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# Control of discontinuous gas exchange in *Samia cynthia*: effects of atmospheric oxygen, carbon dioxide and moisture

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## SUMMARY

**The evolution of discontinuous gas exchange (DGE) in insects is highly controversial. Adaptive hypotheses which have obtained experimental support include a water savings mechanism for living in dry environments (hygric hypothesis), a reduction in oxidative damage due to a high-performance oxygen delivery system (oxidative damage hypothesis), and the need for steep intratracheal partial pressure gradients to exchange gases under the hypercapnic and/or hypoxic conditions potentially encountered in subterranean environments (chthonic hypothesis). However, few experimental studies have simultaneously assessed multiple competing hypotheses within a strong inference framework. Here, we present such a study at the species level for a diapausing moth pupa, *Samia cynthia*. Switching gas conditions from controlled normoxic, normocapnic and intermediate humidity to either high or low oxygen, high or low moisture, elevated carbon dioxide, or some combination of these, revealed that DGE was abandoned under all conditions except high oxygen, and high or low gas moisture levels. Thus, support is found for the oxidative damage hypothesis when scored as maintenance of DGE. Modulation of DGE under either dry or hyperoxic conditions suggested strong support for the oxidative damage hypothesis and some limited support for the hygric hypothesis. Therefore, this study demonstrates that the DGE can be maintained and modulated in response to several environmental variables. Further investigation is required using a strong-inference, experimental approach across a range of species from different habitats to determine how widespread the support for the oxidative damage hypothesis might be.**

Key words: trachea, respiration, metabolic rate, cyclic gas exchange, ventilation rate.

## INTRODUCTION

Most tracheated arthropods regulate gas exchange by means of spiracles, which are subject to neuromuscular control. For arthropods, the most distinct and curious pattern of gas exchange is discontinuous gas exchange (DGE), which is typically observed by recording the $CO_2$ emission of an individual animal. The DGE is characterised by prolonged periods of no gas exchange [a closed spiracle (C) phase], a fluttering period in which spiracles open and close rapidly and limited gas exchange is detectable [a flutter (F) phase], and finally a large open period in which gases are exchanged rapidly [an open spiracle (O) phase], in some instances aided by abdominal or muscular contractions. The DGE pattern is thought to have evolved independently at least five times in the Insecta (Marais et al., 2005) (see also Klok et al., 2002) and several hypotheses (mostly adaptive, but with one non-adaptive explanation) (Chown and Holter, 2000) have been proposed to explain the origin and maintenance of DGE (Lighton, 1996; Chown et al., 2006). The major adaptive hypotheses are: the hygric hypothesis – DGE acts as a water savings mechanism; the chthonic hypothesis – DGE facilitates gas exchange under hypoxic and/or hypercapnic conditions; the chthonic–hygric hypothesis, a combination of the two former hypotheses – but specifically that DGCs are an adaptation to reduce respiratory water loss under conditions of either hypoxia or hypercapnia or both; and the oxidative damage hypothesis – DGE is a consequence of the need to remove accumulated $CO_2$, followed by the demand for reduced oxygen toxicity (Hetz and Bradley, 2005).

Although many tests of these hypotheses have been undertaken, they remain controversial, largely because of considerable differences in the approach adopted. For example, in the case of the significance of respiratory modulation of water loss, including the modulation of elements of the DGE [the hygric hypothesis originally proposed by Buck and Keister (Buck and Keister, 1955)], much of the work has been experimental, examining one or a few species. Frequently, these studies have concluded either that respiratory water loss is too insignificant to be the subject of selection, that DGE is abandoned just when it is most required, or that the predictions of the hygric hypothesis are not supported (e.g. Hadley and Quinlan, 1993; Lighton and Berrigan, 1995; Chown and Holter, 2000; Hetz and Bradley, 2005; Lighton and Turner, 2008) (reviewed by Lighton, 1998; Chown, 2002; Quinlan and Gibbs, 2006). By contrast, explicitly comparative studies, examining either respiratory water loss more generally, or the likelihood that DGE may be responsible for modulating it, have tended to support the idea that respiratory water loss is sufficiently significant to be the focus of natural selection (Zachariassen et al., 1987; Addo-Bediako et al., 2001) and that DGE may contribute to its modulation (e.g. Duncan and Byrne, 2000; Duncan and Dickman, 2001; Chown and Davis, 2003). In consequence, considerable controversy continues to surround the significance of respiratory water loss in insects and the extent to which DGE may be responsible for its modulation (e.g. Gibbs and Johnson, 2004; Lighton and Turner, 2008).

Although this controversy must partly be based on genuinely dissimilar ways in which insects from different taxa respond to

various environmental conditions (Lighton, 1998), and the likelihood that several factors may select simultaneously for DGE (Chown, 2002), it is probably also the consequence of the way in which tests of the alternative hypotheses have been undertaken: more specifically, the predominant, single hypothesis testing approach. While the importance of a strong inference approach to testing alternative hypotheses in evolutionary physiology is now widely appreciated (e.g. Huey et al., 1999; Angilletta et al., 2006; Deere and Chown, 2006; Bozinovic et al., 2007), and has been repeatedly called for in the context of the hypotheses proposed to explain the origin and maintenance of DGE (Chown et al., 2006; Quinlan and Gibbs, 2006), only two studies have adopted such an approach, with only one of these controlling for phylogenetic non-independence (Marais et al., 2005; White et al., 2007). Both investigations have been comparative, rather than experimental, illustrating the ongoing difficulty of adopting a strong inference approach in experimental work, despite the acknowledged importance of experimental manipulation for addressing questions in this field (Lighton, 2007). Indeed, most experimental studies tend to address only a single hypothesis and are frequently characterized by the manipulation of only a single environmental variable thought to be significant in influencing DGE (e.g. Chown and Holter, 2000; Lighton and Turner, 2008), often neglecting potential confounding effects of the treatment. For example, since temperature has a positive, non-linear relationship with saturation deficit, experimental manipulation of temperature may inadvertently alter relative humidity (RH), thus confounding the interpretation of the effects of temperature on DGE modulation. Insect spiracles are sensitive to the hydration state of both the animal and the surrounding atmosphere. Partially dehydrated tsetse flies (*Glossina fuscipes*; Diptera, Glossinidae) show increases in spiracular control (Bursell, 1957), whereas in *Aedes* (Diptera, Culicidae) spiracles can respond to the relative humidity of ambient air (Krafsur, 1971). Consequently, it seems possible that insects that have been shown to abandon DGE at high temperatures (e.g. Chappell and Rogowitz, 2000) may have responded to humidity changes or to interactions between humidity and temperature, rather than to temperature alone (see also Chown, 2002).

