with our flow-through measurements.

We analyzed data using two separate two-way analysis-of-covariance models (ANCOVA). Both models included pattern of gas exchange and mating stage as independent variables, with starting mass as a covariate; observed patterns of gas exchange were discontinuous, cyclic and continuous. The dependent variable in each model was the respiratory water loss:CO₂ release ratio and metabolic rate, respectively. Ants were assayed at different times after collection, so we also included date as a covariate in both models. Time in the laboratory did not have a significant effect in either model, indicating that our results were not affected by acclimatization to laboratory conditions. Thus, we dropped date from our final models. Statistical analyses were performed using JMP 4.0 software (SAS Institute, Cary, NC, USA).

Results

Comparison of water loss and CO₂ loss across patterns of gas exchange

Individual ants exhibited one of three general patterns of gas exchange (Fig. 1). The first pattern resembled classical

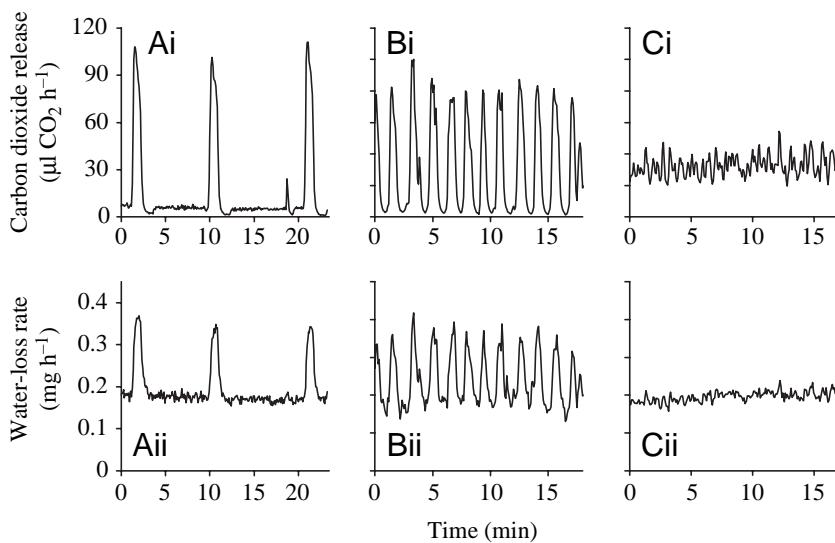


Fig. 1. Recordings of CO₂ release (Ai–Ci) and water loss (Aii–Cii) from three alate female *P. barbatus*. Similar recordings were obtained for mated females and foundresses.

discontinuous gas exchange (Fig. 1Ai), with a cycle length of 5–10 min ($N=9$ ants). Five of these were alate queens, two were newly mated dealate queens, and two were queens excavated from their incipient nests 2 days after mating. Other individuals performed cyclic CO₂ release with a cycle length of 30 s to 2 min (Fig. 1Bi; $N=25$ ants). Both patterns could be considered to be discontinuous gas-exchange, but we treated them separately because of differences in their cycle lengths and because CO₂ release rarely decreased to zero in cyclic breathers. Our conclusions remained the same when these groups were combined (not shown). Peaks in water loss corresponded to simultaneous CO₂ release for all individuals performing DGC and cyclic gas exchange (Fig. 1Aii,Bii). Lastly, 28 ants released CO₂ continuously with no evidence of cycles (Fig. 1Ci). Overall, the number of individuals using each mode of gas exchange did not differ across the three mating stages (Table 1; 3×3 contingency test, $G=3.96$, $P>0.1$) (Sokal and Rohlf, 1995).

Previous studies have quantified respiratory water loss during cyclic gas exchange by assuming that water loss when

the spiracles are closed represents cuticular transpiration. Respiratory water loss is then calculated by subtracting cuticular water loss from total water loss. This procedure cannot be used for continuous breathers, however, because the spiracles never close completely. We therefore developed a new method to distinguish between cuticular transpiration and respiratory water loss that can be applied to any pattern of gas exchange. We plotted water-loss rate against CO₂ release for each individual using the 5 s time-averaged values over the 30 min respiratory run. Regression analysis of these data

yielded a significant positive relationship in each case (Fig. 2). The slope of each regression line estimates the hygric cost of gas exchange for that individual, i.e. the incremental increase in water loss associated with CO₂ release.

It must be noted that the CO₂ and water-vapor detectors were arranged in series in our respirometer. Under our experimental conditions, a given parcel of air reached the CO₂ sensor about 2 s before it reached the water vapor sensor. Thus, signals from these sensors were slightly out of phase, but correction for this effect (by using a weighted average of consecutive humidity readings) did not significantly affect the calculated regression lines. We therefore used the raw data in our analyses.

