The Journal of Experimental Biology **198**, 1613–1620 (1995)
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ANT BREATHING: TESTING REGULATION AND MECHANISM HYPOTHESES WITH HYPOXIA

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Accepted 6 March 1995

Summary

Using normoxic and hypoxic flow-through respirometry, we investigated the regulation of the closed-spiracle (C) and the nature of the fluttering-spiracle (F) phases of the discontinuous gas-exchange cycle (DGC) of the ant *Camponotus vicinus*. We predicted that as ambient O_2 concentrations declined, DGC frequency would increase, because C phase duration would decrease (reflecting earlier hypoxic initiation of the F phase) and F phase duration would shorten (reflecting nitrogen accumulation), if convective mass inflow caused by a negative pressure gradient across the spiracles, rather than by diffusion, is the dominant F phase gas-exchange mechanism. C phase duration decreased with declining ambient O_2 concentrations, as predicted. In contrast, DGC frequency decreased and F phase duration increased with decreasing ambient O_2 concentrations. This was opposite to the expected trend if gas exchange in the F phase was mediated by convection, as is generally hypothesized. We therefore cannot disprove that F phase gas exchange was largely or purely diffusion-based. In addition, our data show equivalent molar rates of H_2O and CO_2 emission during the F phase. In contrast, during the open-spiracle phase, the duration of which was not affected by ambient O_2 concentration, far more H_2O than CO_2 was lost. We discuss these findings and suggest that current hypotheses of F phase gas-exchange mechanisms and function in reducing respiratory water loss in adult insects may require revision.

Key words: ant, *Camponotus vicinus*, discontinuous ventilation, hypoxia, metabolic rate.

Introduction

Discontinuous external gas exchange is now known to occur in many adult insects, as well as in the diapausing lepidopteran pupae in which it was first discovered (Kestler, 1978, 1980, 1985; Lighton, 1988*a*,*b*; Lighton, 1990, 1991*a*, 1992; Lighton and Lovegrove, 1990; Lighton *et al*. 1993*a*,*b*; Lighton and Wehner, 1993). Cyclic discontinuities in external gas exchange have been referred to as discontinuous ventilation cycles or DVCs (Lighton, 1988*a*), using the term 'ventilation' *sensu lato* to denote whole-organism gas exchange, as opposed to mitochondrial respiration. However, this term can cause confusion because 'ventilation' usually denotes convective, as opposed to diffusive, gas exchange. To avoid such confusion, we here use the broader expression 'discontinuous gas-exchange cycle' or DGC (other terms in general use, e.g. closed–flutter–open or closed–flutter–ventilate, do not emphasize the cyclic nature of the phenomenon and assign putative spiracular states that may not occur in all cases; Lighton, 1991*a*). Although the existence of the DGC has been documented and partly characterized in several species of insects, little attention has been paid to several important aspects of the DGC. Notable among these are the regulation of the closed-spiracle or C phase and the nature of the fluttering-spiracle or F phase (for reviews of the insect DGC, see Miller, 1981; Kestler, 1985; Slama, 1994; Lighton, 1994).

The C phase is initiated when the insect's spiracles constrict, effectively eliminating or severely reducing external gas exchange. At the start of the C phase, endotracheal O_2 concentrations are close to atmospheric (Levy and Schneiderman, 1966). At the end, endotracheal O_2 partial pressures fall to approximately 3–5 kPa and, in lepidopteran pupae at least, this hypoxia triggers central nervous system (CNS)-mediated fluttering, or rapid partial openings, of the spiracles. The ultimate evolutionary rationale for the DGC is widely hypothesized to be reduction of respiratory water loss (Lighton *et al.* 1993*b*; Lighton, 1994, and references therein; but see Hadley and Quinlan, 1993; Lighton and Berrigan, 1995). Plainly the C phase represents a *reductio ad absurdum* in this respect. Other things being equal, C phase prolongation is the most powerful method of reducing respiratory water loss. The factors that modulate C phase duration are therefore of some significance. In spite of this, direct data on C phase regulation in adult insects are lacking, and we assume that similar modulatory factors are at work in diapausing pupae and adult insects.

A similar situation exists with respect to the F phase. In diapausing pupae, significant negative endotracheal pressures develop by the end of the C phase (Burkett and Schneiderman, 1974; Slama, 1988), and there has also been sporadic

documentation of negative endotracheal pressures in adult insects (Kestler, 1985; Lighton *et al.* 1993*a*). During the F phase, this negative pressure is generally thought to provide the insect with sufficient O_2 for mitochondrial respiration *via* convective mass flow into the tracheal system, which also retards outward diffusion of H_2O, making the F phase an important factor in reducing respiratory water loss rates (see Kestler, 1985). But it stands to reason that diffusion must also play an important role in F phase gas exchange because of the huge (approximately 16–18 kPa) partial pressure gradient for O_2 and the smaller but still significant (approximately 4–6 kPa) gradient for CO_2 (Levy and Schneiderman, 1966). On the basis of these partial pressure gradients, a respiratory exchange ratio (RER) of approximately 0.2–0.3 would be predicted during the F phase, and such RERs have been directly measured (Lighton, 1988*a*). In contrast, removal of ambient air with no inward or outward diffusive component, as predicted by a purely convective model, is equivalent to a tiny reduction in flow rate and as such is difficult or impossible to detect by flow-through respirometry, which contradicts experimental data (Lighton, 1994). Yet strangely, in spite of its importance to recent theoretical analyses of insect gas exchange (Snyder *et al.* 1995), the hypothesis that convection plays a more important role than diffusion during the F phase of adult insects has not been explicitly tested.

