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To DGC or not to DGC: oxygen guarding in the termite *Zootermopsis nevadensis* (Isoptera: Termopsidae)

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Summary

The ability of some insects to engage in complex orchestrations of tracheal gas exchange has been well demonstrated, but its evolutionary origin remains obscure. According to a recently proposed hypothesis, insects may employ spiracular control of gas exchange to guard tissues against long-term oxidative damage by using the discontinuous gas-exchange cycle (DGC) to limit internal oxygen partial pressure (P_{O_2}). This manuscript describes a different approach to oxygen guarding in the lower termite *Zootermopsis nevadensis*. These insects do not display a DGC but respond to elevated oxygen concentrations by restricting spiracular area, resulting in a transient decline in CO_2 emission. High internal CO_2 concentrations are then maintained; restoring normoxia results in a transient reciprocal increase in CO_2 emission caused by release of excess endotracheal CO_2. These changes in spiracular area reflect active guarding of low internal O_2 concentrations and demonstrate that regulation of endotracheal hypoxia takes physiological priority over prevention of CO_2 build-up. This adaptation may reflect the need to protect oxygen-sensitive symbionts (or, gut bug guarding). Termites may eschew the DGC because periodic flushing of the tracheal system with air may harm the obligate anaerobes upon which the lower termites depend for survival on their native diet of chewed wood.

Key words: termite, *Zootermopsis nevadensis*, trachea, spiracle, oxygen, symbiont.

Introduction

The discontinuous gas-exchange cycle or DGC of insects and other tracheate arthropods is probably the most unusual and dramatic gas-exchange strategy known among terrestrial animals (see reviews by Kestler, 1985; Lighton, 1996, 1998; Chown and Nicolson, 2004; Chown et al., 2005). Not surprisingly, speculation is rife about its evolutionary genesis. Some favor the hygric hypothesis (reduction of respiratory water loss; see review by Lighton, 1996) and others the chthonic hypothesis (decoupling of O_2 and CO_2 exchange, and enhanced ability to exchange gases in hypercapnic and hypoxic environments; Lighton, 1996). Recently Hetz and Bradley (2005) proposed an ingenious third theory, namely avoidance of O_2 toxicity: the insect guards its interior against the toxic effects of oxygen by restricting access to its tracheal system, particularly in the C (closed-spiracle) phase and later in the F (fluttering-spiracle) phase of the DGC, during which internal P_{O_2} is regulated at <4 kPa (see reviews by Kestler, 1985; Lighton, 1996, 1998; Chown and Nicolson, 2004; Chown et al., 2005). We refer below to any spiracle-control strategies that limit internal P_{O_2} as oxygen guarding.

The Hetz-Bradley hypothesis is tenable if, in fact, the DGC creates a *milieu interieur* with a lower mean P_{O_2} than alternative means of gas exchange. But paradoxically, a delayed oxygen exposure penalty is created while P_{O_2} is maintained at a low level of ca. 4 kPa during the DGC's F phase. During the F phase, thanks to stringent spiracular control, CO_2 escapes at <25% of production rate (Lighton, 1988 and references therein). Periodic flushing of this accumulated CO_2 is therefore necessary, and in fact this flushing – known as the open-spiracle (O) or burst phase – is in large part the operational definition of the DGC. As a side-effect of flushing the CO_2 produced during the C and F phases, in the O phase internal P_{O_2} rises to near-atmospheric levels throughout the tracheal system (Hetz and Bradley, 2005 and references therein).

Obtaining comparative data that bear on the oxygen-guarding hypothesis is difficult. While it is interesting that *Drosophila* endowed with extra superoxide dismutase and catalase copies show a small but significant increase in lifespan (see review by Sohal et al., 2002), (a) the effect is minimal if wild-type, rather than genetically compromised, flies are used in the experiments (*ibid*), (b) overexpression of antioxidants may actually have deleterious effects (*ibid*), and (c) most oxygen toxicity experiments are short-term and make the assumption that evolutionary fitness and individual longevity are coterminous. Thus current oxygen toxicity data are not

helpful in resolving the evolutionary origins of the DGC. Other comparative data are not helpful either. Most insects are capable of extremely high rates of oxidative catabolism (Suarez et al., 1997 and references therein), yet to our knowledge no evidence suggests an interspecific lifespan penalty among the more metabolically active insect taxa. Combined with the fact that the distribution of the DGC is discontinuous across clades of tracheate arthropods (Lighton, 1996; Klok et al., 2002; Chown et al., 2005), we currently lack any evidence, comparative or otherwise, of a relationship between the DGC, longevity and oxygen guarding in an adaptive and operational rather than an inferential context.

