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Reactive oxygen species production and discontinuous gas exchange in insects

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While biochemical mechanisms are typically used by animals to reduce oxidative damage, insects are suspected to employ a higher organizational level, discontinuous gas exchange mechanism to do so. Using a combination of real-time, flow-through respirometry and live-cell fluorescence microscopy, we show that spiracular control associated with the discontinuous gas exchange cycle (DGC) in *Samia cynthia* pupae is related to reactive oxygen species (ROS). Hyperoxia fails to increase mean ROS production, although minima are elevated above normoxic levels. Furthermore, a negative relationship between mean \dot{V}_{CO_2} and mean ROS production indicates that higher ROS production is generally associated with lower \dot{V}_{CO_2} . Our results, therefore, suggest a possible signalling role for ROS in DGC, rather than supporting the idea that DGC acts to reduce oxidative damage by regulating ROS production.

Keywords: discontinuous gas exchange; periodic ventilation; adaptation; metabolism; oxidative damage; trachea

1. INTRODUCTION

The oxygenation of Earth's atmosphere marked a dramatic change in the course of evolution. Increases in oxygen concentration enabled the utilization of the oxygen rich, transmembrane communication proteins necessary for multi-cellular eukaryotic life [1], high metabolic rates and a variety of novel metabolic reactions [2]. High oxygen levels eventually led to the rise of complex life forms [3] and their consequent terrestrial expansion [4]. Later rises in oxygen levels further promoted diversification [5], with the recurrence of similar major events such as the evolution of flight in new taxa [6].

However, the benefits of life in an oxygen-rich atmosphere have not been realized without costs, of which cellular-level damage by reactive oxygen species (ROS; also known as free radicals) is perhaps the most significant. ROS, such as the superoxide and hydroxyl ions, react with a wide variety of molecular species, causing considerable molecular damage and interfering with cellular function [7]. ROS are also central to the oxidative damage hypothesis of ageing and play a role in numerous human diseases [8].

Like other multi-cellular eukaryotes, insects employ several biochemical mechanisms to limit damage caused by ROS, including the enzymes superoxide dismutase, glutathione reductase and catalase [9–11] and, in some insects, globins have also been implicated in ROS regulation [12,13]. However, unlike many other organisms, insects have an invaginated, tracheal respiratory system,

of which the finest branches reach deep into the tissues, delivering oxygen almost directly to the mitochondria [14,15]. While this system—owing to its very high efficiency—is able to fuel some of the highest mass-specific metabolic rates recorded among animals [14], it is also a likely source of increased ROS. In consequence, insects are thought to make use of two other, higher level mechanisms to limit oxidative damage. The first is fluid filling of the tracheolar tips during low-oxygen demand to reduce oxygen toxicity [16]. The second, and more controversial mechanism, is whole-organismal control of breathing, which is thought to have led to a unique, cyclic breathing pattern known as the discontinuous gas exchange cycle (DGC) [17].

In insects, the exchange of gases between the atmosphere and tracheal system takes place via valve-like spiracles. In many quiescent species, this exchange is discontinuous [18], and can be divided into three phases based on spiracle behaviour [19]. During the closed (C) phase, the spiracles are closed, oxygen is depleted from the endotracheal space and pressure within the tracheal system declines. The next, flutter (F) phase is triggered by declining PO_2 levels and, via partial spiracular opening, oxygen levels are typically regulated at 2–4 kPa [20]. Eventually, accumulation of CO_2 results in complete spiracular opening, known as the open (O) phase, which may be accompanied by ventilation of the tracheal system. The pattern is then repeated, leading to the DGC. Not all insects show evidence of DGC at rest [18] and several major hypotheses have been proposed to explain the evolutionary origins and maintenance of these cycles and why they are restricted in their taxonomic distribution [19,21,22]. While some authors prefer a single evolutionary explanation, others have suggested

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that DGC may have arisen and are maintained for several reasons [19], among which is the limitation of oxidative damage.

The oxidative damage hypothesis for DGC [17] proposes that this pattern has arisen to reduce oxidative damage. During the C phase, oxygen partial pressure is rapidly reduced, and, in the moth *Attacus atlas*, during the F phase, it is closely regulated at *ca* 4 kPa irrespective of variation in external oxygen partial pressures ranging from 5 to 50 kPa (normoxic conditions at sea level are *ca* 21 kPa). It is this tight regulation of intratracheal PO₂, along with the known cellular-level damage caused by normoxic concentrations of O₂ [23], which underlie the oxidative damage hypothesis [17,19].

However, whether insects do actually limit oxidative damage using higher-level mechanisms is unknown. While three studies [17,24,25] have provided evidence for the oxidative damage hypothesis based on gas exchange dynamics, no studies have shown that spiracular control can limit oxidative damage via regulation of ROS production, independently of the cellular-level mechanisms that already exist to do so. Thus, although the pattern of maintenance of internal partial pressure is consistent with the oxidative damage hypothesis, the chain of reasoning from DGC to oxidative damage is missing a critical, causal link. Indeed, it has been suggested that current oxygen toxicity data cannot be used to support the oxidative damage hypothesis for DGC [26].