Here, to address this notable absence of a strong inference, multi-factorial experimental approach in the field we examine the predictions of several alternative hypotheses proposed to explain DGE by manipulating ambient oxygen partial pressure, carbon dioxide partial pressure and relative humidity. Specifically, we test simultaneously the predictions of the hygric, chthonic, hybrid and oxidative damage hypotheses using a three-way study design, separating the effects of atmospheric humidity, partial pressure of carbon dioxide ($P_{CO_2}$) and oxygen ($P_{O_2}$) on the modulation of DGE. We make four predictions for whole-organism responses to address the relative importance of each of the primary adaptive hypotheses (Table 1). (1) If the hygric hypothesis is the key driver of DGE, DGE should be present under low humidity but should be abandoned at high humidity since it is no longer required for conservation of respiratory water. Moreover, if present, the DGE should be modulated such that a positive relationship should be found between DGE cycle frequency and ambient relative humidity (i.e. longer closed phases in drier conditions). Specifically, DGE should be tightly regulated under dry conditions. (2) If the chthonic hypothesis accounts for DGE, the effects of experimental manipulation of $P_{O_2}$ or $P_{CO_2}$, but not ambient humidity, should be evident. Specifically, under high $P_{CO_2}$ and/or low $P_{O_2}$ DGE should be evident, but under low $P_{CO_2}$ and/or high $P_{O_2}$ DGE should be abandoned. Humidity should make little difference to DGE prevalence. (3) The chthonic–hygric hypothesis makes the prediction that $P_{CO_2}$ and $P_{O_2}$ as well as atmospheric humidity changes will influence DGE modulation. Specifically, under low relative humidity conditions and high $P_{CO_2}$ and/or low $P_{O_2}$ DGE will occur. In all other cases DGE should be abandoned. (4) The oxidative damage hypothesis makes the prediction that the alteration of atmospheric water and $P_{CO_2}$ should have little effect on DGE prevalence. When ambient $P_{O_2}$ is reduced below intra-tracheal $P_{O_2}$, however, DGE should be abandoned. Varying ambient humidity should also have little or no effect on DGE occurrence or on the modulation of cycle frequency. Furthermore, DGE cycle frequency should decline at higher $P_{O_2}$.

Table 1. Predictions for discontinuous gas exchange hypotheses addressed in the present study

| Hypothesis | $\Delta H_2O$ | $\Delta P_{CO_2}$ | $\Delta P_{O_2}$ |
|---|---|---|---|
| DGE present? | | | |
| Hygric | Yes | * | – |
| Chthonic | * | Yes | Yes |
| Chthonic–hygric | Yes | Yes | Yes |
| Oxidative damage | * | * | Yes |
| DGE modulation: C-phase duration | | | |
| Hygric | – | ≈ | ≈ |
| Chthonic | ≈ | + | – |
| Chthonic–hygric | – | + | – |
| Oxidative damage | ≈ | ≈ | + |

DGE, discontinuous gas exchange.
For DGE present, the asterisk indicates DGE occurrence might not change or is not specifically required for the hypothesis. For modulation of DGE: C-phase duration, – indicates a reduction in C-phase duration; ≈ indicates no change; + indicates an increase in C-phase duration with the respective changes in gas conditions.

## MATERIALS AND METHODS

### Animals

*Samia cynthia* Drury 1773 (Lepidoptera: Saturniidae) were obtained as eggs from local breeders and reared on *Ailanthus altissima*. Caterpillars were maintained at 20°C (9 h:15 h L:D photoperiod) to induce diapause (Pammer, 1966). Two weeks after pupation they were stored in a refrigerator at 6°C and 70–80% RH for the duration of the experiments. Diapausing pupae were used in respirometry experiments 8–12 weeks after pupation.

To overcome problems associated with manipulations of $CO_2$, such as potential failure of the infra-red gas analyzer to detect DGE under high experimental $CO_2$ treatment conditions, and also to distinguish accurately flutter (F) from open (O) and closed (C) phases (Wobschall and Hetz, 2004), we make use of simultaneous $CO_2$ and intratracheal pressure recordings.

### Respirometry

Flow-through respirometry of carbon dioxide release rates was undertaken in *Samia cynthia* pupae using a two channel respirometry system equipped with a two channel differential infra-red carbon dioxide analyzer (URAS 14, range 0 to 100 p.p.m., ABB, Frankfurt, Germany). The air was first driven through the reference cell before it entered the analyzer cell of the URAS 14. The output signal of the $CO_2$ analyser was converted into nmol $min^{-1}$ $g^{-1}$ fresh live body mass using the following equation:

$$\dot{M}_{CO_2} = \frac{f_{CO_2} \times P_{hyd} \times \dot{V}_{gas}}{R \times T \times m_{animal}} , \quad (1)$$

where $\dot{M}_{CO_2}$ is the carbon dioxide release rate in nmol $min^{-1}$ $g^{-1}$; $f_{CO_2}$ is the fractional concentration of carbon dioxide (signal from

the analyzer); $P_{hyd}$ is the hydrostatic pressure within the air stream (kPa); $\dot{V}_{gas}$ is the flow rate of gas in ml min$^{-1}$ STPD (standard temperature and pressure dry); $R$ is the gas constant (8.314 J mol$^{-1}$ K$^{-1}$); $T$ is temperature (283.16 K); and $m_{animal}$ is the animal fresh live body mass (g).

Because pilot studies showed that the duration of respiratory cycle at 6°C was 4–8 h, 10°C was chosen as a compromise between a temperature that maintained diapause and one that gave a shorter cycle duration (Hetz, 2007). The temperature of the respirometry chambers were therefore maintained at 10°C by a custom-made Peltier-cooling unit with a computer-controlled feedback (accuracy ±0.1°C). Two individuals were measured simultaneously using 144 ml min$^{-1}$ flow rates produced by two Woesthoff pumps (5KM3O3/a-F; H. Woesthoff GmbH, Bochum, Germany). The pump action of the gas-mixing pumps generated slight pressure fluctuations in the flow-through setup that were recorded by the pressure transducers. Therefore, pressure dampers (silicon gloves located inside 5 l glass jars) were inserted after the Woesthoff pumps to buffer the baseline of the differential pressure transducers. One pump was used to control the gas composition while the second pump was used to split the airstream and drive it at equal flow rates through the two respirometry chambers (see Fig. 1). The internal dead space of the respirometry chamber (after factoring in the average pupal size) was estimated to be ~12 ml. Thus, at the flow rate used the chamber would be flushed approx 12 times per minute resulting in adequate temporal resolution.