Hypotheses concerning the termination of the C phase and the role of diffusion in the F phase can be efficiently, if indirectly, tested by altering ambient O_2 concentrations. Ambient O_2 concentration determines the maximum molar quantity of O_2 available at the start of each C phase, and the consensus C phase termination hypothesis therefore predicts that C phase duration will shorten with declining O_2 concentrations. Ambient O_2 concentration also affects the amount of O_2 transported by convection or diffusion into the tracheae during the F phase. If convection predominates in the F phase, then in accordance with the Hagen–Poiseuille equation (see Loudon, 1989), O_2 ingress rates during hypoxia will rapidly fall to insupportably low levels because increasing nitrogen accumulations will impede negative pressure generation. This will force premature termination of the F phase and thus increase DGC frequency (Snyder *et al.* 1995). Outward diffusion of nitrogen, which would otherwise partially counteract this process (see Kestler, 1985), will not occur in a purely convective model (Snyder *et al.* 1995). In contrast, if diffusion predominates in the F phase, hypoxia must induce an increase in trans-spiracular conductance to compensate for low ambient O_2 concentrations by effectively increasing the area term of the Fick equation. This increased conductance must also elevate CO_2 emission levels during the F phase, thus delaying the hypercapnic initiation of the open-spiracle phase (O phase) and *decreasing* DGC frequency.

We report here the modulation of the C phase and the primary mechanism of the F phase in an ant *Camponotus vicinus* (Mayr), using flow-through respirometry combined with direct mass measurement at O_2 concentrations ranging from ambient (21 %) down to 8 %. In doing so, we test two main hypotheses. First, that the termination of the C phase in adult insects is triggered chiefly by endotracheal hypoxia. And second, that the primary mechanism of O_2 ingress during the F phase in adult insects is inward convection. The first hypothesis is disproved if the duration of the C phase increases or remains constant as ambient O_2 concentration is reduced. The second hypothesis is disproved if the duration of the F phase increases or remains constant as ambient O_2 concentration is reduced, as a result of which DGC frequency will remain constant or decrease (see also Discussion).

Materials and methods

Animals

We collected a subcolony of *Camponotus vicinus* (Mayr) from a riverine forest habitat in Red Butte Canyon, Salt Lake County, Utah, USA. This large-bodied, polymorphic ant is widespread in the American southwest, occurring in habitats ranging from xeric to mesic, with a tendency towards increased cuticular pigmentation in representatives from more mesic habitats. The cuticular pigmentation in our sample was a dark, translucent yellow. We maintained our ants in a culture room at 24±2 °C on a 12 h:12 h L:D cycle, and fed them on sugar water and chopped mealworms *ad libitum*.

Experimental animal preparation

We normally utilized female alates for our research because their large body mass (>100 mg) aided in preparation and because female alates have traditionally been neglected in physiological studies relative to workers, in spite of their far greater importance to colony survival, especially in the crucial foundation stages (Lighton *et al.* 1993*b*; Lighton and Berrigan, 1995; S. Rissing, personal communication).

We prepared an alate for measurement by cold anesthetization, then decapitation. Following decapitation, we carefully sealed the wound with low-melting-point wax and fixed the alate by the extreme tips of its tarsi to a cardboard pad (8 mm×15 mm) saturated with low-melting-point wax.

Decapitation was necessary because direct mass measurements (see below) are exquisitely sensitive to movement-induced artifacts. Decapitation exerted a profoundly calming influence on the ants while leaving their cyclic gas exchange characteristics minimally affected relative to their intact motionless state (see also Lighton, 1992; Lighton *et al.* 1993*a*,*b*; and the Discussion). In particular, all of the ants displayed clearly differentiated closed, flutter and open-spiracle phases, thus yielding the comparative data we required. Only data from ants that showed normal gas-exchange cycles within 30 min of decapitation and that continued to cycle regularly for the duration of the recording were used.

Respirometry and mass measurement

Flow-through CO_2-based respirometry was performed as described elsewhere (Lighton, 1990, 1991*b*), utilizing a Sable Systems TR-3 respirometry system (Sable Systems, 476 E South Temple, Salt Lake City, UT 84111, USA). We modified the respirometry system slightly to vary O_2 concentration by injecting N_2 at a controlled flow rate into the CO_2-free incurrent airstream; the initial O_2 concentration was 20.95±0.02 %. Post-mixing O_2 concentration, measured to a resolution of 0.01 % with an Ametek S-3A O_2 analyzer, was continuously recorded during each experiment.

Mass loss measurements were made simultaneously, as described elsewhere (Lighton *et al.* 1993*b*). Briefly, we modified a Cahn C-32 ultramicrobalance by extending one scale pan with a 150 mm fine-gauge hot-drawn Nichrome hangdown. This hangdown extended through a laser-aligned passage into a chamber (volume ca. 2500 ml) flushed at a rate of 250 ml min^{-1} with CO_2-free air of precisely known O_2 concentration. This rate of flow was independent of O_2 concentration. A much smaller respirometer chamber (volume 40 ml) accommodated a lightweight pan on which the ant was placed. The pan was suspended from the hangdown. Air was withdrawn from the weighing chamber and into the analysis system at a rate of 75 ml min^{-1}, controlled by a Sierra Instruments mass flow controller.