Compared to possible avoidance of long-term oxygen toxicity, respiratory adaptations to avoid short-term oxygen toxicity would constitute a powerful assay of oxygen-guarding gas-exchange strategies. Finding evidence of respiratory adaptations that mitigate short-term oxygen toxicity might then bear directly on the adaptive significance of the DGC in ameliorating longer-term oxygen stress. However, short-term oxygen toxicity in normoxia is not known to be a limiting factor for any insect.

The same is not true, however, of symbiotic microorganisms on which some insect taxa depend for access to carbon. The lower termites (class isopoda, families Kalo-, Masto-, Hodo-, Serri- and Rhinotermiditae) are a good example. The guts of these insects contain symbiotic microbes, including cellulose-digesting protists that they depend upon for survival (Cleveland, 1924). These protists, and many other gut microorganisms, are true anaerobes, dying when termites are exposed to oxygen at high concentrations and/or partial pressures (Cleveland, 1925; Messer and Lee, 1989), or to normoxia outside of the protection of the gut environment (Trager, 1934). Exact measurements of the oxygen tolerance of protists in pure culture are not available, but their culture requires an anaerobic medium supplemented with a reducing agent (Yamin, 1978, 1981). Acetogenic spirochetes isolated from the termite gut were able to tolerate transient exposure to low concentrations of O_2, but ceased growth in the presence of $\geqslant$1% O_2 (Graber and Breznak, 2004).

It has been demonstrated that oxygen penetrates up to 200 μm into the lumen of hindguts removed from the termite, and can significantly impact carbon and electron flow in this system (Brune et al., 1995; Tholen and Brune, 2000). The ability of the insect host to impact oxygen concentrations in the gut has yet to be explored, but may provide a demonstrable incentive for stringent oxygen guarding in the short term (minutes to hours), as well as a putative requirement for longer-term oxygen guarding *sensu* Hetz and Bradley (2005). Their gas-exchange strategies are therefore arguably more relevant to the possible evolution of oxygen-guarding respiratory strategies than are those of insects without short- as well as long-term requirements for oxygen guarding.

Few studies have been undertaken on termite gas-exchange kinetics, and those that exist (Shelton and Appel, 2000b, 2001a–c) show no evidence of a DGC. Rather, they show continuous gas exchange, sometimes with brief, more or less regular, increases in CO_2 output. In no case does the CO_2 output fall to the near-zero or very low levels diagnostic of the DGC's C or F phases, and the occasional CO_2 peaks are far smaller than those of the DGC's O phase. Expressing termite gas exchange in terms of coefficient of variation (CV=standard deviation/mean), typical termite CVs are 15–50% (Shelton and Appel, 2000b, 2001a–c) *vs* >200% for a typical DGC (Lighton, 1990). Thus the gas-exchange kinetics of termites are nearly flat-line and the occasional periodicities do not reflect discontinuities of the kind that characterize the DGC. This neither strictly continuous nor even remotely discontinuous form of gas exchange has been widely documented in other insects (Lighton, 1996; Williams and Bradley, 1998; Lighton et al., 2004; see especially Klok and Chown, 2005, who refer to it as 'Cyclic Gas Exchange' or CGE). CGE has also been found in non-hexapod tracheate arthropods (Lighton, 2002; Lighton and Joos, 2002).

We tested two hypotheses regarding oxygen guarding in individual dampwood termites, *Zootermopsis* sp.

First, we hypothesized that if the DGC exerts a significant overall oxyprotective effect, the termites would switch from CGE to the DGC under hyperoxic conditions. This hypothesis assumes that termites are phylogenetically and physiologically capable of generating the DGC. Phylogenetically, we infer that this is the case from the fact that the closely related clade of the roaches (dictyoptera) are capable of expressing the DGC (Kestler, 1985; Marais and Chown, 2003), as are other closely related clades such as the orthoptera (Hadley and Quinlan, 1993). Physiologically, the DGC is very widespread, if patchily distributed, among tracheate arthropods. This argues that most such arthropods are capable of the necessary spiracular control and of responding directly to changes in tracheal oxygen levels (see also Chown and Holter, 2000).

Our second hypothesis addressed the question of oxygen guarding in CGE specifically. It has recently been discovered that insects expressing CGE initially react to elevated ambient oxygen levels by constricting their spiracles (Lighton et al., 2004), suggesting that even in insects expressing CGE, internal P_{O_2} is controlled in a manner analogous to that F phase of the DGC, though at an unknown level. The resulting spiracular constriction causes external CO_2 output to decline briefly on exposure to hyperoxia (Lighton et al., 2004). At this juncture two mutually exclusive oxygen regulation strategies exist. Tracheal P_{O_2} may rise (no oxygen guarding), or it may be guarded at or close to its normoxic level. We hypothesized that if oxygen guarding took place in the termites, prolonged hyperoxia would elevate tracheal P_{O_2} until sufficient outward CO_2 flux occurred in spite of the spiracular constriction required to control tracheal P_{O_2} at its regulated, low level. Because of the resulting higher tracheal P_{CO_2}, oxygen guarding would result in a transient efflux of CO_2 following re-establishment of normoxia. The regulatory alternative – that tracheal P_{CO_2} would be controlled at normal levels and that tracheal P_{O_2} would therefore be permitted to rise – would cause no compensatory rise in CO_2 emission levels after normoxia was re-established. Lighton et al. (2004) did not distinguish

between these post-hyperoxic strategies because their experiment terminated hyperoxic exposure with exposure to anoxia in order to determine maximal spiracular throughput.