The experimental treatments included changes of ambient humidity, ambient oxygen and ambient carbon dioxide levels. Humidity was set by bubbling the air through two moistening chambers held at 4.7°C or 9.7°C with a temperature controlled water jacket to give either 70% or 97% RH, respectively. Low relative humidity (<5% RH, 'dry' treatment) was obtained by bypassing the moistening chambers and directing the airstream through a column of Drierite (W. A. Hammond, Xenia, OH, USA) and silica gel. Oxygen and carbon dioxide partial pressures were modified using the Woesthoff gas-mixing pumps. The reference air (from outside the building) was bubbled through two 20 l tanks each containing 21 of 5 mol l$^{-1}$ NaOH and Drierite in order to remove ambient $CO_2$ and water. Nitrogen or oxygen was mixed with the reference air to obtain hypoxic or hyperoxic gas mixtures. Dry carbon dioxide, oxygen and air, or carbon dioxide, nitrogen and air were mixed to obtain hypercapnic and hypocapnic gases, respectively, according to the following equation for binary gas mixtures (see Eqn 1 for an example of setting the oxygen partial pressure):

$$P_{O_2} = \frac{(f_{O_2}^{gas1} \times \dot{V}_{gas1}) + (f_{O_2}^{gas2} \times \dot{V}_{gas2})}{\dot{V}_{gas1} + \dot{V}_{gas2}} \times (P_h^{air} - P_{H_2O}), \quad (2)$$

where $P_{O_2}$ is the oxygen partial pressure (kPa); $f_{O_2}^{gas1}$ is the fractional concentration of oxygen in gas1 (0 for $N_2$, 0.209 for air and 1 for $O_2$); $\dot{V}_{gas1}$ is the flow rate of gas1 (ml min$^{-1}$); $P_h^{air}$ is the hydrostatic pressure [air pressure and perfusion pressure (usually less than 0.01 kPa)] of air flowing through the respirometer (in kPa; readout from pressure sensor in URAS 14); and $P_{H_2O}$ is the water vapour pressure (kPa) calculated from the Magnus equation (Magnus, 1844) for the saturation water partial pressure above a water surface with given temperature $T$ (°C):

$$P_{H_2O} = 0.61078 \times 10^{\left(\frac{7.5T}{237.3T}\right)}. \quad (3)$$

For setting hypoxic and hyperoxic gas mixtures for gas1 usually air was used whereas for gas2 pure oxygen was used to set hyperoxic and pure nitrogen was used to set hypoxic gas mixtures. To set hypercapnic carbon dioxide mixtures while leaving the oxygen partial pressure at ambient level the above gas mixture was fed into another Woesthoff pump in order to set the partial pressures of two gases (e.g. oxygen and carbon dioxide) independently.

The oxygen partial pressure was verified by directing the airstream from the outlet of the carbon dioxide analyzer into an Ametek S-3II/A oxygen analyser (Applied Electrochemistry, USA). All data were sampled at 0.25 s frequency and recorded at 1 s intervals to a computer hard disk using a customized recording program in TurboLab 4.03 (Bressner Technology, Germany).

### Tracheal pressure

Tracheal pressure is a tool to monitor the action of the spiracles (Wobschall and Hetz, 2004), especially if no carbon dioxide release can be recorded (e.g. at high ambient $CO_2$ concentrations). We therefore recorded the tracheal pressure with the help of a differential pressure transducer. One port of the pressure transducer was

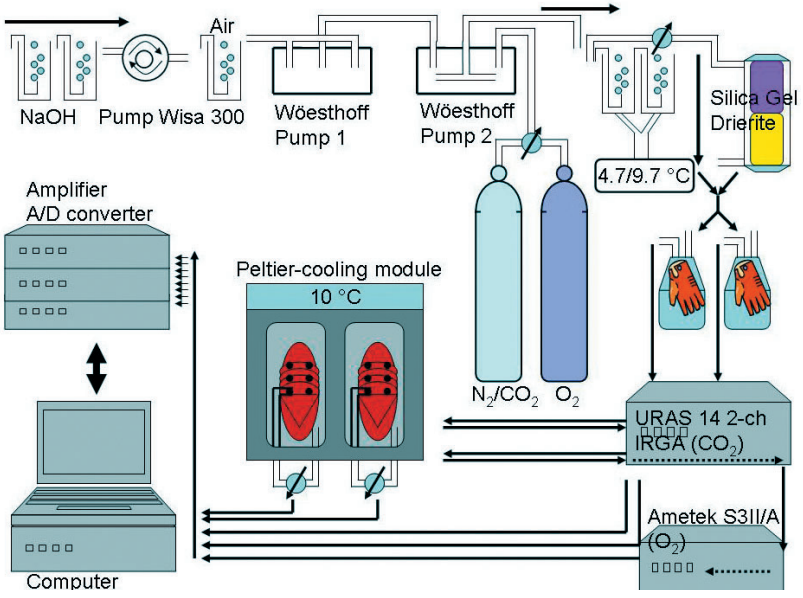


Fig. 1. Schematic diagram of the experimental setup employed during these experiments. Direction of airflow to the gas analyser is indicated by solid arrows. (For further details see Materials and methods.)