By recording the mass readings at 20-bit resolution and employing digital filtration, we extended the effective resolution of the balance from 100 ng to approximately 20 ng when sampling at 5 s intervals. Rate of mass loss was then obtained by differentiating the mass recordings against time in hours. Mass loss measurements are closely equivalent to direct water loss measurements made by infrared gas analysis (Lighton *et al.* 1993*b*). Consequently the term 'water loss' is used below when referring to our mass loss measurements (see Kestler, 1985; Lighton, 1992, for details). Because we were interested only in comparing water loss rates over the DGC, we subtracted cuticular (defined as C phase) water loss rates from the recordings, leaving only the respiratory water loss component.

During a typical run, we varied O_2 concentration from 21 to 6 % in five discrete steps (21, 15, 12, 10, 8 and 6±0.2 % O_2). At least one, and usually 3–5, DGCs were recorded at each O_2 concentration at which the ant cycled, after a 1–2 DGC equilibration period, depending on DGC frequency at the concentration used. This protocol was chosen because it minimized acclimation periods to hypoxic conditions (see Discussion). A typical recording lasted about 6 h and consisted of simultaneous resordings of rate of CO_2 production ($\dot{V}_{CO_2}$), rate of H_2O loss ($\dot{V}_{H_2O}$) and ambient O_2 concentration data. All experiments were carried out at room temperature (25±1 °C) in an air-conditioned laboratory.

Statistics

DATACAN V software was used for all data acquisition and analysis of the resulting recordings. Summary statistics were analyzed with software written by J.R.B.L. and validated against SYSTAT version IV (Wilkerson, 1988). Means are accompanied by standard deviations (S.D.) and sample sizes (*N*) and are compared using Student's *t*-test. Regressions are calculated by least mean squares, compared using analysis of covariance (ANCOVA) and tested for significance using analysis of variance (ANOVA).

Results

Metabolic parameters

Using the techniques described above, our decapitated ant preparations cycled reliably for 12 h or more (Fig. 1) with a slight reduction in $\dot{V}_{CO_2}$ and DGC frequency as the run progressed, even in normoxic conditions. This 'winding-down' effect was, however, minimal compared with the effects of hypoxia (Fig. 2). We succeeded in producing eight headless preparations (mean original body mass 107.4±7.1 mg, mean head mass 10.6±0.36 mg) that cycled normally within 30 min of decapitation and remained in good condition for the duration of the recording. Typical data from a complete recording are shown in Fig. 2. At an ambient O_2 concentration of 21 %, mean $\dot{V}_{CO_2}$ was 0.178±0.031 ml g^{-1} h^{-1} at 25 °C (*N*=8). This corresponds to a mean standard metabolic rate (SMR, assuming RQ=0.73; Lighton, 1992) of 143.5±32.5 μW.

$\dot{V}_{CO_2}$ decreased by 2.8±0.6 μl g^{-1} h^{-1} for every 1 % decrease in O_2 tension below 21 %. This corresponded to a decrease of approximately 20 % in $\dot{V}_{CO_2}$ as ambient O_2 concentrations decreased from 21 to 8 %. It is premature, however, to regard *C. vicinus* as a metabolic conformer that does not show a critical O_2 concentration (at least in the range 8–15 % O_2) above which $\dot{V}_{O_2}$ and, hence, $\dot{V}_{CO_2}$ are constant. Estimating the magnitude and significance of such effects was complicated by our experimental design, which concentrated on C and F phase regulation on an individual cycle basis only, thus controlling for any larger-scale changes in $\dot{V}_{CO_2}$. Reducing

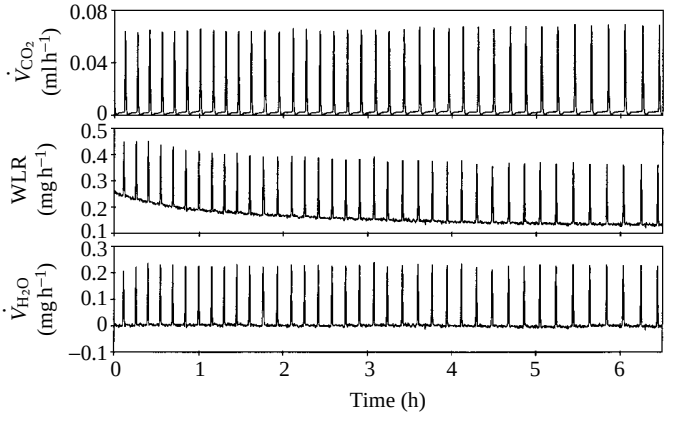

Fig. 1. Typical normoxic recording of $\dot{V}_{CO_2}$ (ml h^{-1}), total water loss rate (WLR; mg h^{-1}) and respiratory water loss rate ($\dot{V}_{H_2O}$, mg h^{-1}). $\dot{V}_{H_2O}$ is derived from the WLR trace by subtraction of the cuticular (interburst) WLR component, using a polynomial baseline correction algorithm. This recording is from a worker ant, mass 30 mg; mean DGC frequency 1.59 mHz, mean $\dot{V}_{CO_2}$ 6.64 μl h^{-1}.