Here, we use manipulation of oxygen concentrations, and a combination of flow-through respirometry and live-cell fluorescence microscopy, which provide simultaneous real-time recordings of gas exchange and ROS production, respectively, to determine whether the fundamental, missing link between spiracular opening and somatic ROS production can be established. *Samia cynthia* (Lepidoptera: Saturniidae) was chosen for this work as a model species that exhibits DGC because moth pupae are a classic model group for the study of insect gas exchange (reviewed in [15,17,24]).

These experiments, therefore, aim to examine the role of free radical production in insect gas exchange pattern modulation. Specifically, we test a single key prediction of one of the major adaptive hypotheses, the oxidative damage hypothesis of DGC, but do not seek to compare the relative importance of the various alternative hypotheses as this was the focus of previous comparative and experimental work (e.g. [17,24,27,28]). We used live, diapausing *S. cynthia* in a novel, real-time dual respirometry and fluorescence microscopy set-up. Briefly, flow-through respirometry was used to monitor gas exchange patterns (measured as carbon dioxide release). Simultaneously, ROS production was measured using fluorescent probes. In a parallel set-up, we also explored the effects of different oxygen levels on the diapausing pupae's spiracular responses and gas exchange rates using intratracheal intubation (following e.g. [24]).

2. MATERIAL AND METHODS

(a) Animals

Diapausing *S. cynthia* pupae were obtained by air freight from a laboratory culture maintained in Germany (SKH laboratory). This species was chosen as a model for DGC because it has been well studied and clearly shows this gas

exchange pattern in diapausing pupae [24,29,30]. During shipping, pupae were transported in insulated containers kept at low temperatures (8°C–10°C). To maintain diapause in the laboratory, pupae were held in the dark, in a climate chamber (YIH DER growth chamber, model LE-539, SCILAB instrument CO Ltd., Taiwan) set to 10°C (\pm 1°C).

(b) Respirometry

Within 3 days of extraction from its cocoon, an individual pupa was placed within the dual respirometry and fluorescence microscopy set-up. This short waiting period ensured that handling the pupae did not result in a break in diapause. Flow-through respirometry was used to monitor gas exchange patterns (measured as carbon dioxide release) [31]. Airflow was regulated at 300 ml min⁻¹ using a mass flow control valve (Sidetrak, Sierra International, USA) connected to a mass flow control box (Sable Systems, Las Vegas, Nevada, USA) and fed through a 60 ml custom-built cuvette to a calibrated Li-7000 infrared gas analyser (LiCor, Lincoln, Nebraska, USA). The analyser was connected to a laptop computer via RS-232 connection to record CO₂ production in parts per million (ppm) using Licor software. A manual switch was used to change between normoxic (21% O₂, balance N₂), hyperoxic (40% O₂, balance N₂) and anoxic (100% N₂) air during the experiments (cf. [31–33]). Normoxic air (generated by an air pump (Hailea, China)) was scrubbed of CO₂ by a column containing soda lime, and water was removed by a column containing 50:50 silica gel: Drierite (WA Hammond Drierite Company Ltd, Ohio, USA). All equipment was housed in an air-conditioned laboratory maintained at, or slightly below, 20°C for the duration of the experiments. To assess spiracular responses to oxygen more generally in *S. cynthia*, using a parallel flow-through respirometry set-up, pupae were subjected to different oxygen levels while recording intratracheal pressure. Average tracheal pressure during the C- and F-phase was used as a parameter to measure the reaction of the pupae's spiracles to different oxygen levels (cf. [24]).

(c) Reactive oxygen species production

The presence of ROS in live pupae was measured using a live-cell fluorescence Olympus IX 81 microscope with Cell^R software (Olympus Biosystems/Soft Imaging System) and a MT-20150 W xenon illumination source (e.g. [34,35]).

Pupae were injected with 100 nM mitochondrial tracker (MitoTracker Green FM, M7514, Molecular Probes, Invitrogen detection technologies) and 300 nM ROS tracker (MitoTracker Red CM-H₂XRos, M7513, Molecular Probes, Invitrogen detection technologies) with balance phosphate buffer solution (PBS). Injection volumes were typically 1–2% of an individual pupae's wet weight, which was assumed to represent 90 per cent of live body mass. *Samia cynthia* pupa used in this experiment had a mean mass of 2.528 ± 0.391 g, and the mass of pupae used for each of the two treatments did not differ ($t_{19} = -0.528$, $p = 0.60$). Furthermore, pupa mass was not correlated with ROS production ($r = -0.090$, $p = 0.70$; electronic supplementary material, figure S1).