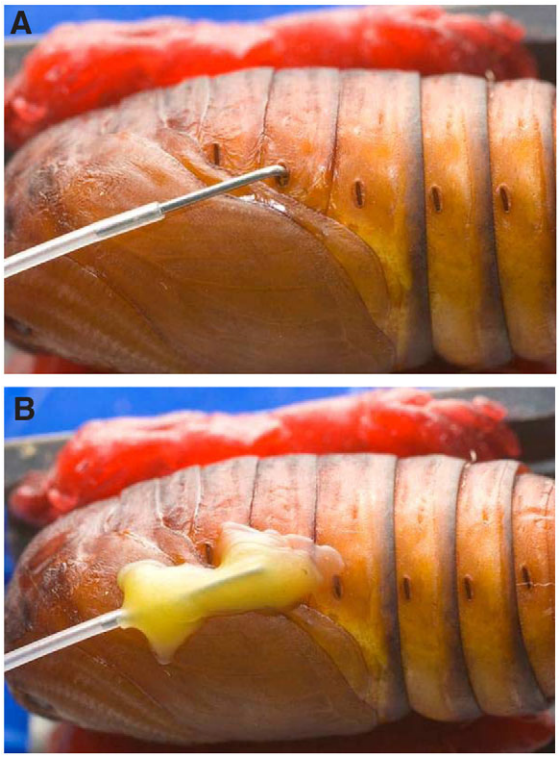


Fig. 2. A *Samia cynthia* pupa during the tracheal intubation process. (A) The steel intubation tube is inserted into the spiracle prior to sealing with wax, and (B) just after wax sealing.

connected to a short piece of polyethylene tubing inserted into the tracheal system *via* a single spiracle (Fig. 2). The surrounding of the spiracle was sealed with wax. The other port of the transducer opened into the respirometry chamber. Pressure was measured with a precision micro pressure transducer (SenSym SDXL010, SensorTechnics, Puchheim, Germany). The pressure sensor electrically consisted of a Wheatstone bridge that was driven with a precision voltage reference of 6.000 V (REF02, Burr Brown, Texas Instruments, Dallas, TX, USA) buffered with a precision operational amplifier (OPA177, Burr Brown). Differential voltage output of the pressure sensor bridge was amplified with a differential amplifier (INA131, Burr Brown). This corresponded to a pressure difference of 504.6 Pa $V^{-1}$. An offset voltage was added to set the pressure range for measuring sub-atmospheric pressure in the tracheal system from –2200 to +317 Pa. Accuracy and stability over 24 h was within 5 Pa (<0.25%) of full scale. This low baseline drift was mainly achieved by attaching the pressure sensor device to the Peltier-cooled respirometer. The pressure recordings were used to verify presence or absence of DGE under altered gas conditions.

All electrical signals from the devices (temperature, tracheal pressure, carbon dioxide release rate, ambient oxygen partial pressure) were amplified with custom-made voltage amplifiers and sampled to the hard disk of a computer *via* an A/D-board (DT2821, Data Translation, Marlboro, MA, USA) and the use of the TurboLab data acquisition software (Stemmer, Puchheim, Germany).

### Analyses

Experimental runs were started with a control period in which pupae were held under controlled standard gas conditions consisting of normoxia (20.9 kPa), normocapnia (<0.1 kPa) and normal rearing humidity (70% RH). The control part of the experiments lasted for at least 10 to 12 h. Subsequently, the gas conditions were switched to treatment conditions (see Table 2 for range of treatments) and gas exchange measured for an additional 6–10 h. The raw data were processed offline using a custom made script running under the TurboLab data acquisition software (Stemmer, Puchheim, Germany) (for details, see Hetz, 2007). Individual pupae were assessed for DGE presence or absence based on the rate of $CO_2$ release ($\dot{M}_{CO_2}$) and intratracheal pressure recordings under control and several experimental treatment conditions. The number of individual pupae that showed DGE under control and treatment conditions relative to the total number of individuals recorded was then used to generate contingency tables which were tested for significance using Fisher's exact test which is robust for small and unequal sample sizes (Zar, 1997). The contingency tables were used to compare DGE presence/absence for predictions of the hygric, oxidative, chthonic and hybrid hypotheses by inference on the basis of the predictions made in the Introduction and Table 1. Thereafter, for all treatments in which individual pupae maintained DGE, we undertook analyses examining potential modulation of individual components of the DGE. Analyses of $\dot{M}_{CO_2}$ variables were undertaken as pair-wise responses of treatment *versus* control conditions within individuals.

Table 2. Contingency table for the presence or absence of discontinuous gas exchange patterns under altered environmental gas conditions

| | DGE present | | DGE absent | | | |
|---|---|---|---|---|---|---|
| Treatment | Control | Treatment | Control | Treatment | $\chi^2$ | *P*-value |
| Hyperoxia | 7 | 5 | 3 | 5 | 0.83 | 0.325 |
| *Hypoxia* | **10** | **0** | **1** | **10** | **17.36** | **<0.0001** |
| Dry | 6 | 6 | 0 | 0 | ‡ | >0.99 |
| Wet | 8 | 8 | 0 | 0 | ‡ | >0.99 |
| Hypercapnia | 3 | 0 | 5 | 8 | 3.69 | 0.100 |
| Hypercapnia 12 kPa $P_{CO_2}$ | **6** | **0** | **1** | **7** | **10.50** | **0.002** |
| Hypoxia + hypercapnia | 2 | 0 | 4 | 6 | 2.4 | 0.227 |
| Hypoxia + hypercapnia + dry | **5** | **0** | **4** | **9** | **6.92** | **0.015** |
| Hypoxia + hypercapnia + wet | **4** | **0** | **5** | **9** | **5.14** | **0.041** |

Hyperoxia=40 kPa $P_{O_2}$; hypoxia=4 kPa $P_{O_2}$; dry=<5% RH; wet=98% RH; hypercapnia=4 kPa $P_{CO_2}$ unless otherwise specified. Significant values based on Fisher's exact test are given in bold type and compare the ratio of individuals showing DGE (discontinuous gas exchange) in the control period (as a proportion of the total tested) relative to the ratio of individuals showing DGE in the treatment period (i.e. in the hyperoxia treatment we ask if the 7/10 pupae showing DGE observed during the control period significantly different from 5/10 pupae showing DGE during treatment conditions).

‡, $\chi^2$ values are infinitely small and cannot be computed in these cases owing to contingency table analysis limitations (i.e. some variation is required). However, it is clear that these groups are not significantly different and one can assume *P*-values>0.99.