The chthonic hypothesis predicts that the ratio of respiratory water loss to CO₂ release is lowest during DGC, that is, the slopes of the regression lines will be lower for individuals performing DGC than for those using other types of gas exchange. Our data did not support this prediction, because the slopes did not vary by pattern of gas exchange (ANCOVA, $F_{2,52}=0.83$, $P>0.4$) or mating stage ($F_{2,52}=0.74$, $P>0.4$), or for the interaction between these two effects ($F_{4,52}=0.19$, $P>0.9$); starting mass was a non-significant covariate ($F_{1,52}=0.09$, $P>0.7$). Across all three patterns of gas exchange, ants lost an average of 1.76 moles of water per mole of CO₂ expelled (Fig. 3).

Respiratory water loss:CO₂ release ratios varied through the gas exchange cycle. Inspection of individual cycles revealed that the ratio (i.e. the slope) was lowest shortly after spiracles opened, when high internal P_{CO_2} would create a large gradient for CO₂ release (Fig. 4). After CO₂ release had peaked, the water loss:CO₂ ratio increased later in the cycle, as internal CO₂ levels declined.

Metabolic correlates of gas-exchange pattern

Metabolic rates differed significantly across patterns of gas exchange (ANCOVA, $F_{2,52}=12.88$, $P<0.0001$; Fig. 5), but not by mating stage ($F_{2,52}=0.096$, $P>0.9$) or the interaction between these two main effects ($F_{4,52}=0.58$, $P>0.6$). *A posteriori*

Table 1. Number of females at each of the three stages of mating that exhibited discontinuous, cyclic, and continuous gas exchange

Female stage	Pattern of gas exchange			Total
	DGC	Cyclic	Continuous	
Unmated	5	8	6	19
Mated pre-excavation	2	8	13	23
Mated post-excavation	2	9	9	20
Total	9	25	28	62

DGC, discontinuous gas-exchange cycle.

For mating stage: unmated, alate female collected from her nest; mated pre-excavation, dealate female captured as she walked from the mating aggregation; mated post-excavation, female excavated from her incipient nest 2 days after mating.

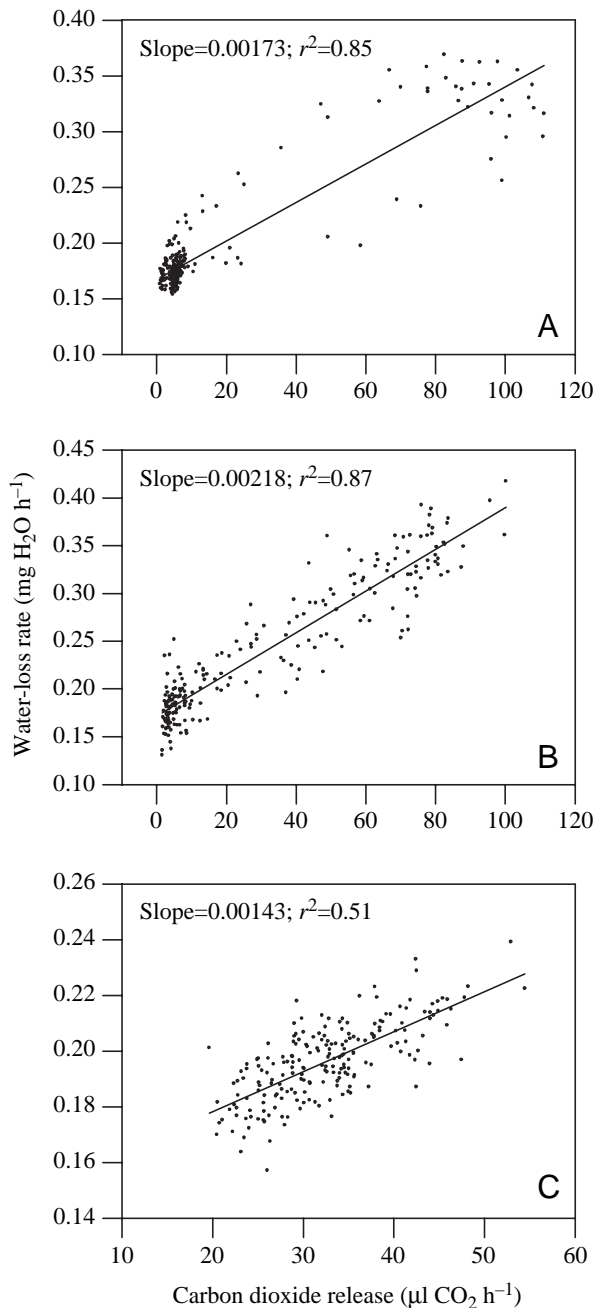


Fig. 2. Plots of water-loss rate vs CO₂ release for the three individuals A–C shown in Fig. 1.

comparisons showed that metabolic rates were lowest for individuals using discontinuous gas exchange, intermediate for individuals using cyclic gas exchange, and highest for individuals using continuous gas exchange (pair-wise Tukey–Kramer test, $P < 0.05$ for all comparisons) (Fig. 5). Starting mass did not affect metabolic rate ($F_{1,52} = 0.030$, $P > 0.8$), presumably because of the limited size range of individuals used in this study.