Testing the two hypotheses outlined above therefore addresses two issues. First, the capability of termites to switch to the DGC under oxygen stress is evaluated. If they do so, then the DGC is likely related at least in part to minimizing internal oxygen exposure. Second, the ability of termites to control their gas-exchange characteristics in a manner compatible with oxygen guarding is evaluated. If they do so, and if they do not engage in the DGC while doing so, it can be argued that other gas-exchange strategies may guard against oxygen toxicity at least as effectively as the DGC – especially when it is recalled that the DGC periodically flushes the tracheal system with normoxic air.

Materials and methods

Animals

Termites *Zootermopsis nevadensis* Hagen were collected from fallen *Pinus ponderosa* logs in the Chilao Recreation area of the Angeles National Forest, California, USA. They were transferred to polyethylene containers together with chips of host wood, kept damp by occasional spritzing with deionized water, and maintained in darkness at 20–22°C. The animals remained alive and apparently healthy throughout the experiments.

Respirometry

We used a Sable Systems International (SSI; www.sablesystems.com; Las Vegas, NV, USA) TR-2 respirometry system, modified to allow the exchange of ambient air with pure oxygen under computer control. The outline of the system is shown in Fig. 1. Briefly, we exchanged moist, CO_2-free air (21% O_2: 79% N_2) for moist, CO_2-free O_2 for the middle 50 min of a 3 h recording of CO_2 emission rate ($\dot{V}_{CO_2}$).

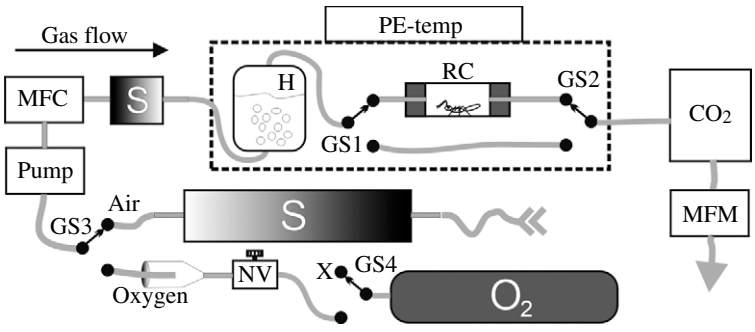

Fig. 1. Conceptual diagram of the experimental setup. PE-temp, Peltier Effect temperature cabinet and controller; H, water reservoir (for air hydration); RC, respirometer chamber; GS1–GS4, gas changeover solenoids under computer control *via* ExpeData; MFC, mass flow control valve and electronics unit; S, scrubber. Large scrubber=Drierite/Ascarite/Drierite for removal of H_2O and CO_2 from normoxic air; small scrubber=Ascarite for removal of trace CO_2 from the selected gas stream. NV, needle valve; CO_2, CO_2 analyzer; MFM, mass flow meter; X, closed (no gasflow). The light emitter and sensor used for detection of activity in the respirometry chamber are not shown. See text for details.

The temperature of the termite and its surroundings was controlled at 15°C by a SSI PELT-5 temperature controller. Data were acquired and analyzed with SSI's ExpeData data acquisition and analysis software, which also controlled baselining and gas switching. In a typical run, the termite was selected at random from the colony, weighed to 0.1 mg (Mettler AG245, Columbus, OH, USA) and placed in a 5 cm^3 respirometer chamber. The chamber was placed in the temperature controlled cabinet, and water saturated, CO_2-free air was diverted past it to establish the zero-CO_2 baseline of the system, using an SSI RM-8 gas flow multiplexer. The ca. 30 ml water reservoir required about 12 h to equilibrate prior to use. A push flow system was used, with a flow rate of 40 ml m^{-1} controlled by a mass flow controller (SSI FC1 controller and Tylan FC-260 mass flow control valve). The mass flow rate at the exit of the system after the CO_2 analyzer was monitored and recorded (SSI SS-3 integrated subsampler and mass flow meter, which also provided the sealed diaphragm pump that pushed the system's gas flow) to detect any leaks or plumbing problems. After 2 min of baseline measurement the air was diverted through the respirometer chamber containing the termite, and the recording was paused for 2 min while the accumulated CO_2 washed out of the system. The recording was restarted, and lasted for a total of just under 3 h including a second baseline at the end. At 65 min into the recording, the air pushed through the system was replaced by water saturated, CO_2-free O_2. After a further 50 min the O_2 was replaced again by water saturated, CO_2-free air. As determined by blank recordings, no change in CO_2 baseline concentration occurred during the gas changes. After the final baseline was taken the recording was repeated, 2–4 times per termite.