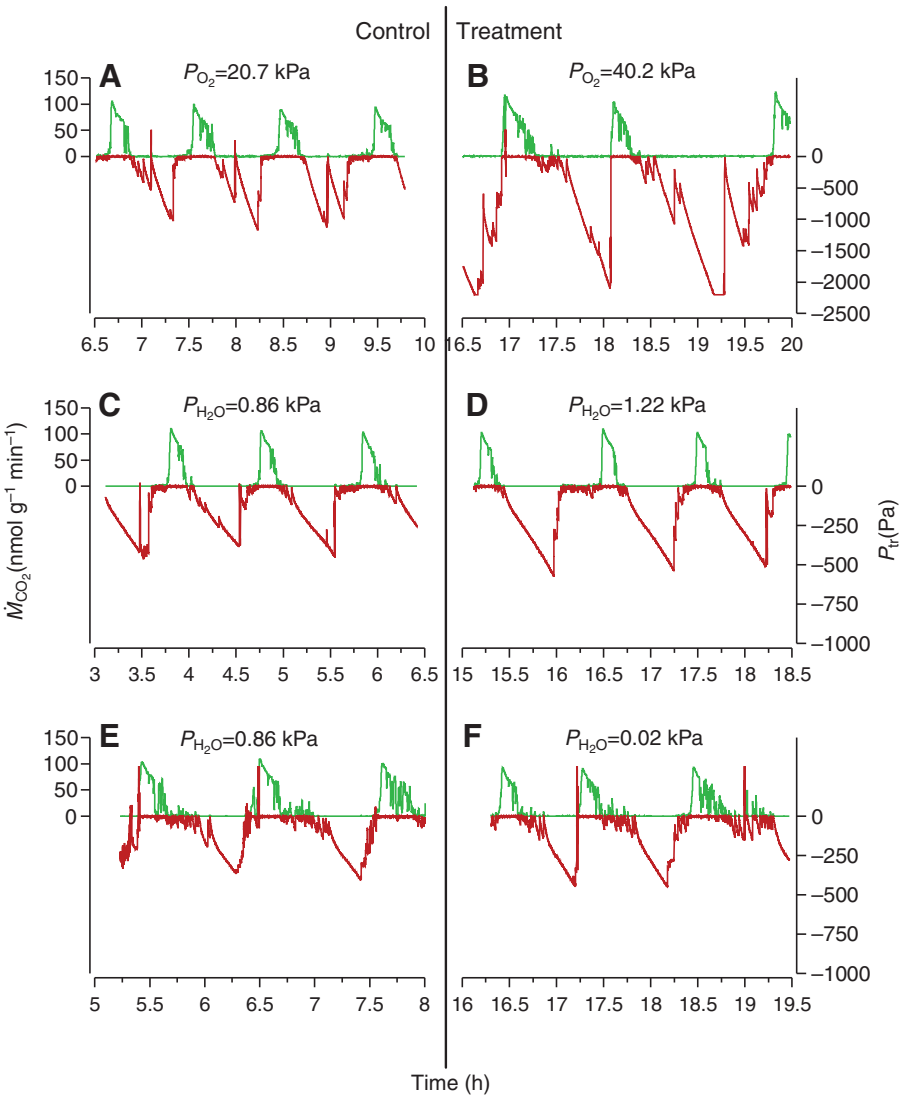


Fig. 3. Examples of traces of carbon dioxide release ($\dot{M}_{CO_2}$, green) and intratracheal pressure ($P_{tr}$, red) under experimental conditions in which DGE was maintained. Hyperoxia, wet and dry experiments during control (A,C,E) and treatment (B,D,F) conditions in *Samia cynthia*. (For full details of conditions during control and treatment periods see Materials and methods.)

For these analyses, data from individuals for which three consecutive bursts were available under both control and treatment conditions were used. We extracted phase duration, average $\dot{M}_{CO_2}$ and the average maximum $\dot{M}_{CO_2}$ for each phase from each of the treatment groups. All parameters were averaged over three bursts within a control or treatment period, and these were compared using a paired *t*-test between control and treatment periods.

## RESULTS

### Presence of discontinuous gas exchange

Discontinuous gas exchange was maintained only under hyperoxic ($P_{O_2}$ 40 kPa), dry (0% RH) and wet (95% RH) conditions (Table 2; Figs 3 and 4). By contrast, no DGE was observed when pupae were subjected to hypoxia ($P_{O_2}$ 4 kPa) or hypercapnia ($P_{CO_2}$ 4 or 12 kPa). Furthermore, any combination of increased moisture, hypercapnia and hypoxia also resulted in the abolition of DGE. Changes in the observed proportion of DGE *versus* non-DGE patterns in moth pupae, compared between control and treatment periods, were significant for hypoxia, hypercapnia, hypoxia+hypercapnia+dry, and hypoxia+hypercapnia+wet treatments (Table 2). In other words, the proportion of individuals showing DGE relative to non-DGE changed under these experimental conditions. However, the hypoxia + hypercapnia treatment effect was non-significant relative to non-DGE patterns in the contingency table (Table 2). Other continuous patterns of gas exchange not constituting a true DGE were present in all treatments except dry and wet conditions suggesting some regulation of gas exchange pattern in these latter two treatments.

### Modulation of discontinuous gas exchange

For the experimental treatments for which DGE was maintained (i.e. hyperoxia, dry and wet; Fig. 3), pair-wise *t*-tests were undertaken to investigate modulation of DGE and phase components [closed (C), flutter (F) and open (O) phases] between treatment and control periods. Statistically, analyses of mean $\dot{M}_{CO_2}$ suggested little modification of any phase of the DGE during hyperoxia, high moisture or low moisture, although there was a substantial though non-significant reduction in the O-phase during hyperoxia (Table 3).

It does appear, however, that some of the marginal effects in our study were probably a consequence of relatively small sample sizes and relatively large inter-individual variation in DGE parameters under treatment conditions. For example, in the hyperoxic treatment (Table 4), the only significant effect was an increase in O-phase duration. However, although duration of the F-phase decreased by nearly 50%, this was non-significant and should probably be attributed to a type II statistical error. Similarly, C-phase duration, although ~1.5 times longer during hyperoxia,