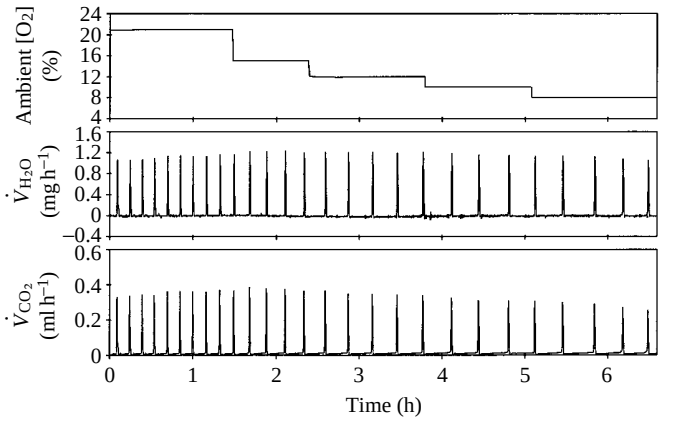

Fig. 2. Typical recording of ambient O_2 concentration (upper trace; percentage), rate of respiratory H_2O loss ($\dot{V}_{H_2O}$; mg h^{-1}) and $\dot{V}_{CO_2}$ (ml h^{-1}) during hypoxic respirometry. Cycling ceased at 6 % O_2 (not shown). Ant mass was 110.4 mg, head mass 9.8 mg, ambient temperature 25.0 °C.

ambient O_2 concentrations from 21 to 6 % in small steps was essential to allow the preparations to equilibrate incrementally from normoxic to hypoxic conditions, but unavoidably introduced a systematic time component that may also have affected SMR. This may account for a significant proportion of the observed decrease in $\dot{V}_{CO_2}$ over each experimental period. We were concerned in this study only with actual $\dot{V}_{CO_2}$ at each ambient O_2 concentration; further data on metabolic regulation *per se* require a randomized design to remove ageing effects.

Discontinuous external gas exchange

As O_2 concentrations decreased, DGC frequency decreased markedly (Fig. 3), falling from 1.7 mHz (DGC duration of 10 min) to 0.8 mHz (DGC duration of 20 min) at O_2 concentrations of 21 and 8 %, respectively. At 6 % O_2, external gas exchange ceased in all but one of our preparations, and the single exception cycled once only. Because at least two cycles are required to yield all of the DGC parameters we analyzed, data from 6 % O_2 were not analyzed further.

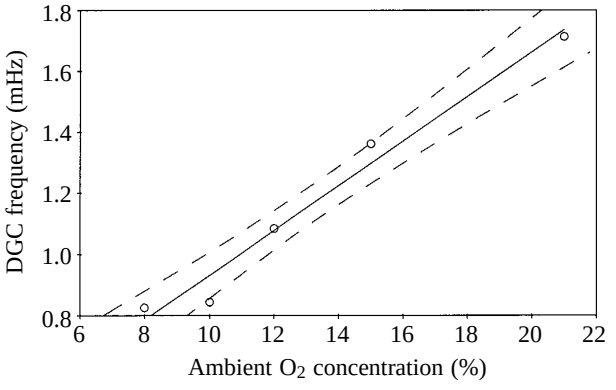

Fig. 3. The effect of ambient O_2 concentration on DGC frequency. Each point is the mean value for eight ants, with 1–5 DGCs per ant at each ambient O_2 concentration. DGCF=0.200+0.0731[O_2], where DGCF is DGC frequency in mHz and [O_2] is ambient O_2 concentration as a percentage. The dashed curves denote the 95 % confidence intervals of the regression. The standard error of the slope is 0.0007. The equation explains 98 % of the variance in DGC frequency (F[1,3]=117.4, P=0.001).

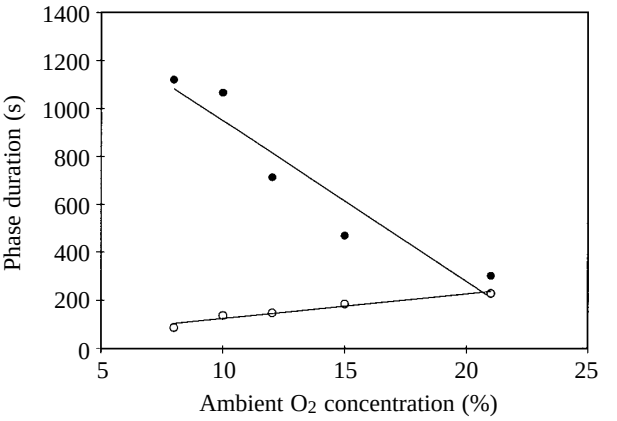

Fig. 4. The effect of ambient O_2 concentration on C and F phase duration (filled and open circles, respectively). Each point is the mean value for eight ants, with 1–5 DGCs per ant at each ambient O_2 concentration. CPD=23.15+10.10[O_2], where CPD is C phase duration in seconds and [O_2] is ambient O_2 concentration as a percentage (r^2=0.95, F[1,3]=52.1, P=0.005). FPD=1621.4+ 67.23[O_2], where FPD is F phase duration in seconds (r^2=0.89, F[1,3]=25.5, P=0.01).

Although DGC duration as a whole increased with decreasing O_2 tension, the three phases of the DGC differed significantly in their responses to hypoxia. O phase duration remained constant at 99.7±4.8 s (P>0.2). The duration of the C phase declined, while increasing F phase duration was entirely responsible for the overall increase in DGC duration with increasing hypoxia (Fig. 4).