Data were analyzed using ExpeData. Respirometry equations were as described previously (Lighton and Turner, 2004). Prior to introduction of O_2, the lowest 30 min of CO_2 emission was located and its mean was used for calculating pre-treatment metabolic rate, expressed as rate of CO_2 production or $\dot{V}_{CO_2}$. After introduction of O_2, the lowest 60 s of CO_2 production was located, from which the low O_2 treatment $\dot{V}_{CO_2}$ was calculated. Next, the lowest section of 15 min, starting at 15 min into the O_2 treatment, was located and the equilibrium O_2 treatment $\dot{V}_{CO_2}$ was calculated. Next, after air was reintroduced, the highest 60 s of CO_2 production within the next 15 min was located, from which the high post-treatment $\dot{V}_{CO_2}$ was calculated. Finally, the lowest section of 15 min, starting 15 min after the introduction of air, was located and the equilibrium post-treatment $\dot{V}_{CO_2}$ was calculated.

Activity was constantly monitored using an SSI AD-1 optical activity detector, and calculated for each of the sections noted above as described in detail elsewhere (Lighton and Turner, 2004). Briefly, any movement by the termite caused fluctuations in detected light levels within the detector. In ExpeData,

the sign-insensitive sum of differences between adjacent data points was calculated. If light levels did not change, this absolute difference sum (ADS) increased very slowly over time owing to random electrical noise in the detection circuitry. If the light level fluctuated significantly because of the termite's movements, the sum increased more rapidly. We therefore employed the rate of change of the ADS *vs* time, i.e. the slope of the linear regression over the selected section of ADS data, as an operational index of activity.

Means are accompanied by standard deviations (S.D.) and sample sizes (*N*). Differences between means were assayed using Student's *t*-test, or by analysis of variance (ANOVA), with significance set at $P<0.05$. Where needed for statistical comparisons, points were digitized from published graphs with ExpeData's DigitEyes image analysis utility. Regressions were calculated by the least-squares method and compared by analysis of covariance (ANCOVA). Regression significance was evaluated with the *F* test. Regression slopes are accompanied by standard errors (S.E.M.).

Results

We obtained data from 21 termites, mean mass 0.0387±0.0187 g (range 0.0122–0.0807 g), mean temperature 15.00±0.04°C. Their mean metabolic rate prior to O_2 treatment, expressed as $\dot{V}_{CO_2}$, was 9.87±4.21 μl h^{-1}. A typical experimental recording is shown in Fig. 2.

Body mass significantly affected $\dot{V}_{CO_2}$ in all stages of the experiment (Fig. 3), explaining nearly 75% of metabolic rate (MR) variance in the case of pre-O_2 treatment $\dot{V}_{CO_2}$. The shared mass scaling exponent of body mass *vs* $\dot{V}_{CO_2}$ across all treatments was 0.688. All of the termites remained intermittently active throughout all runs.

After switching to pure O_2, the termites showed a pronounced transient decline in $\dot{V}_{CO_2}$ to 25.8±3.4% (± S.E.M.) of pre-treatment $\dot{V}_{CO_2}$ (Fig. 4). This ~75% decline was highly significant ($F_{[1,19]}=56.7$, $P<10^{-8}$). Expressed factorially, the decline (to 0.31±0.06 of pre-treatment levels) was not correlated with body mass ($P>0.2$). (Note that factorial statistics were calculated as the simple quotient of two data sets, whereas percentages or ratios not explicitly defined as factorial were calculated from the slope of the relevant linear