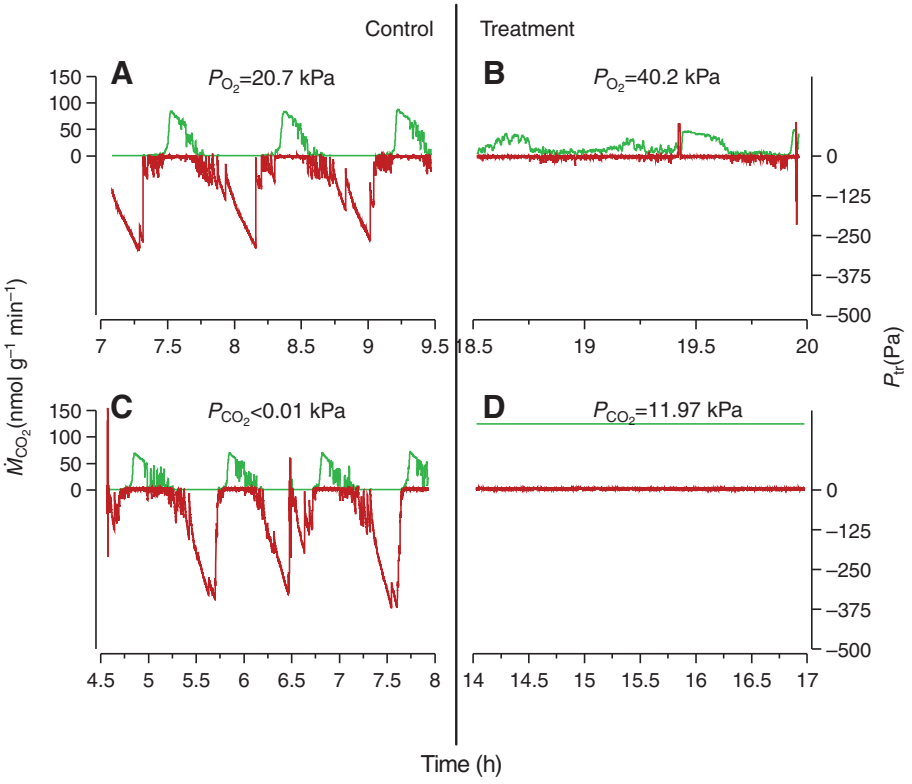


Fig. 4. Examples of traces of carbon dioxide release ($\dot{M}_{CO_2}$, green) and intratracheal pressure ($P_{tr}$, red) under experimental conditions in which discontinuous gas exchange (DGE) was not maintained. Hypoxia and hypercapnia experiments during control (A,C) and treatment (B,D) conditions in *Samia cynthia*. (For full details of conditions during control and treatment periods see Materials and methods.)

was marginally non-significant (Table 4; specifically, total DGE duration increased nearly twofold yet this variation was statistically non-significant). A *post-hoc* power test for the C-phase duration result suggests that β=0.401 (α 2-tailed=0.05). If this effect size remained constant as more individuals were added, at least 30 individuals would be required to raise the power to 0.8, which might be considered a suitably rigorous, although somewhat arbitrary, level of statistical power (Di Stefano, 2003).

For average duration of each phase, hyperoxia resulted in non-significantly longer C-phases, non-significantly shorter F-phases, and significantly longer O-phases (Table 4). Across other treatment groups, no effects were detected on average duration which might be indicative of DGE burst modulation (Table 4). For the total DGE duration (i.e. the time taken for C, F and O phases combined), wet and dry treatments appeared to have little significant influence on *S. cynthia*. By contrast, hyperoxia appeared to increase total DGE duration substantially, but with considerable variation among bursts and among individual pupae, and hence this effect was statistically non-significant (Table 4).

Maximum $\dot{M}_{CO_2}$ during each phase suggested a possible marginal effect of hyperoxia indicating an increase in peak burst volumes during the O-phase relative to normoxic conditions (Table 4), probably as a consequence of remaining in a C-phase for longer and thus increasing intratracheal $P_{CO_2}$. By contrast, the dry treatment clearly resulted in a significant reduction in peak O-phase $\dot{M}_{CO_2}$, although other phases appeared unaffected (Table 5). This small, but significant reduction was accompanied by a significant decline in O-phase burst volume (area under the $\dot{M}_{CO_2}$ curve) in dry compared with normal rearing (70%) humidity conditions, from 741.4±160.8 to 690.1±176.9 nmol $CO_2$ g$^{-1}$ ($t_5$=3.326; $P$<0.021).

Table 3. Summary of statistics and results from paired (dependent) *t*-tests of phases of discontinuous gas exchange for average rate of $CO_2$ release for experimental treatments in which discontinuous gas exchange was maintained

| | | Control | | | Treatment | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Treatment | Phase | $\dot{M}_{CO_2}$ (nmol g$^{-1}$ min$^{-1}$) | s.d. | *N* | $\dot{M}_{CO_2}$ (nmol g$^{-1}$ min$^{-1}$) | s.d. | *N* | *t* | d.f. | *P*-value |
| Hyperoxia | C | 0.584 | 0.263 | 3 | 0.584 | 0.260 | 3 | 0.020 | 2 | 0.986 |
| | F | 2.218 | 1.307 | 3 | 0.675 | 0.641 | 3 | 2.159 | 2 | 0.163 |
| | O | 39.814 | 10.043 | 3 | 30.386 | 9.167 | 3 | 3.785 | 2 | 0.063 |
| Wet | C | 0.337 | 0.055 | 6 | 0.310 | 0.049 | 6 | 1.631 | 5 | 0.164 |
| | F | 1.188 | 0.335 | 6 | 1.140 | 0.472 | 6 | 0.426 | 5 | 0.688 |
| | O | 29.784 | 9.933 | 6 | 30.646 | 8.383 | 6 | 0.369 | 5 | 0.728 |
| Dry | C | 0.368 | 0.043 | 5 | 0.436 | 0.182 | 5 | 0.741 | 4 | 0.500 |
| | F | 1.323 | 0.601 | 5 | 2.031 | 2.576 | 5 | 1.135 | 4 | 0.320 |
| | O | 31.384 | 9.518 | 5 | 30.024 | 9.786 | 5 | 0.511 | 4 | 0.636 |

Phases: C, closed; F, flutter; O, open; $\dot{M}_{CO_2}$, rate of $CO_2$ release; d.f., degrees of freedom.
In no cases was a significant effect detected at $P$=0.05.