Under normoxic conditions, O phase CO_2 emission volumes were 24.4±5.5 μl g^{-1} and H_2O loss was 75.6±20.6 μg g^{-1} (N=112 measurements on eight ants). These are statistically equivalent to previous measurements made on workers of this species (Lighton, 1992; $P\geqslant$0.1), but the somewhat higher H_2O losses and lower CO_2 emissions resulted in a higher ratio of H_2O lost:CO_2 emitted during the O phase (3.07±0.27) than that of worker ants (P=0.05). Hypoxia increased O phase H_2O loss volumes (Fig. 5), but did not affect O phase CO_2 emission volumes or rates, which remained constant at 25.8±10.4 μl g^{-1} and 0.939±0.031 ml g^{-1} h^{-1}, respectively (P>0.3).

Reflecting the decline in overall $\dot{V}_{CO_2}$, C phase $\dot{V}_{CO_2}$ (probably trans-cuticular in origin; see Lighton and Wehner, 1993) decreased slightly but significantly as ambient O_2 concentration declined (Fig. 6). In contrast, F phase $\dot{V}_{CO_2}$ increased markedly as ambient O_2 concentration declined (Fig. 6), in spite of the decrease in overall $\dot{V}_{CO_2}$. As ambient O_2 concentration decreased from 21 to 8 %, rising F phase $\dot{V}_{CO_2}$ and declining overall $\dot{V}_{CO_2}$ combined to elevate F phase $\dot{V}_{CO_2}$ from 31 to 56 % of overall $\dot{V}_{CO_2}$ (Fig. 7).

C phase $\dot{V}_{H_2O}$ was the operational baseline of the H_2O loss

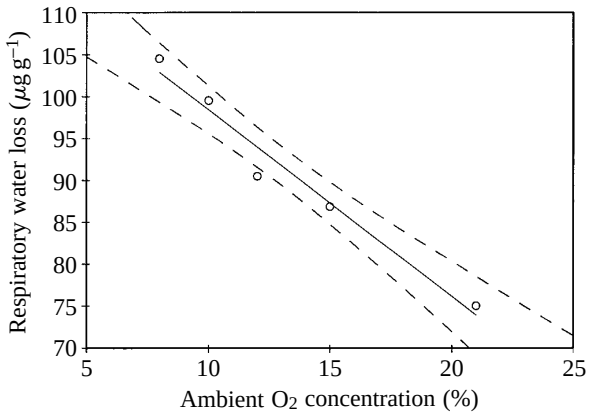

Fig. 5. The effect of ambient O_2 concentration on respiratory H_2O loss during the O phase. Each point is the mean value for eight ants, with 1–5 DGCs per ant at each ambient O_2 concentration. OWL=120.7−2.229[O_2], where OWL is respiratory H_2O loss in $\mu g\,g^{-1}$ and [O_2] is ambient O_2 concentration as a percentage. The dashed curves denote the 95 % confidence intervals of the regression. The standard error of the slope is 0.237. The equation explains 97 % of the variance in respiratory water loss during the O phase (F[1,3]=88.2, P=0.002).

trace and so was not correlated with ambient O_2 concentration. Perhaps because of the greater noise in the $\dot{V}_{H_2O}$ measurements, ambient O_2 concentration exerted no obvious effect on F phase $\dot{V}_{H_2O}$ (P>0.2). However, by subtracting molar C phase $\dot{V}_{H_2O}$ from molar F phase $\dot{V}_{H_2O}$ for each DGC, the noise in the system could be controlled to a greater extent. Under these circumstances, the increase in molar water loss rate during the F phase with increasing hypoxia was significant, in spite of the considerable noise in the signal (Fig. 8). Not surprisingly, in view of the lower noise of the CO_2 measurement system, the

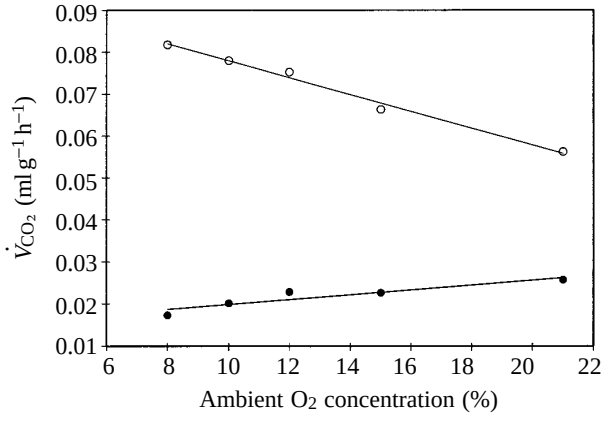

Fig. 6. C phase and F phase $\dot{V}_{CO_2}$ ($C\dot{V}_{CO_2}$, filled circles; $F\dot{V}_{CO_2}$, open circles, ml $g^{-1}\,h^{-1}$) as a function of ambient O_2 concentration. Each point is the mean value for eight ants, with 1–5 DGCs per ant at each ambient O_2 concentration. $C\dot{V}_{CO_2}$=0.0141+0.00058[O_2], where [O_2] is ambient O_2 concentration as a percentage (r^2=0.86, F[1,3]=18.5, P=0.02). $F\dot{V}_{CO_2}$=0.0981−0.0020[O_2] (r^2=0.99, F[1,3]=289, P<0.001).