The green MitoTracker was used to identify areas of functioning mitochondria, as this probe fluoresces in all live mitochondria regardless of mitochondrial membrane potential. The red ROS MitoTracker occurs as a non-fluorescent-reduced rosamine until it is oxidized by ROS generated by the mitochondria, resulting in detectable changes in

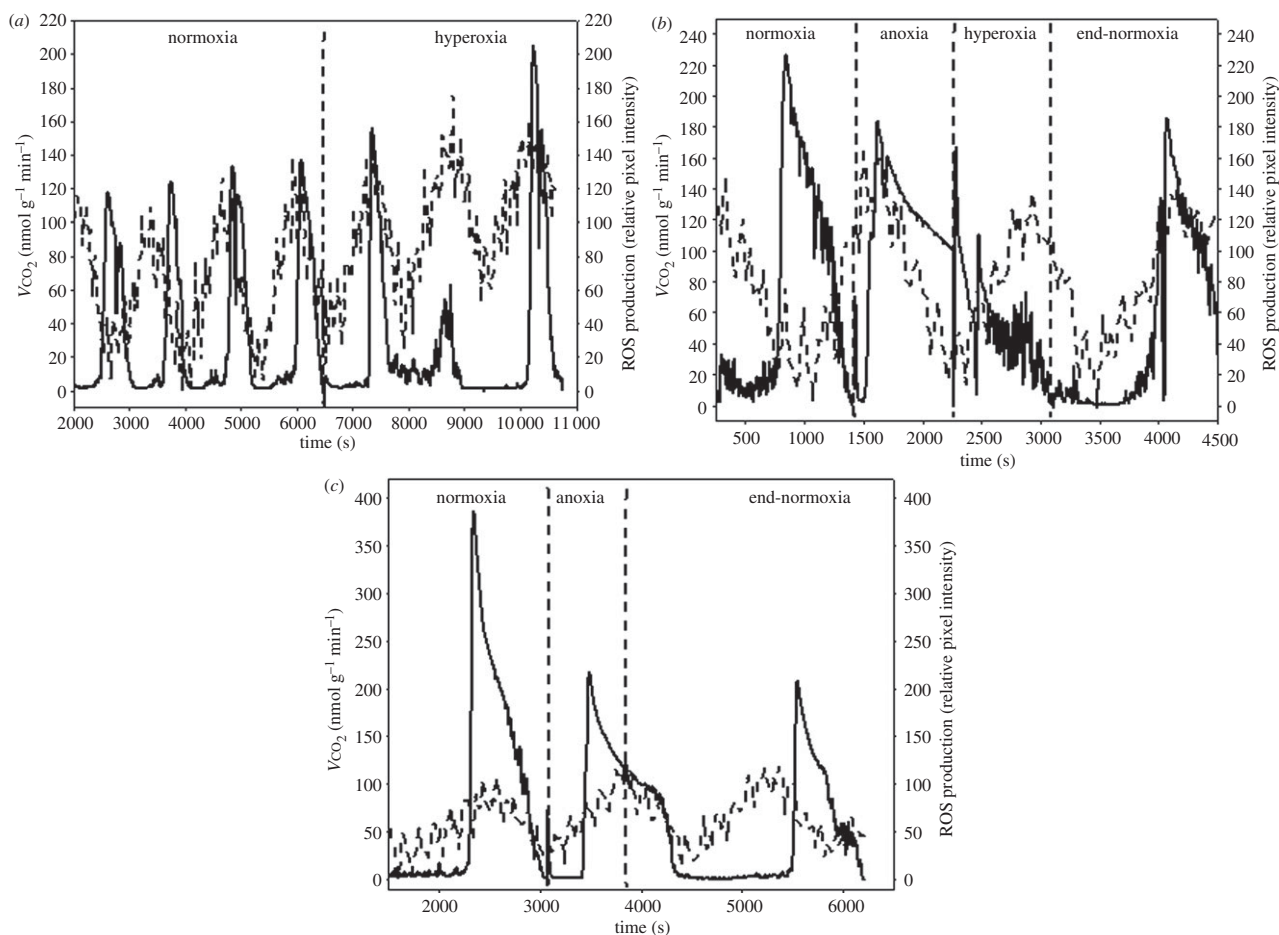


Figure 1. Simultaneous measurement of \dot{V}_{CO_2} and reactive oxygen species (ROS) under different gas conditions during discontinuous gas exchange in a *S. cynthia* pupa. (a) Normoxic (ca 21% O₂) and hyperoxic (40% O₂) conditions. No significant changes in ROS production (expressed in units of relative pixel intensity) are evident with the switch to hyperoxia, but the discontinuous gas exchange pattern is modulated by increasing the closed (C) phase duration and open (O) phase burst volume. (b) Normoxic–anoxic–hyperoxic–normoxic (NAHN) trial. (c) Normoxic–anoxic–normoxic (NAN) trial. (a–c) Solid line, \dot{V}_{CO_2} ; dashed line, ROS.

fluorescent intensity (electronic supplementary material, figure S2), without evidence for cytotoxicity [36].

A custom built 60 ml respirometry cuvette, with a flat window to reduce light diffraction effects, was mounted on the Olympus IX 81 microscope stage. TxRED and FITC excitation filters (at 10X magnification) were used to identify ROS and mitochondrial signals, respectively. ROS was quantified as change in relative pixel intensity in Cell^R software. Because most ROS production is thought to occur within mitochondria [11,37], an area in the region of the flight muscle was selected, using the green mitochondrial tracker to focus on an area of actively functioning mitochondria. Any effects that the cuticle may have had on experimental ROS production and its detection were assumed to be similar across all individuals.