Table 4. Summary statistics and results from paired (dependent) *t*-tests of phases of discontinuous gas exchange for the duration of the experimental treatments in which discontinuous gas exchange was maintained

| | | Control | | | Treatment | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Treatment | Phase | Duration (s) | s.d. | *N* | Duration (s) | s.d. | *N* | *t* | d.f. | *P*-value |
| Hyperoxia | C | 1568.1 | 306.8 | 3 | 3238.4 | 415.8 | 3 | 1.694 | 2 | 0.232 |
| | F | 894.8 | 230.7 | 3 | 375.4 | 223.5 | 3 | 4.080 | 2 | 0.055 |
| | **O** | **1248.3** | **356.4** | **3** | **4329.4** | **3101.6** | **3** | **8.175** | **2** | **0.015*** |
| Wet | C | 1663.2 | 458.0 | 6 | 1649.3 | 461.9 | 6 | 0.444 | 5 | 0.675 |
| | F | 613.4 | 351.1 | 6 | 624.5 | 386.7 | 6 | 0.093 | 5 | 0.929 |
| | O | 2013.8 | 1245.1 | 6 | 1891.9 | 1121.1 | 6 | 1.008 | 5 | 0.360 |
| Dry | C | 1456.7 | 504.3 | 5 | 1590.7 | 423.6 | 5 | 1.904 | 4 | 0.130 |
| | F | 619.7 | 405.6 | 5 | 650.2 | 337.5 | 5 | 0.339 | 4 | 0.752 |
| | O | 1570.4 | 386.0 | 5 | 1544.7 | 295.3 | 5 | 0.120 | 4 | 0.910 |
| Total DGE | | | | | | | | | | |
| Hyperoxia | | 3711.2 | 339.3 | 3 | 7943.3 | 3670.5 | 3 | 2.046 | 2 | 0.177 |
| Wet | | 4290.4 | 1475.2 | 6 | 4165.8 | 1260.1 | 6 | 0.732 | 5 | 0.497 |
| Dry | | 3646.7 | 420.6 | 5 | 3785.5 | 280.9 | 5 | 0.501 | 4 | 0.643 |

Phases: C, closed; F, flutter; O, open; d.f., degrees of freedom; s.d., standard deviation.
*$P<0.05$. Statistically significant variation within a DGE phase between control and treatment is highlighted in bold type.

## DISCUSSION

Because hypercapnia and/or hypoxia did not result in maintenance of DGE in *Samia cynthia*, the predictions of the chthonic and hybrid hypotheses were not supported. By contrast, DGE was maintained under high, but not low (i.e. equivalent to intratracheal $P_{O_2}$) oxygen conditions, so failing to reject the predictions of the oxidative damage hypothesis (Hetz and Bradley, 2005). Although somewhat surprising, the increased O-phase duration detected in the pupae under hyperoxic conditions (Table 4) can be explained by increased $CO_2$ build-up during extended C-phases, thereby requiring more time to restore haemolymph pH balance when spiracles open under hyperoxia. Owing to the maintenance of DGE in pupae when switched from normal to high ambient moisture conditions the predictions of the hygric and the hybrid hypotheses also appear to have been falsified. If water conservation is an important factor favouring the maintenance of DGE, the abolition of DGE under high moisture conditions might be expected, yet even after increasing moisture content in the air stream, DGE was maintained. However, the reduction in maximum $\dot{M}_{CO_2}$ observed under dry conditions is indicative of some support for the hygric hypothesis, especially if it is assumed that respiratory water loss is positively related to $\dot{M}_{CO_2}$ during the O-phase. This is not an altogether unreasonable assumption based on previous simultaneous recordings of $\dot{V}_{CO_2}$ and $\dot{V}_{H_2O}$ in DGE and non-DGE insects [in particular, see Fig. 3 in Lighton et al. (Lighton et al., 1993)] (see also Lighton and Berrigan, 1995; Chown and Davis, 2003; Gibbs and Johnson, 2004; Lighton et al., 2004; Schilman et al., 2005; Gray and Chown, 2008). Moreover, O-phase burst volume showed a significant reduction under dry conditions, in keeping with the finding that reduced respiratory water loss rates are associated with simultaneous declines in both O-phase duration and $\dot{M}_{CO_2}$ in other insect species (e.g. Chown and Davis, 2003). Unfortunately, in this study the declines in water loss could not be confirmed by direct measurement because in pilot trials we struggled to distinguish the animal's water loss from the background signal under high humidity conditions. Nonetheless, a range of studies has examined the conditions thought to promote DGE using $\dot{V}_{CO_2}$ data only (e.g. Lighton, 1990; Lighton, 1991; Duncan and Dickman, 2001; Duncan et al., 2002a).

Table 5. Summary of statistics and results from paired (dependent) *t*-tests of phases of discontinuous gas exchange for average maximum rate of $CO_2$ release for experimental treatments in which discontinuous gas exchange was maintained

| | | Control | | | Treatment | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Treatment | Phase | Max. $\dot{M}_{CO_2}$ (nmol g$^{-1}$ min$^{-1}$) | s.d. | *N* | Max. $\dot{M}_{CO_2}$ (nmol g$^{-1}$ min$^{-1}$) | s.d. | *N* | *t* | d.f. | *P*-value |
| Hyperoxia | C | 6.165 | 1.188 | 3 | 3.713 | 3.283 | 3 | 1.905 | 2 | 0.197 |
| | F | 12.636 | 4.345 | 3 | 4.551 | 1.674 | 3 | 2.603 | 2 | 0.121 |
| | O | 90.413 | 5.096 | 3 | 118.817 | 15.706 | 3 | 3.276 | 2 | 0.082 |
| Wet | C | 1.122 | 0.384 | 6 | 0.861 | 0.273 | 6 | 2.454 | 5 | 0.058 |
| | F | 5.284 | 1.131 | 6 | 5.589 | 1.164 | 6 | 0.494 | 5 | 0.642 |
| | O | 89.988 | 12.878 | 6 | 91.473 | 12.754 | 6 | 0.933 | 5 | 0.394 |
| Dry | C | 1.237 | 0.824 | 5 | 2.258 | 2.216 | 5 | 1.433 | 4 | 0.225 |
| | F | 5.960 | 1.412 | 5 | 9.027 | 5.357 | 5 | 1.405 | 4 | 0.233 |
| | **O** | **91.138** | **23.215** | **5** | **84.845** | **20.074** | **5** | **3.324** | **4** | **0.029*** |

Phases: C, closed; F, flutter; O, open; $\dot{M}_{CO_2}$, rate of $CO_2$ release; d.f., degrees of freedom; s.d., standard deviation.
*$P<0.05$. Statistically significant variation in a discontinuous gas exchange phase between control and treatment is highlighted in bold type.