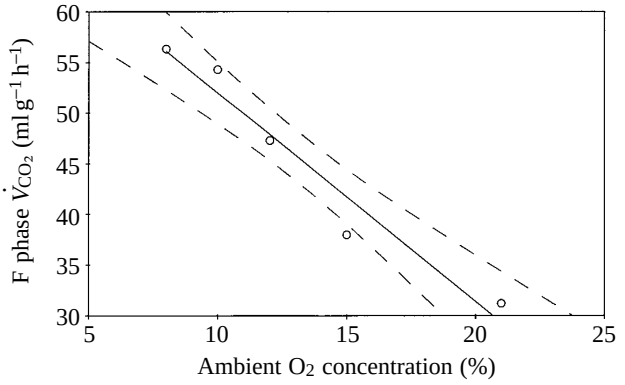

Fig. 7. The effect of ambient O_2 concentration on the percentage of overall CO_2 attained by F phase $\dot{V}_{CO_2}$ (FPC). Each point is the mean value for eight ants, with 1–5 DGCs per ant at each ambient O_2 concentration. FPC=72.6–2.058[O_2], where [O_2] is ambient O_2 concentration as a percentage. The dashed curves denote the 95 % confidence intervals of the regression. The standard error of the slope is 0.275. The equation explains 95 % of the variance in DGC frequency (F[1,3]=56.2, P=0.005).

increase in molar $\dot{V}_{CO_2}$ from the C to the F phase was highly significant (Fig. 8). Expressing rates in molar terms allows direct comparison between H_2O and CO_2 emission rates. As ambient O_2 concentrations decreased, the increase of molar F phase $\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$ relative to C phase levels shared a common slope and intercept (Fig. 8). During the F phase, therefore, molar rates of respiratory CO_2 and H_2O loss were

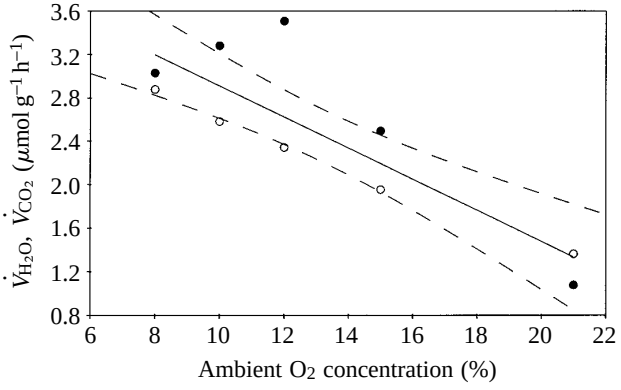

Fig. 8. The effect of ambient O_2 concentration on the increase in $\dot{V}_{H_2O}$ (filled circles) and $\dot{V}_{CO_2}$ (open circles) from the C to the F phase. Each point is the mean value for eight ants, with 1–5 DGCs per ant at each ambient O_2 concentration. $\dot{V}_{H_2O}$=4.93−0.171[O_2], where $\dot{V}_{H_2O}$ is the increase from C phase $\dot{V}_{H_2O}$ to F phase $\dot{V}_{H_2O}$ in μmol $g^{-1}\,h^{-1}$ and [O_2] is ambient O_2 concentration as a percentage (r^2=0.80, F[1,3]=11.7, P=0.04). $\dot{V}_{CO_2}$=3.75−0.116[O_2], where $\dot{V}_{CO_2}$ is the increase from C phase $\dot{V}_{CO_2}$ to F phase $\dot{V}_{CO_2}$ in μmol $g^{-1}\,h^{-1}$ (r^2=0.99, F[1,3]=463, P<0.001). By ANCOVA, the two lines share a common slope [F[1,6]=1.20, P(different slopes)=0.3] and a common intercept [F[1,7]=3.87; P(different intercepts)=0.09]. The common slope is −0.143; the common intercept is 4.342. The common regression line is shown. The dashed curves denote the 95 % confidence intervals of the common regression.

equivalent. This is in contrast to the O phase, where the molar ratio of H_2O:CO_2 lost (at normoxia) is 3.81±0.33, which differs significantly from 1.0 (P<0.001; see also Discussion).

Discussion

Standard metabolic rate

Ant SMRs are usually measured when the ant is in an intermittently active state. This results in a systematic overestimation of nearly twofold compared with inactive ants (Lighton and Wehner, 1993). Decapitated ants provide an excellent means of controlling for activity by virtue of their profoundly passive disposition. In a previous investigation, it was found that the DGCs and SMRs of completely inactive intact and decapitated *Cataglyphis bicolor* of the same body mass were almost exactly equivalent (Lighton *et al.* 1993*a*). This is not surprising in view of the decentralized nature of spiracular control in insects (Miller, 1964, 1981; Kaars, 1981). In the present investigation, the equation of Lighton and Wehner (1993; SMR=1143$M^{0.933}$, where SMR is in μW and body mass M is in g) predicts an SMR of 142.1 μW at 25 °C for an ant of 107 mg live body mass. Our measured SMR of 143.5±32.5 μW at 21 % O_2 and 25 °C is statistically equivalent to this estimate and suggests that the head's contribution to overall metabolic flux is small, at least during inactivity. During intense mandibular activity, overall gas-exchange rates can increase by as much as 31-fold (Roces and Lighton, 1995) and it may be for this reason that CO_2 sensors exist in the heads of some insects (see Miller, 1964). However, the relevance of such sensors to insects at rest is doubtful.