Discussion

The chthonic hypothesis proposes that discontinuous gas-

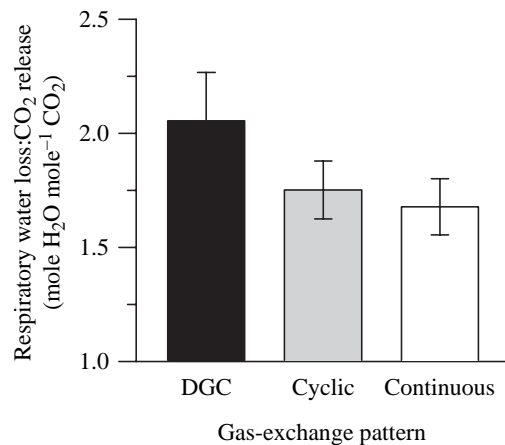


Fig. 3. Ratios of respiratory water loss to CO₂ release, calculated from the slopes of plots such as those in Fig. 2. Data are means \pm S.E.M. For N values, see Table 1.

exchange cycles originated in insects from hypercapnic environments, and were adaptive because they allowed CO₂ to be released while minimizing respiratory water loss (Lighton and Berrigan, 1995; Lighton, 1996, 1998). Alternatively, insects could simply open their spiracles for extended periods to allow gas exchange, but this pattern "... may impose an unacceptable water loss penalty" (Lighton and Berrigan, 1995, p. 528). Although these authors do not make specific predictions, we interpret the chthonic hypothesis to imply that DGC will minimize the ratio of respiratory water loss to CO₂ release. For DGC to be advantageous, this ratio must be lowest in individuals that perform DGC, relative to those using other modes of gas exchange. If this is the case, and if individual variation is repeatable (Marais and Chown, 2003) and has a genetic basis, then DGC may have been maintained and modified by natural selection in other habitats as a means of water conservation. *Pogonomyrmex barbatus* is a burrowing insect from an arid habitat, and water stress is an important factor in the success of new colonies (Johnson, 2000b). Queens of this species therefore provide an excellent test of the chthonic hypothesis.

A critical experimental problem is distinguishing respiratory water loss from cuticular transpiration across all modes of gas exchange. Cuticular transpiration is easily quantified during the closed phase of the gas-exchange cycle in insects performing DGC, and respiratory water loss can be measured by integrating water-loss peaks during the open phase. However, this approach does not work if insects breathe continuously. Our regression method allows us to estimate respiratory water loss no matter which gas-exchange pattern is used. The slope of water loss:CO₂ release plots quantify how much water is lost per molecule of CO₂. The chthonic hypothesis would be supported if these slopes were lowest for individuals that used discontinuous gas exchange.

In queens of *P. barbatus*, the ratio of respiratory water loss to CO₂ release did not vary as a function of gas-exchange pattern. No matter which pattern was used, 1.5–2 molecules of

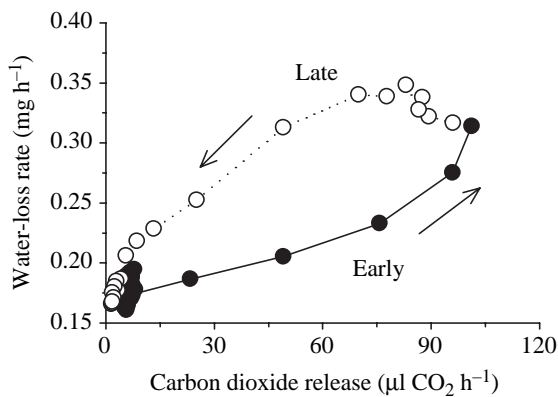


Fig. 4. The relationship between CO₂ release and water loss during a single gas-exchange cycle. Filled symbols indicate data recorded as CO₂ release, which increased early in the cycle, including the C (closed) and F (flutter) phases; open symbols indicate the rest of the cycle, as CO₂ release declined after reaching its peak.