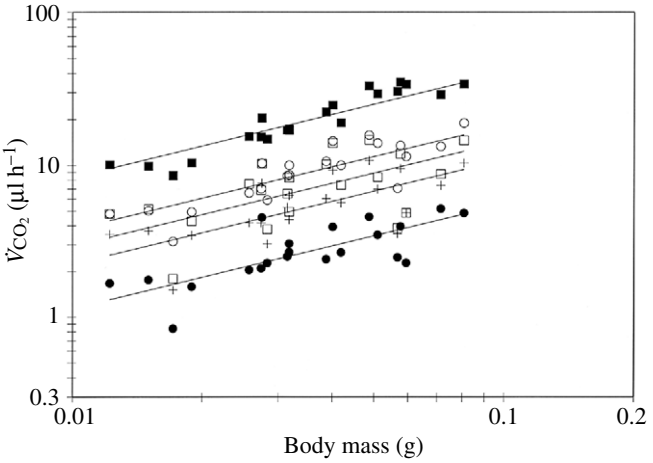

Fig. 3. Mass scaling of rate of CO_2 emission ($\dot{V}_{CO_2}$ in μl h^{-1}) in *Zootermopsis nevadensis* prior to hyperoxia (open circles), shortly after exposure to hyperoxia (closed circles), ca. 30 min after exposure to hyperoxia (open squares), shortly after re-establishment of normoxia (closed squares) and ca. 30 min after re-establishment of normoxia (crosses); $N=21$, all at 15°C. By ANCOVA, all lines share a common slope or mass scaling exponent of 0.688($F_{[4,95]}=0.59$; $P>0.3$). The intercepts, however, differed significantly ($F_{[4,99]}=109.7$, $P<10^{-12}$). The mass scaling coefficients are, respectively, 89.7, 27.0, 69.8, 197.7, and 53.1 with $\dot{V}_{CO_2}$. in units of μl h^{-1}.

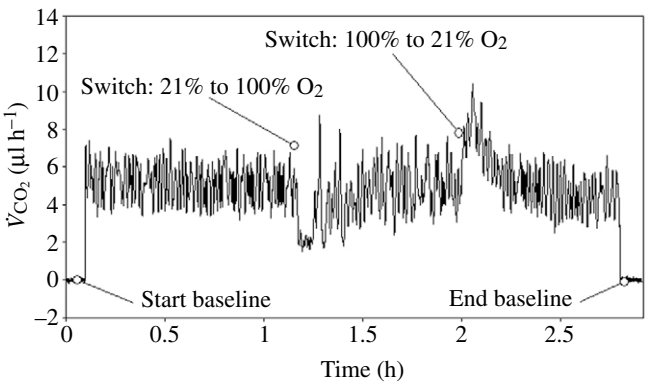

Fig. 2. Typical recording, showing the response of a termite to hyperoxia (switch to 100% O_2; see indicator on graph) with a subsequent return to normoxia (switch to 21% O_2; see indicator on graph) at 15°C. The oxygen guarding response, resulting in a transient decline of CO_2 emission rates, is clearly visible, as is a compensatory transient emission of CO_2 after re-establishment of normoxia. The body mass of this termite was 0.0122 g. Baselines are shown at the start and end of the recording. See text for details.

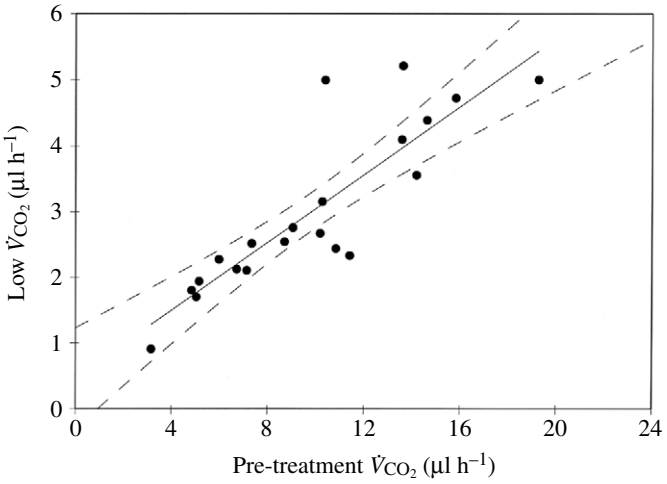

Fig. 4. The relation between equilibrium, pre-treatment $\dot{V}_{CO_2}$ (μl h^{-1}) and the lowest 60 s of hyperoxic $\dot{V}_{CO_2}$ in the dampwood termite *Zootermopsis nevadensis*; $N=21$, at 15°C. Pre-treatment $\dot{V}_{CO_2}$ explains 75% of hyperoxic $\dot{V}_{CO_2}$ variance; hyperoxic $\dot{V}_{CO_2}=0.46+0.258\pm0.034$ (pre-treatment $\dot{V}_{CO_2}$), $F_{[1,19]}=56.7$, $P<10^{-6}$.