(d) Experimental manipulation

Intact whole pupae were subjected to different ambient gases to induce spiracle opening, which resulted in acute exposure to either anoxia or hyperoxia. Weighed moth pupae were injected with trackers, inserted into the respirometry cuvette and DGC and ROS production were simultaneously monitored. Cotton wool was used to ensure that the animal was held tightly against the viewing window, although occasional body movements did cause images to blur. In cases of high activity, where the mitochondrial tracker trace was affected

by the movement, pupae were discarded from the analysis. After tracker injection, and being placed in the respirometry cuvette, animals were allowed to recover for at least 4 h until they settled into a regular DGC pattern under normoxic conditions before the ROS recording started and gases were manipulated.

Since all pupae were capable of modulating spiracle opening in response to changes in ambient PO₂ during the trial intubation experiments, a series of gas switches (normoxia, anoxia, hyperoxia, normoxia; NAHN) [31] were used to induce oxidative damage and quantify the protection afforded by keeping spiracles closed. The gas protocol involved a period of normoxia during which baseline ROS production was estimated. This was followed by a switch, typically undertaken during the C-phase of the DGC, to 100 per cent N₂, which usually results in full opening of all spiracles to improve gas exchange (see [31,32]), and which was found here too (figure 1b,c). After the \dot{V}_{CO_2} peak during anoxia, \dot{V}_{CO_2} values were carefully monitored until the value fell to approximately half the peak \dot{V}_{CO_2} value. At this point, 100 per cent N₂ was replaced with 40 per cent O₂ (balance N₂). Although this oxygen concentration is high, it has been used previously to examine DGC responses to hyperoxia [28]. The transition to anoxia typically elicits full spiracular opening [31], the equivalent of surgically intubating all of the animal's spiracles. At this time, the pupa has been starved of oxygen during the anoxic

period, and therefore the first intake of air after the hypoxic phase is hyperoxic air, which is likely to challenge the pupa's respiratory system. After an individual pupa's \dot{V}_{CO_2} trace had returned to zero, indicating that all spiracles were once again closed, hyperoxic gas was switched to normoxic air to allow for the pupa to recover and resume normal gas exchange. Thus, pupae with open spiracles were exposed to, and actively ventilated during hyperoxia for approximately 10–15 min. The NAHN trials were performed at 300 ml min^{-1} flow rate to ensure rapid gas switching inside the cuvette. At this flow rate, the response times for the respirometry system (including the cuvette) was less than 40 s. The washout time required to reach 1 per cent of the initial amount of CO_2 in the cuvette ($5 \times$ time constant), with the time constant calculated from volume and flow rate in the experimental set-up, was 240 s. To investigate the likelihood of oxidative damage by normoxic air, the gas switches were repeated excluding the hyperoxic period. Effectively this was a normoxic–anoxic–normoxic trial (NAN) (as in [31]). Furthermore, the end-normoxic period of a NAN trial (i.e. the period of normoxia after the anoxia) will act as a control for the hyperoxic period in the NAHN trial since the effects of reoxygenation with normoxic air could be compared with that of hyperoxic air. The NAN trial was conducted under the identical respirometry set-up and flow-rate conditions as the NAHN trial.

(e) Data analysis

Cell[^]R imaging software (Olympus Biosystems/Soft Imaging System) was used to analyse the images obtained during fluorescence microscopy and extract relative pixel intensity values for the two fluorescent dyes. EXPEDATA v. 1.1.25 (Sable Systems) was used to baseline correct and analyse both the respirometry and the ROS data. Respirometry data were converted from parts per million (ppm) to $\text{nmol g}^{-1} \text{ min}^{-1}$ for each individual (see [29]).

Within each experimental group, only animals for which both gas exchange patterns and ROS production were available were used in analyses. A minimum of 14 individuals were run for each experimental manipulation, with at least 10 successful trials obtained. Independently for each treatment trial (NAHN and NAN), analyses of ROS production (MitoTracker Green FM subtracted from the changing intensity of MitoTracker Red) were conducted as Wilcoxon matched pairs or dependent sample *t*-tests for different gas treatments within each individual (e.g. normoxic versus hyperoxic; following e.g. [24]). To assess changes in mean total \dot{V}_{CO_2} and mean ROS production during each gas phase of both the NAHN and NAN trials independently, a Kruskal–Wallis ANOVA followed by a multiple comparisons of mean ranks post hoc test was conducted. Changes in ROS production were calculated relative to normoxic values for each individual as a percentage, producing an estimate of relative ROS change within an individual across NAHN or NAN treatment. These values were grouped for each gas phase of each trial and analysed using Levene's test for homogeneity of variance. All statistical tests were performed using STATISTICA v. 9 (StatSoft, Inc.). All values are presented as arithmetic means \pm 1 s.d.