Discontinuous external gas exchange

The O phase

At normoxic levels, our external gas-exchange data are essentially equivalent to those reported previously for decapitated workers of *C. vicinus* (Lighton, 1992), although the DGC frequency and $\dot{V}_{CO_2}$ of our sample of alates was significantly lower, reflecting their larger masses (P<0.05). As noted in previous investigations, far more H_2O than CO_2 is lost during the O phase. Presumably this reflects the fact that most of the CO_2 emitted during each O phase must diffuse from the hemolymph prior to entering the tracheal system and leaving *via* the spiracles, whereas H_2O can evaporate directly and rapidly from the tracheolar respiratory surfaces. In the F phase, however, endotracheal CO_2 concentrations may be high enough to eliminate or reduce this effect (see below).

The significant increase in O phase respiratory water loss with hypoxia is rather interesting (Fig. 5). Reduction of ambient O_2 concentration cannot explain this effect directly. Presumably, the increase in O phase H_2O loss stems both from increased spiracular conductance and from an increase of moist tracheolar surface area as ambient O_2 concentration decreases (see Wigglesworth, 1972). Any effect of hypoxia on the tracheoles must be confined to the O and early C phase because the longest phase of the DGC (F) is characterized by constant endotracheal O_2 concentrations of approximately 3 % (see Levy and Schneiderman, 1966). Conceivably, the increase in O phase water loss rate could also result from more intense ventilatory activity, such as hemolymph pressure pulsations (Slama, 1994), although the existence of such phenomena in ants is questionable (Lighton, 1994).

The C phase mechanism

The hypothesis that the C phase ends, and the F phase is initiated, when endotracheal O_2 tensions fall to a critical point can readily be tested. Assuming that endotracheal volumes and the critical O_2 tension remain constant with decreasing ambient O_2 concentrations, C phase duration should be directly and linearly related to ambient O_2 concentration. The null hypothesis – that ambient O_2 concentration has no effect on C phase duration – is plainly false (Fig. 4).

The F phase mechanism

During the F phase, O_2 enters the insect at a rate equivalent to tissue-level $\dot{V}_{O_2}$ (Levy and Schneiderman, 1966). The exact mechanism of O_2 ingress is, of course, controversial. Three quite distinct possibilities are likely: that the F phase is mediated by convection alone (Snyder *et al.* 1995), by diffusion alone (Kanwisher, 1966) or by a combination of the two (see Kestler, 1985). Which of these three hypotheses is least contradicted by our experimental evidence?

If the F phase is mediated by convection alone, then as ambient O_2 concentration decreases and less O_2 is obtained *via* convection, the F phase should terminate prematurely because of excessive nitrogen accumulation (Snyder *et al.* 1995). On the contrary, F phase duration *increases* markedly with decreasing ambient O_2 concentration (Fig. 4), so our data conclusively disprove this hypothesis. A *completely* convective F phase would, by definition, also lack a measurable diffusive component and, therefore, would not be detectable by flow-through respirometry in the first place (Lighton, 1994). The F phase is, however, easily detected by this technique (Figs 1, 2; see also Lighton *et al.* 1993*a* for validation by direct spiracular observation).

If, however, the F phase has a significant diffusive component, it should be detectable by flow-through respirometry, which is clearly the case (Figs 1, 2). Furthermore, in a diffusive F phase, a decrease in ambient O_2 concentration requires either an increase in the effective diameter of the fluttering spiracles or (more likely) a change in the duty cycle of the fluttering to favor the open rather than the closed position. In any event, this increase in effective spiracular conductance allows sufficient O_2 to enter the tracheal system *via* the shallower concentration gradient to satisfy mitochondrial demands. Simultaneously, it must necessarily permit proportionately more CO_2 (and N_2 and H_2O) to escape from the tracheal system. The release of significant volumes of CO_2 will delay the hypercapnic trigger that initiates the O phase, thus lengthening the F phase. This is precisely what we observed (Figs 4, 5).

The increase in absolute diffusion rates through the spiracles as ambient O_2 concentration decreases can be demonstrated in

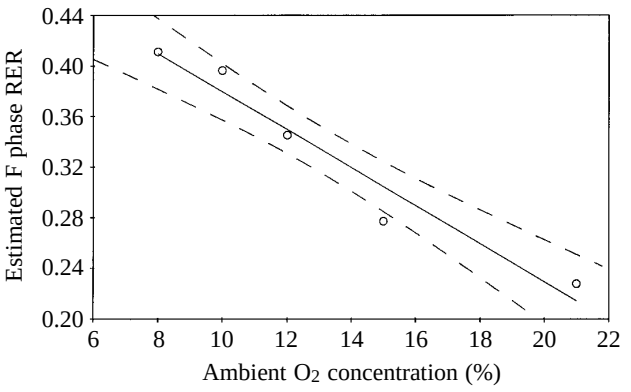

Fig. 9. The effect of ambient O_2 concentration on the estimated respiratory exchange ratio (RER, or short-term $\dot{V}_{CO_2}/\dot{V}_{O_2}$) during the F phase. This RER reflects diffusive O_2 uptake only. Each point is the mean value for eight ants, with 1–5 DGCs per ant at each ambient O_2 concentration. RER=0.530−0.0150[O_2], where [O_2] is ambient O_2 concentration as a percentage. The dashed curves denote the 95% confidence intervals of the regression. The standard error of the slope is 0.0020. The equation explains 95% of the variance in DGC frequency (F[1,3]=56.2, P=0.005).