Of course, it would be remiss of us not to point out that several of the marginal effects in our study were probably a consequence of relatively small sample sizes, which, in turn, were constrained by equipment and experimental duration. Similarly, relatively large inter-individual variation in DGE parameters under treatment conditions, which was not obvious in earlier pilot trials, may have compounded these effects. Experimental design and future tests of adaptive hypotheses should attempt to account for, and understand factors contributing to, this variation at the individual and species level. However, it is clear that statistical power is always going to be a limiting factor in such multi-factor experimental designs. If, for example, a full-factorial experimental design were to be used in which four levels are investigated within each of four main experimental treatments (e.g. humidity manipulation which includes humidities of 5, 20, 60 and 80%), based on the present statistical power (Results) in which approximately 30 individuals might be required per treatment group, a total of ~480 individuals would be required. This obviously presents a considerable logistic constraint.

Bearing the above caveats in mind, it appears that avoidance of oxidative damage, and to a lesser extent, retention of water, play significant roles in determining the presence and form of DGE in the pupae of this moth species. Obtaining support for the oxidative damage hypothesis in *S. cynthia* is perhaps unsurprising considering that a related species, *Attacus atlas*, has provided rigorous experimental data in favour of the oxidative damage hypothesis (Hetz and Bradley, 2005). Nonetheless, it does indicate that, at least for pupae of this group of insects, the oxidative damage hypothesis has support, especially since a strong inference, experimental approach has been unable to discount it, but has proved able to reject predictions of most of the competing hypotheses. Moreover, since the predictions of this hypothesis were also partially supported in the comparative study of White et al. (White et al., 2007), it is clear that the extent to which avoidance of oxidative damage might select for current maintenance of DGE requires further, careful scrutiny in other groups.

Similarly, the hygric hypothesis has received considerable support across a wide range of species and studies (e.g. Kestler, 1985; Lighton et al., 1993; Vogt and Apple, 2000; Duncan et al., 2002b; Chown and Davis, 2003; Marais et al., 2005; White et al., 2007), including the pupae of other moth species (Levy and Schneiderman, 1966). Thus, finding support for it here is not surprising either, although this outcome contrasts strongly with some recent statements concerning the demise of the hygric hypothesis (Lighton and Turner, 2008).

Importantly, the finding that the predictions of both the hygric and oxidative damage hypotheses cannot be rejected re-emphasizes the idea that DGE and the modulation of its underlying components may be a response to several different environmental factors (Chown, 2002). The independent origin of this gas exchange pattern in several higher taxa (Klok et al., 2002; Lighton and Joos, 2002; Marais et al., 2005) also provides some support for this idea, although conceivably DGE could have arisen as an independent response to a single factor across all of the taxa. In consequence, besides the variety of investigative approaches adopted, multiple pathways to DGE might also constitute a sound reason why most of the hypotheses proposed to explain its maintenance continue to engender support, and why this support seems to vary so consistently from one group to the next (e.g. in ants, predictions of the chthonic hypothesis and its variations cannot be rejected, whereas in moth pupae the predictions of the oxidative damage hypothesis cannot be rejected). Nonetheless, on present evidence the hygric and oxidative damage hypotheses clearly have the broadest support, especially since neither comparative nor experimental strong-inference approaches have been able to reject their predictions (see also White et al., 2007). However, it should be noted that these tests all examine hypotheses for the present maintenance of DGE (Chown et al., 2006). Given that several of the hypotheses continue to engender support, it may well be the case that a variety of conditions select for maintenance or further modulation of DGE, but that its origin is non-adaptive. Chown and Holter (Chown and Holter, 2000) proposed that DGE may be a non-adaptive consequence of the interactions between the $CO_2$ and $O_2$ regulation systems that together determine spiracle opening and gas exchange under conditions of minimal demand. Their hypothesis has yet to be the subject of careful scrutiny.

The present data also provide some grounds for questioning a number of the conclusions reached by Sláma et al. (Sláma et al., 2007). In particular, they claim that the DGEs identified by Lighton (Lighton, 1996) could be an artefact resulting from exposure of insects to a dry air stream. Clearly, the full DGE pattern (C-, F- and O-phases) is maintained in *S. cynthia* under dry and humid conditions, although some modulation of its form takes place. In consequence, Sláma et al.'s (Sláma et al., 2007) conjecture is not supported here, although whether this is the case also for other species, besides the termites they discuss, is not clear. Second, they claim that continuous adjustment of the form of $CO_2$ release, dependent on environmental conditions, means that previous hypotheses concerning the maintenance of DGE have poor physiological substantiation. Our simultaneous measurements of $\dot{M}_{CO_2}$ and tracheal pressure provide little support for the notion that different conditions, and especially dry *vesus* humid conditions, result in very different $CO_2$ sequestration and release patterns.

In conclusion, to date, the majority of experimental investigations have not adopted a strong inference approach and have most commonly found support for the oxidative damage (Hetz and Bradley, 2005), chthonic-hygric (Lighton and Berrigan, 1995) and chthonic hypotheses (Lighton and Turner, 2008). By contrast, recent comparative studies have typically found support either for the hygric hypothesis or the oxidative damage hypothesis (e.g. Marais et al., 2005; White et al., 2007). The results of the present study are therefore significant for several reasons. First, the study constitutes the first strong inference-based, experimental assessment of the predictions of the adaptive hypotheses proposed to explain the maintenance of DGE. Second, by employing intratracheal pressure measurement, this study is also the first to assess DGE presence or absence under varying $P_{CO_2}$, $P_{O_2}$ and $P_{H_2O}$ conditions. Such an approach has not previously been adopted most probably because of the technical limitations of specific gas analysers, e.g. operating against high gas concentration ('background') conditions [though see Sláma et al. (Sláma et al., 2007) for an alternative approach]. Third, support for the oxidative damage hypothesis was found with some limited support for the hygric hypothesis. Clearly, several questions remain, most notably the likely effect of short-term plasticity on the DGE responses of organisms to altered conditions, the likely role of $CO_2$ sequestration and release in affecting DGE, and the role played by interacting setpoints in giving rise to DGE. These questions have been only either discussed briefly or investigated in a small set of taxa (Chown and Holter, 2000; Sláma et al., 2007; White et al., 2007), and therefore not given nearly sufficient consideration in experimental and theoretical work to date. Nonetheless, the present results, in conjunction with those of Hetz and Bradley, Marais et al. and White et al. (Hetz and Bradley, 2005; Marais et al., 2005; White et al., 2007), suggest that the death of the hygric and oxidative damage hypotheses has been declared

prematurely by Lighton and Turner (Lighton and Turner, 2008). Neither is ready for interment and the funeral must be called off.

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