3. RESULTS

Under normoxic conditions, gas exchange in diapausing pupae of the moth *S. cynthia* was discontinuous (figure 1). ROS production typically followed a cyclic pattern with

variations that are related to variation in spiracular opening as measured using whole animal \dot{V}_{CO_2} (figure 1*a–c*). Analyses of both \dot{V}_{CO_2} pattern and ROS production frequency during three control runs (normoxic air only) showed that the mean frequencies for both parameters are statistically indistinguishable ($t_2 = 0.763$, $p = 0.53$; 0.847 ± 0.092 and 0.790 ± 0.112 mHz, respectively). However, a lag was typically found between peak \dot{V}_{CO_2} and peak ROS production, with ROS production increasing during the C and F phases (figure 1*a*). On average, there was a negative correlation between average \dot{V}_{CO_2} and average ROS production (electronic supplementary material, figure S3) indicating that higher ROS production was generally associated with lower \dot{V}_{CO_2} and the C and F phases (overall fit: $y = 91.2232 - 0.3837 \times x$; $r = -0.3394$, $p = 0.0012$).

Exposure of pupae to different ambient oxygen levels (cf. [24]) led to a significant reaction in spiracle activity. In hyperoxia, the pupae closed their spiracles for longer periods which in turn led to significantly lower tracheal pressures. On average, intratracheal pressure decreased nearly fivefold when pupae were switched from 21 to 40 per cent oxygen levels (mean decrease 4.86 ± 6.30 Pa). Hyperoxia therefore resulted in a significant reduction in tracheal pressure which in most cases was accompanied by an extension of the C and F phases, and increased burst volumes, of the DGC ($Z_7 = 2.678$, $p < 0.01$).

As the spiracular reaction to oxygen was confirmed in these experiments, spiracular opening in the pupae was manipulated by altering gas concentrations from normoxia to anoxia to hyperoxia and back to normoxia (NAHN trial, figure 1*b* and electronic supplementary material, figure S2 and video S1), or from normoxia to anoxia to normoxia (NAN trial, figure 1*c*) over the course of several hours. The NAN trial served as a manipulation control to assess the effects to experimental handling on the pupae.

Results of both NAHN and NAN trials show that anoxia caused \dot{V}_{CO_2} to increase, indicating that spiracles were held open (NAHN: Kruskal–Wallis $H_3 = 28.99$, $p < 0.0001$ and NAN: Kruskal–Wallis $H_2 = 20.87$, $p < 0.0001$) without a subsequent increase in mean total ROS production (NAHN: Kruskal–Wallis $H_3 = 0.68$; $p = 0.88$ and NAN: Kruskal–Wallis $H_2 = 1.03$, $p = 0.59$; figure 2). The NAHN trial revealed that hyperoxia after anoxia caused \dot{V}_{CO_2} to drop rapidly, indicative of spiracle closure (figure 2; electronic supplementary material, tables S1 and S2). Mean minimum ROS production during the NAHN trial increased significantly during hyperoxia in comparison with the other gas phases (hyperoxia versus normoxia: $Z_{10} = 2.191$, $p < 0.05$; hypoxia versus anoxia: $Z_{10} = 1.988$, $p < 0.05$; hyperoxia versus end-normoxia: $Z_{10} = 2.803$, $p < 0.01$; tables 1 and 2). For both NAHN and NAN trials, the return to normoxia resulted in \dot{V}_{CO_2} and ROS production values that were not significantly different to initial normoxic values (figure 2, tables 1 and 2 and electronic supplementary material, tables S1 and S2).

When mean ROS production is calculated as a percentage change relative to normoxia levels within individual pupae, for both NAHN and NAN trials, no significant differences were detected among gas phases (Kruskal–Wallis $H_4 = 2.057$, $p = 0.72$; figure 3*a*). However, it is clear that in both trials, anoxia causes a similar reduction in relative mean ROS production (NAHN: -18.66 ± 37.36 ; NAN: -8.08 ± 42.80 ; as a percentage change in