other ways. For example, direct calculation of the respiratory exchange ratio or RER (short-term $\dot{V}_{CO_2}/\dot{V}_{O_2}$) from typical tracheal *versus* external gas concentrations during the F phase under normoxic conditions, assuming simple diffusion, predicts an RER of approximately 0.2–0.3 (see Lighton, 1988*a*). The estimated RER of our normoxic ants was 0.23±0.05 (N=8; calculated from F phase $\dot{V}_{CO_2}$ and whole-DGC $\dot{V}_{O_2}$ which, in turn, was estimated from whole-DGC $\dot{V}_{CO_2}$ and respiratory quotient, RQ, assumed to be 0.73; Lighton, 1992). As expected in a diffusion-based system, this figure increases as ambient O_2 concentrations drop and the trans-spiracular O_2 concentration gradient becomes shallower, necessitating an increase in spiracular conductance (Fig. 9). Note that our experimentally determined normoxic RER accurately matches the RER estimated for a purely diffusive F phase. Clearly, it is not parsimonious to propose that processes other than diffusion are essential components of the F phase, in the case of *C. vicinus* at least.

To a good approximation, the rates of diffusion of CO_2 and H_2O differ according to the inverse square of their molecular masses (Graham's Law). The ratio of $\dot{V}_{CO_2}/\dot{V}_{H_2O}$ during the F phase should be $(1/\sqrt{44})/(1/\sqrt{18})$, or 1.6, if these rates are purely diffusive and the partial pressure gradients are similar (2–6 kPa CO_2; Levy and Schneiderman, 1966, J. Harrison, personal communication; and 3 kPa H_2O at 25 °C, assuming endotracheal saturation and desiccating external conditions). As shown in Fig. 8, molar F phase $\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$ were equivalent, i.e. their ratio does not differ significantly from 1.0 and is not itself a function of ambient O_2 concentration. The actual ratio of F phase $\dot{V}_{CO_2}/\dot{V}_{H_2O}$ is 0.651±2.235 (N=112 DGCs measured in eight ants), which is obviously not significantly different from 1.6 (t=0.4; P>0.4). There is, nevertheless, a slight tendency for greater emission rates of H_2O than CO_2 during the F phase. The somewhat slower diffusion of CO_2 presumably reflects the fact that CO_2 diffuses from the hemolymph into the tracheal system, whereas H_2O evaporates directly from the respiratory surfaces, before either gas can effuse through the spiracles. However, this effect is not statistically significant. We therefore cannot disprove the hypothesis that diffusion alone adequately explains the observed relative rates of CO_2 and H_2O release during the F phase.

Our findings may not apply to all systems that exhibit a DGC, in particular diapausing lepidopteran pupae, in which negative endotracheal pressure is definitely generated during the F phase (e.g. Slama, 1988; but see Kanwisher, 1966). Moreover, in such pupae, F phase duration decreases with decreasing ambient oxygen concentration (Schneiderman and Williams, 1955; Levy and Schneiderman, 1966), as predicted for a purely convective F phase (Snyder *et al.* 1995). The role of convection in the F phase of adult insects, in contrast, remains unclear. It is known that at least some insects develop negative endotracheal pressures during the F phase (Kestler, 1985; Lighton *et al.* 1993*a*), making inward mass flow eminently feasible. However, we currently have no compelling evidence that convection plays a significant role during the F phase in adult insects; it certainly did not appear to do so in our experiments.

Further, we have demonstrated that molar rates of CO_2 and H_2O loss during the F phase are equivalent within experimental error. The observation that approximately equivalent amounts of H_2O and CO_2 are lost during the F phase calls into question its role in reducing respiratory H_2O loss (see also Lighton and Berrigan, 1995). However, during the O phase the rate of H_2O loss, relative to CO_2 emission, increases approximately fourfold over F phase levels. It follows that the resistance to outward diffusion imposed by spiracular fluttering must diminish the significance of rates of CO_2 diffusion from the hemolymph into the tracheae (slower) *versus* rates of water evaporation from the tracheal and tracheolar surfaces (faster). Consequently, less water is lost than would be the case in the absence of spiracular control, of which the O phase is an extreme example (Lighton *et al.* 1993*b*).

Our findings are rather disconcerting in view of the widely held belief, centered around the role of inward convective air flow during the F phase, that the DGC evolved as a mechanism for reducing rates of respiratory water loss (Snyder *et al.* 1995; and see reviews by Miller, 1981; Kestler, 1985; Lighton, 1994; Slama, 1994). Of course, our findings do not imply that inward convective mass flow is necessarily insignificant in all insects; it certainly appears to play an important role in some cockroaches (Kestler, 1985) and moth pupae (see Slama, 1988). Our data nevertheless strongly suggest that the adult insect DGC is not necessarily dependent on convection during the flutter phase and that the effects of the DGC on respiratory water loss rates are more subtle than has been generally supposed.

This study is also relevant to the field biology of ants, which generally dwell in underground nests. It is in such nests that

most species of ants spend the vast majority of their time. Although we are not aware of any fully satisfactory measurements of O_2 concentrations within ant nests, it is reasonable to suppose that poor ventilation combined with high population densities and soil microbial activity may conspire to produce naturally hypoxic conditions. The effects that such conditions may exert on the external gas exchange characteristics of the nest's inhabitants have been the subject of some speculation (Lighton and Berrigan, 1995) and constitute a worthwhile subject for further research.

We are most grateful to the US National Science Foundation (grants BSR 9006265 and IBN 9306537 to J.R.B.L.) and to the David and Lucille Packard Foundation (Fellowship to J.R.B.L.) for financial support, and to David Berrigan, Jon Harrison, Paul Kestler and Barbara Joos for helpful discussions.

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