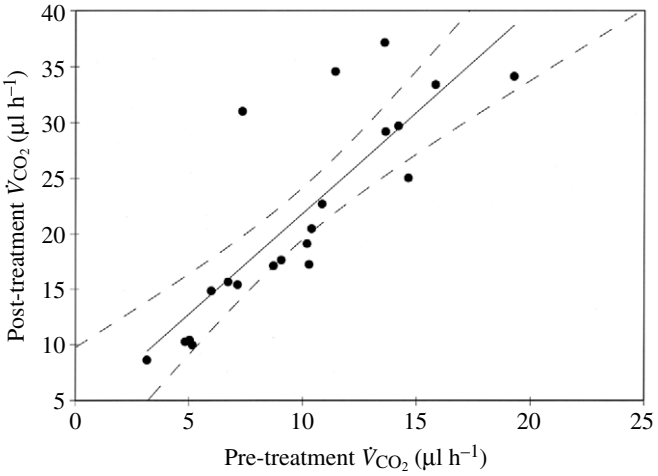

Fig. 5. The relation between equilibrium, pre-treatment $\dot{V}_{CO_2}$ (µl h^{-1}) and the equilibrium $\dot{V}_{CO_2}$ during prolonged exposure to hyperoxia in the dampwood termite *Zootermopsis nevadensis*; N=21, at 15°C. Pre-treatment $\dot{V}_{CO_2}$ explains 79% of equilibrium $\dot{V}_{CO_2}$ variance during hyperoxia; equilibrium hyperoxic $\dot{V}_{CO_2}$=0.33+0.802±0.094 (pre-treatment $\dot{V}_{CO_2}$), $F_{[1,19]}$=72.9, $P<10^{-6}$. The slope is marginally significantly below 1.0 (P<0.05). See text for details.

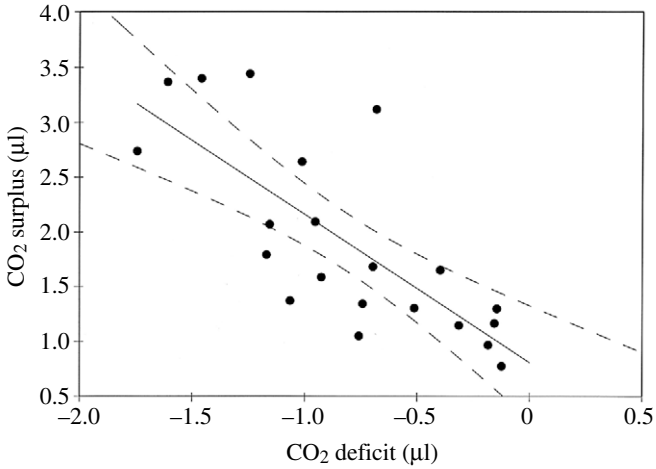

Fig. 7. The relation between the volume of CO_2 withheld immediately following exposure to hyperoxia (CO_2 deficit), and the volume (µl) of CO_2 emitted immediately after restoration of normoxia (CO_2 surplus), N=21, at 15°C. CO_2 deficit explains 58% of CO_2 surplus variance. CO_2 surplus=0.81–1.353±0.264 CO_2 deficit; $F_{[1,19]}$=26.2, $P<10^{-6}$. The slope of this relation does not differ significantly from 1.0 (P=0.1). See text for details.

regression, which will be equivalent to the factorial statistic only where the intercept is exactly zero.) Within approximately 15 min of continued hyperoxia $\dot{V}_{CO_2}$ returned to 80.2±9.4% (± S.E.M.) of normal (pre-treatment) levels, a value barely significantly less than pre-hyperoxic levels (P=0.05). Activity indices were unchanged (P>0.4).

After switching back from pure O_2 to air, the termites showed a dramatic, transient increase in $\dot{V}_{CO_2}$ to 181.8±27.3% (± S.E.M.) of pre-treatment $\dot{V}_{CO_2}$ (Fig. 5). This increase was highly significant ($F_{[1,19]}$=44.4, $P<10^{-6}$). Expressed factorially, this increase (to 2.31±0.63 times pre-treatment levels) was not correlated with body mass (P=0.35). No increase in the activity index occurred; in fact the activity index was slightly but not significantly lower than pre-treatment levels during the CO_2 output peak. Within 15 min after the peak, $\dot{V}_{CO_2}$ dropped to 57.6±5.0% (± S.E.M.) of pre-treatment levels (Fig. 6), a significant decrease ($P<10^{-3}$). The activity index during post-treatment recovery did not differ significantly from pre-treatment levels (P>0.3). Within ca. 45 min (by the start of the next pre-treatment $\dot{V}_{CO_2}$ measurement) $\dot{V}_{CO_2}$ had returned to pre-treatment levels.