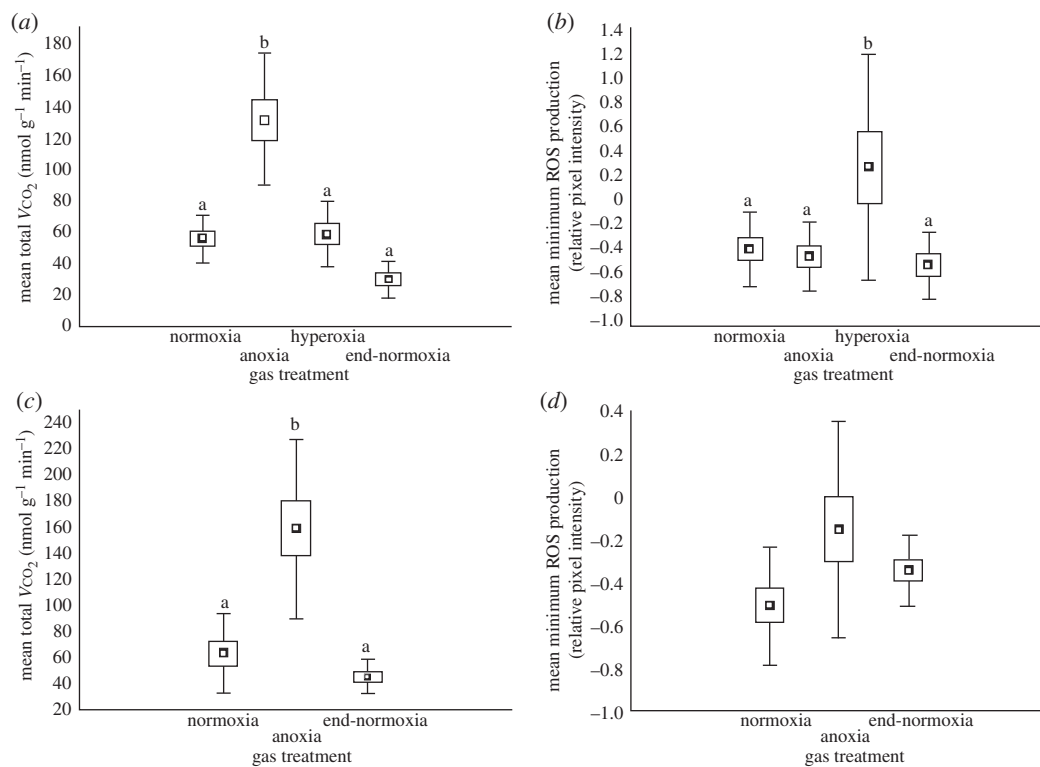


Figure 2. Box plots showing mean (box = s.e., whiskers = s.d.) total \dot{V}_{CO_2} and ROS production, during different gas phases, for (a,b) the normoxic–anoxic–hyperoxic–normoxic (NAHN) and (c,d) normoxic–anoxic–normoxic (NAN) trials. In both trials, anoxia causes an increase in mean total \dot{V}_{CO_2} owing to opening of the spiracles ((a) Kruskal–Wallis $H_3 = 28.99$, $p < 0.0001$ and (c) Kruskal–Wallis $H_2 = 20.87$; $p < 0.0001$; letters indicate significant different groups), but has no effect on ROS production ((b) Kruskal–Wallis $H_3 = 0.68$, $p = 0.88$ and (d) Kruskal–Wallis $H_2 = 1.03$, $p = 0.59$). During hyperoxia, \dot{V}_{CO_2} falls (a) while ROS increases slightly (b). Note that axes are scaled differently for clarity.

Table 1. Mean values (with standard deviation in brackets) of ROS production in normoxic–anoxic–hyperoxic–normoxic (NAHN) and normoxic–anoxic–normoxic (NAN) trials for *S. cynthia*. The NAHN trial indicates that hyperoxic gas alters the mean ROS production relative to the other gas conditions, while NAN results indicate that the stress of manipulation does not affect ROS values during different gas conditions. rpi, relative pixel intensity.

variable	normoxia (rpi)	anoxia (rpi)	hyperoxia (rpi)	end-normoxia (rpi)
NAHN trial: $n = 10$; mean mass = 2.554 ± 0.379 g				
mean total ROS	1.080 (0.752)	0.766 (0.560)	1.312 (1.368)	1.018 (0.786)
mean maximum ROS	2.442 (1.243)	2.170 (1.532)	2.199 (1.624)	2.365 (1.107)
mean minimum ROS	-0.382 (0.295)	-0.438 (0.272)	0.281 (0.908)	-0.508 (0.270)
NAN trial: $n = 11$; mean mass = 2.504 ± 0.419 g				
mean total ROS	1.217 (0.692)	1.150 (0.895)		1.136 (0.615)
mean maximum ROS	2.842 (1.204)	2.289 (1.174)		2.654 (1.125)
mean minimum ROS	-0.514 (0.278)	-0.158 (0.505)		-0.349 (0.163)

pixel intensity, mean \pm s.d.; $t_{19} = 0.60$, $p > 0.5$). The relative change in mean ROS during hyperoxia in the NAHN trial, by comparison with the relative change in end-normoxic ROS of the NAN trial, was not significant though clearly elevated (NAHN: 40.51 ± 102.89 ; NAN: $-2.65 \pm 42.80\%$; $t_{19} = -1.31$, $p = 0.2$). Relative end-normoxic ROS production for both trials were identical ($t_{19} = 0.01$, $p = 0.99$). Thus, although mean minimum ROS production during hyperoxia was elevated by comparison with the other treatments, overall mean values were not. Importantly, however, hyperoxia also significantly increased the variance of ROS production during the NAHN trial by comparison with all of the other gas treatments (Levene's $F_4 = 6.07$, $p < 0.001$, figure 3b).