water were lost with each molecule of CO₂. This value is higher than that observed in the carpenter ant *Camponotus vicinus* (Lighton and Garrigan, 1995), presumably due in part to the higher experimental temperature (and therefore higher tracheal water-vapor pressure) in our experiments. More importantly, the absence of differences between ants using different patterns of gas exchange contradicts an important prediction of the chthonic model. We note, however, that DGC could reduce respiratory water loss from *P. barbatus*, if the ants opened their spiracles for shorter periods of time. During the early portion of the open phase, high CO₂ levels in the tracheal system resulted in a steeper gradient for CO₂ flux and a lower ratio of water:CO₂ release (Fig. 4). Late in the open phase, the CO₂ gradient had decreased, but the gradient for water loss would have remained the same. Water loss:CO₂ release ratios were relatively high during this period. If ants had closed their spiracles sooner, the amount of water lost per CO₂ would have been lower. Thus, *P. barbatus* queens appear to open their spiracles longer than they 'should' to conserve water. We conclude that these ants do not regulate gas exchange so as to conserve water, and that DGC do not reduce respiratory water loss relative to other patterns of gas exchange. Similarly, Kanwisher (1966) noted that *Hyalophora* pupae had longer open phases than necessary for effective gas exchange.

One could argue that our results do not directly test the chthonic hypothesis, because our respirometry measures were not performed under hypercapnic conditions. Unfortunately, high CO₂ levels saturate the LI-6262 detector and interfere with its ability to detect water vapor (M. C. Quinlan, personal communication), so we were unable to perform these experiments. We predict that, if such measurements were technically feasible, the ratio of respiratory water loss to CO₂ release would be higher, because the gradient for water loss would be unchanged, whereas the gradient for CO₂ release would decline. Assuming that metabolic rates remain the same, the frequency of cyclic gas exchange would increase as the net

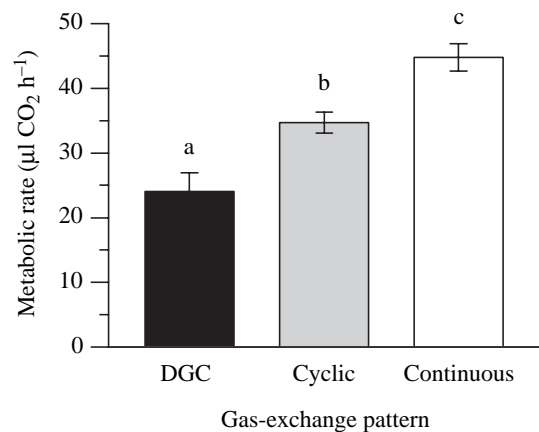


Fig. 5. (A) Metabolic rates of ants using different patterns of gas exchange. Significant differences are indicated by the letters a–c: a<b<c. Data are means \pm S.E.M. For *N* values, see Table 1.

amount of CO₂ lost during the open phase decreased. The more important issue is whether respiratory water loss:CO₂ release ratios would differ among ants using different patterns of gas exchange. We find no reason to think that the chthonic hypothesis would be better supported under hypercapnia, because only the magnitude of the CO₂ gradient would have changed from our acapnic experimental conditions.

We conclude that selection to reduce respiratory water loss in burrowing insects cannot explain the origin of DGC. What then is the explanation? An alternative formulation of the chthonic model proposes that DGC serves to decrease tracheal oxygen levels, thereby increasing the gradient for O₂ uptake when the spiracles open (Lighton, 1996, 1998). Our experiments do not test this idea directly, but we note that CO₂ production and O₂ consumption are tightly coupled *via* aerobic respiration. Both gases are transported during the gas exchange cycle, and gas exchange in other ants occurs primarily by diffusion rather than convection (Lighton and Berrigan, 1995). Thus, O₂ uptake and water loss will also be tightly coupled. In addition, insects in general are still able to maintain their metabolic rates in hypoxic conditions (Hoback and Stanley, 2001) and may lose the ability to perform DGC under hypoxia (Chown and Holter, 2000), when it would be most valuable for O₂ uptake. Discontinuous gas exchange has also been proposed as a mechanism to reduce parasitism (Shelton and Appel, 2001) or to protect insects from oxidative stress by reducing internal O₂ levels (Bradley, 2000), but these hypotheses have not been tested.

Alternatively, DGC may not be adaptive, but may simply arise from the interaction of CO₂ and O₂ gas exchange setpoints and their effects on spiracular regulation (Chown and Holter, 2000). One implication of this hypothesis is that cycle frequency increases with metabolic rate, such that gas exchange becomes continuous at the highest metabolic rates. Consistent with this, continuous breathers had the highest metabolic rates, those exhibiting rapid cycling had intermediate metabolic rates, and ants performing classical discontinuous gas exchange had

the lowest metabolic rates (Fig. 5). This non-adaptive explanation for the origin of DGC does not preclude its retention and modification for adaptive reasons. Discontinuous gas exchange will still aid in water conservation, because water loss is reduced when the spiracles are closed. Even so, DGC is not necessary for survival in arid environments (Lighton and Berrigan, 1995; Quinlan and Lighton, 1999).

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