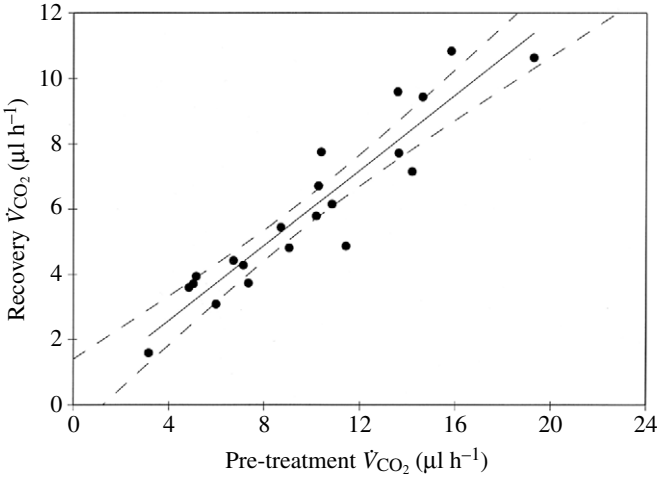

Fig. 6. The relation between equilibrium, pre-treatment $\dot{V}_{CO_2}$ (µl h^{-1}) and the equilibrium recovery $\dot{V}_{CO_2}$ after returning to normoxia (following prolonged exposure to hyperoxia) in the dampwood termite *Zootermopsis nevadensis*; N=21, at 15°C. Pre-treatment $\dot{V}_{CO_2}$ explains 87% of recovery $\dot{V}_{CO_2}$ variance during hyperoxia; recovery $\dot{V}_{CO_2}$=0.28+0.576±0.050 (pre-treatment $\dot{V}_{CO_2}$), $F_{[1,19]}$=133, $P<10^{-6}$. See text for details.

Clearly, $\dot{V}_{CO_2}$ dropped after exposure to hyperoxia and then increased after re-initiation of normoxia. By integrating the $\dot{V}_{CO_2}$ data *vs* time relative to pre-treatment $\dot{V}_{CO_2}$, a measure of total CO_2 output deficit (during hyperoxia) and CO_2 output surplus (during re-initiation of normoxia) in volume units can be made. Expressed in this fashion, variance in CO_2 output deficit explains nearly 60% of the variance in CO_2 output surplus (Fig. 7). If the two were exactly equal, the slope of the relation would be 1.00; the measured slope [1.35±0.26 (± S.E.M.)] does not differ significantly from 1.00 (P>0.1). The intercept in this relation [0.80±0.57 µl (± S.E.M.)] does not differ significantly from 0.

Discussion

Metabolic rates

Because of persistent low levels of activity in our sample of termites, their metabolic rates are somewhat higher than would have been the case had they remained inactive. For the same

species of termite Shelton and Appel (2000b) report a $\dot{V}_{CO_2}$ at 15°C of 5.91 μl h^{-1} (calculated from data in their table 1), which is lower than the value we obtained, though not significantly so (t=0.92; P>0.35).

We were surprised that our sample of termites remained active despite being placed in water-saturated air at a relatively low temperature. It is possible that placing our termites in water-saturated air may have stimulated a higher level of activity than would have been the case in dry air, analogous to the findings of Gibbs et al. (2003) in hygrophilic drosophilids. Dampwood termites are never naturally exposed to completely dry air (as used by Shelton and Appel, 2000b), and exposure to it may have elicited a 'freeze' response equivalent to that reported by Gibbs et al. (2003) in xerophilic drosophilids. This argument is reinforced by the fact that Shelton and Appel (2000b) report almost exactly the consensus $\dot{V}_{CO_2}$ expected for a motionless, flightless insect of that mean mass (5.56 μl h^{-1}, assuming a Q_{10} of 2.0, mean mass 0.0334 g; Lighton et al., 2001).

Exposure to dry air may also explain the semi-cyclic gas exchange reported by Shelton and Appel (2000b) in 60% of their sample of dampwood termites at 15°C. We did not observe consistent CO_2 emission cyclicity in our sample of termites. A useful index of the degree of spiracular control displayed by an insect at rest is the coefficient of variation or CV of $\dot{V}_{CO_2}$, defined as $\dot{V}_{CO_2}$ S.D./mean. The CV of $\dot{V}_{CO_2}$ can vary from near 0% for continuous gas exchange to >200% in insects displaying the DGC (Lighton, 1990). The CV of $\dot{V}_{CO_2}$ in our sample of termites was 15.7±4.5%, which is equivalent to the 'acyclic' *Z. nevadensis* termites described by Shelton and Appel (2000; 18.1% at 15°C, digitized from their Fig. 3, t=0.5, P>0.4). The two laboratories used similar SSI TR-2 systems with similar flow rates, making their CV figures directly comparable. Shelton and Appel (2000b) did not distinguish between the $\dot{V}_{CO_2}$ values of dampwood termites undergoing cyclic and acyclic gas exchange; it is possible that their acyclic termites displayed $\dot{V}_{CO_2}$ values more similar to those of our sample of termites.

Clearly, the possible interactions between ambient gas hydration and gas exchange in termites merit further study. We nevertheless consider water-saturated gases and cool temperatures to have been the ideal ambient conditions for our experiments. They more closely approximated the presumptive natural conditions within the galleries of dampwood termite colonies, and eliminated osmotic stress as a possible complicating factor when interpreting our results.

Oxygen guarding: Mechanisms

We have shown that termites undergoing continuous gas exchange actively reduce spiracular area under hyperoxic challenge, as has been shown in other insects (Lighton et al., 2004). New to this investigation, we have demonstrated that maintaining internal hypoxia on a prolonged basis (tens of minutes) is accompanied by extended internal hypercapnia, and thus that the homeostatic imperative of maintaining internal hypoxia outweighs that of maintaining elevated levels of internal CO_2. The internal hypercapnia to which we refer is inferred both from the transient depression of $\dot{V}_{CO_2}$ following exposure to hyperoxia, and from the transient elevation of $\dot{V}_{CO_2}$ following the re-establishment of ambient normoxia. It is especially clearly shown by the hypercapnic load, which is released in its entirety on re-exposure to normoxia (Fig. 7). Thus, insects that do not engage in the DGC defend internal hypoxia, and not internal P_{CO_2}, against changes in ambient gas concentrations.