4. DISCUSSION

Little consensus has emerged on the main reason for the evolutionary origin and maintenance of DGC, and multiple independent origins suggest that it evolved for different reasons in various taxa and was then pressed into service to meet other demands [18,19,38]. While a variety of alternative explanations exist, both species-level experimental data and comparative studies have come out strongly in favour of water loss and the prevention of oxidative damage as the primary underlying reasons for DGC [27,28,39]. However, matters have become increasingly more complicated. In particular, a recent proposal has DGC functioning to effect an energy savings in species that need to downregulate energy-demanding systems and

Table 2. Results of paired *t*-tests comparing ROS variables within normoxic–anoxic–hyperoxic–normoxic (NAHN) and normoxic–anoxic–normoxic (NAN) trials in *S. cynthia*. Significant *p*-values are indicated in bold.

comparison	NAHN		NAN	
	Z_9	<i>p</i> -value	Z_{10}	<i>p</i> -value
normoxic versus anoxic				
mean ROS	1.580	0.114	0.800	0.424
maximum ROS	0.764	0.445	2.490	0.013
minimum ROS	0.420	0.674	1.511	0.131
normoxic versus hyperoxic				
mean ROS	0.561	0.575		
maximum ROS	0.866	0.386		
minimum ROS	2.191	0.029		
normoxic versus end-normoxic				
mean ROS	0.663	0.508	1.067	0.286
maximum ROS	0.153	0.878	1.1511	0.131
minimum ROS	1.172	0.241	1.689	0.091
anoxic versus hyperoxic				
mean ROS	1.070	0.285		
maximum ROS	0.153	0.878		
minimum ROS	1.988	0.047		
anoxic versus end-normoxic				
mean ROS	0.968	0.333	0.089	0.929
maximum ROS	0.866	0.386	2.317	0.021
minimum ROS	0.764	0.445	1.245	0.213
hyperoxic versus end-normoxic				
mean ROS	1.172	0.241		
maximum ROS	0.663	0.508		
minimum ROS	2.803	0.005		

particularly the central nervous system (especially the brain) [21]. Such downregulation then probably means a handing over of respiratory control to the segmental ganglia so resulting in the typical DGC [40]. The DGC could actually then be considered a water-profligate, energy savings strategy, where modulation of the phases to effect water conservation is necessary [22]. Thus, the reduction of respiratory water loss might not be a primary explanation for the origin of the DGC, but rather a reason for modulation thereof, as has been found in previous work [27,39].

In a similar vein, to date the crucial, causal link between DGC and the limitation of ROS production has been missing: that is a demonstration that ROS production does indeed rise if intratracheal oxygen concentration is not regulated by spiracle behaviour. Our simultaneous, real-time recordings of gas exchange and ROS production are, unfortunately, equivocal in this regard. While a strong relationship clearly does exist between the frequencies of \dot{V}_{CO_2} and ROS production, their cycles are out of phase (electronic supplementary material, figure S3). Mean ROS production was also largely unaffected by the experimental treatments. However, mean minimum ROS production did increase during experimentally induced hyperoxia, with this estimate likely to be conservative, especially in comparison with end-normoxia in the NAHN trial. Reperfusion with normoxic air after anoxia has been shown to cause physiological damage in *Drosophila melanogaster* [41] and many vertebrate species [42]. Therefore, it is likely that even during the NAN trial, ROS may have been produced

in the end-normoxic phase, making the NAHN end-normoxia period similarly conservative.

How might these equivocal findings be interpreted? The increase in minimum ROS production, and the changing variance in the system does suggest that ROS production and the system responsible for the removal thereof are significantly affected by experimentally induced hyperoxia. That experimental manipulation *per se* does not influence the variance is clear from the NAN trial, where the variance in changes in ROS production relative to normoxia remained low despite the different gas treatments (NAN SD range: 34–43). By contrast, in the NAHN trial, variance of the relative ROS production was high—indeed the variation in this treatment was, on average, three to five times greater than that of the other treatments in the full set of experiments (NAHN SD range: 20–103). Rising variance and changing skewness are strong indicators of critical transitions in homeostatic [43], biological feedback [44] and ecological systems [45,46]. In physiological systems, elevated variance accompanies the onset of epileptic seizures and also asthma attacks [47,48]. Indeed, in our experiments skewness was also significantly affected. The hyperoxic period in the NAHN trials had a significantly skewed frequency distribution (skew \pm s.e.: 1.344 ± 0.687) relative to the anoxic or end-normoxic period (anoxia: -0.345 ± 0.687 , $t_9 = 2.452$, $p < 0.05$, end-normoxia: -0.419 ± 0.687 ; $t_9 = 2.559$, $p < 0.05$). However, in the NAN trials, the end-normoxic period (0.869 ± 0.661) was not significantly different from the anoxic period (0.377 ± 0.661 ; $t_{10} = 0.745$, $p > 0.5$). Thus, the increase in variance of relative ROS production during hyperoxia in the NAHN trial might be interpreted as suggesting that the intrusion of oxygen via the tracheal system had stressed, and perhaps at least briefly, overwhelmed typical higher-level and cellular-level defences, which may then have led to overexpression of the systems that typically deal with ROS.

Had the \dot{V}_{CO_2} and ROS production cycles been in phase, this outcome may have provided strong support for the oxidative damage hypothesis. The marker we used, CM-H₂XROs, fluoresces upon oxidation by ROS and this method has been demonstrated to detect repeatable differences in ROS among treatments reliably [36,49]. Therefore, the absence of a response in mean ROS production under the experimental conditions could be interpreted as an increase in ROS production, but removal of most of the ROS by cellular-level mechanisms before it had been reduced by the marker. Such mechanisms include the ROS scavengers superoxide dismutase, glutathione reductase and catalase [9–11], and possibly one or more globins that have also been implicated in ROS regulation [13,50].