Our investigation raises some questions. Primarily, what are the actual endotracheal P_{O_2} and P_{CO_2} levels during hyperoxia? Our results show that oxygen guarding exists, but do not quantify its effectiveness. On *a priori* grounds it is logical to assume that the limiting factor in oxygen guarding is the degree to which internal hypercapnia can be tolerated. In this respect, quantifying responses to graded hyperoxia would be interesting. We know from studies of the DGC (see reviews mentioned in the Introduction) that in insects expressing the DGC, internal P_{CO_2} can rise to at least 4–5 kPa before the O phase is initiated, yielding an equivalent partial pressure gradient across the spiracles in normal air. Higher P_{CO_2} values are unlikely on physiological grounds. Even in normoxia the partial pressure gradient of CO_2 is dwarfed by that of O_2, which is ca. 16–18 kPa at sea level (*ibid*.). This imbalance means that continuous gas exchange cannot be based on steady-state diffusion alone, even in normoxia. Outward diffusion of CO_2 must therefore be augmented by periodic increases in spiracular area (as in CGE) or by bulk transfer mechanisms such as intermittent convection. Although this field is starting to attract attention (Lighton et al., 2004; Klok and Chown, 2005), it remains seriously understudied. The kinetics of the DGC are far better understood than those that underlie less dramatic but arguably more widespread gas-exchange strategies in tracheate arthropods.

Oxygen guarding and the DGC

Our findings are not incompatible with those of Hetz and Bradley (2005). However, in the case of our termites (and likely in the cases of other continuous gas exchangers; Lighton et al., 2004), internal hypoxia presumably comparable to that of the F phase of the DGC is maintained and actively guarded on a continuous basis, without periodically flooding the tracheal system with O_2 as occurs during the open-spiracle or O phase of the DGC (Hetz and Bradley, 2005). Although the requisite long-term P_{O_2} measurements remain to be made in insects with continuous gas exchange, it cannot be shown on the basis of present knowledge that continuous gas exchange imposes a tissue O_2 exposure penalty relative to the DGC when taking into account periodic flushing with normoxic air – a definitional requirement of the DGC.

This begs the question of the ultimate selective pressures responsible for maintaining a low internal P_{O_2} in insects. The Hetz-Bradley 'oxidative damage hypothesis' (see Chown et al., 2005 for discussion) is certainly worthy of consideration and, though defined in terms of the DGC, may be applicable *sensu lato* to continuous gas exchangers as well in light of our

findings. Hetz and Bradley (2005), however, reasonably limit their consideration of oxidative damage to the insect's somatic tissues.

We suggest that internal hypoxia in insects may have selective correlates that have not previously been considered. These are brought into clear conceptual relief by the subject animal of the present investigation. Termites cannot survive without their symbiotic gut flora, which are therefore truly symbiotic rather than merely commensal (Cleveland, 1924, 1925). Termite gut flora, especially the protists essential for cellulose digestion, are able to thrive only in hypoxic environments (Yamin, 1978, 1981; Trager, 1934). Thus their host animals confer not only the benefit of a continuous supply of cellulose, but that of a hypoxic environment. Thus the question is whether internal hypoxia preadapted termites for inhabitation by protists, or whether the maintenance of internal hypoxia evolved as a response to the selective benefits created by normoxia-sensitive symbionts – among the termites, and perhaps also in other insect taxa.

The success of termites throughout most of this planet's ecosystems is a clear example of the importance of symbiotic microorganisms. Benefits conferred by microorganisms in other insect taxa may not be as dramatic, but are likely to be significant, as is the case with higher vertebrates (Hooper and Gordon, 2001; Fooks and Gibson, 2002; Guarner and Malagelada, 2003). We suggest that the selective advantages of gut bug guarding, or supplying an hypoxic environment to gut symbionts, may in part explain tracheal oxygen guarding, and may be a hitherto unrecognized adaptive factor in the evolution of insect gas exchange. For example, it is possible that an initial selective pressure for gut bug guarding – maintaining internal hypoxia in order to facilitate the growth of anaerobic gut microorganisms – may have been among the selective factors that allowed the evolution of the degree of spiracular control required for the DGC. It is even possible that the periodic flushing with outside air characteristic of the DGC might, far from maintaining strict internal hypoxia, actually constitute a mechanism in some insect taxa for modulating the growth of oxygen-sensitive micro-organisms, whether intra- or extracellular.

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