However, the lag between \dot{V}_{CO_2} and ROS production cycles suggest an alternative explanation—that spiracular control has no effect on the levels of ROS production in a manner consistent with the expectations of the oxidative damage hypothesis (i.e. increasing ROS during or just after the O phase). This could occur for two potential reasons. First, if fluid filling in the tracheoles reacted as fast (or faster) as the CM-H₂XROs marker as a response to hyperoxia then we might not expect any signal change. Alternatively, if the variation in spiracle activity does not directly translate into tracheal system oxygen levels owing to some stratification within the tracheal system, one

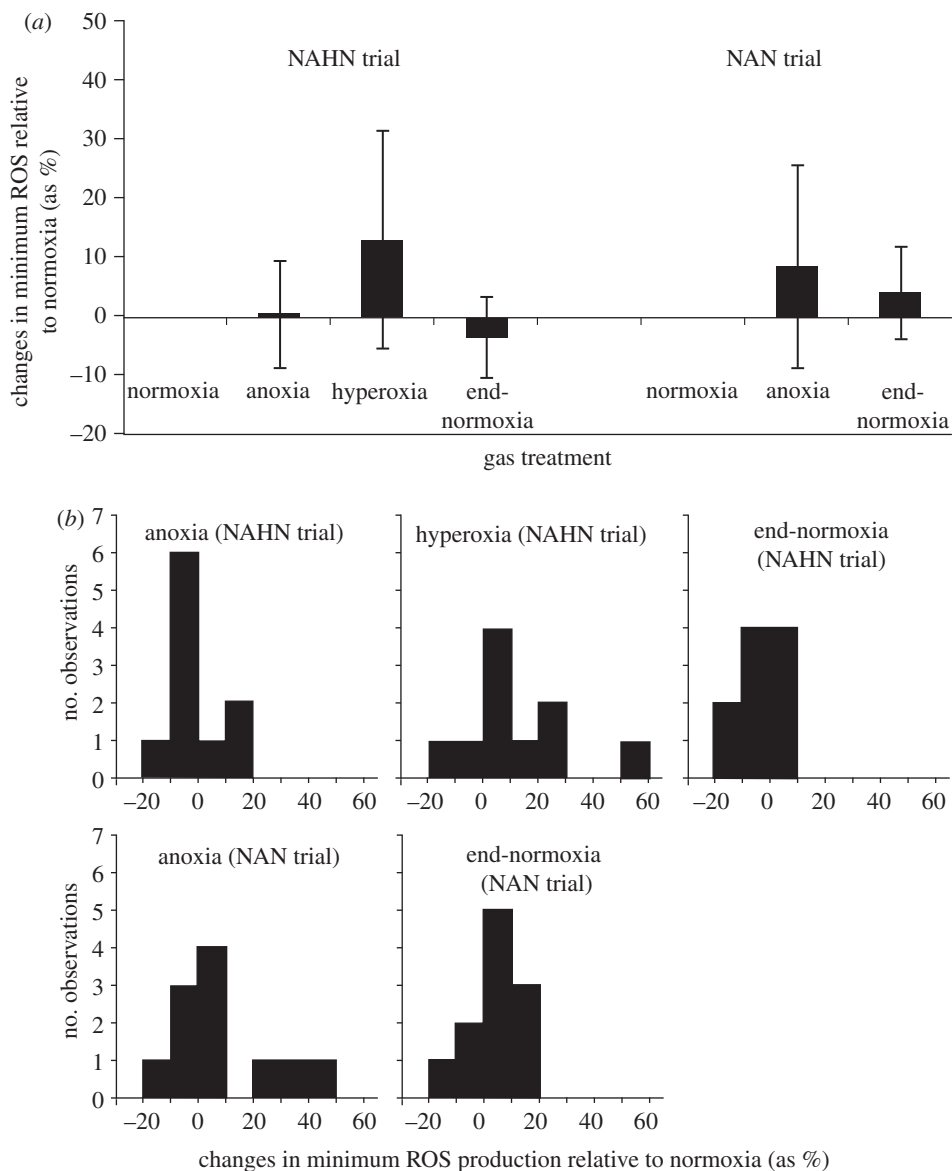


Figure 3. (a) Box plots showing mean (whiskers = s.d.) changes in ROS production relative to normoxic values, during each gas phase, for both NAHN and NAN trials. Differences between the gas trials were not significant (Kruskal–Wallis $H_4 = 2.057$, $p = 0.72$). (b) Histograms showing the variance of the mean changes in relative ROS production, which was significant and a result of the hyperoxia treatment (Levene's $F_4 = 6.07$, $p < 0.001$).

might not expect a change in mitochondrial ROS production. In both of these cases, the link between DGC and ROS production would not be established, leaving the chain of reasoning underlying the oxidative damage hypotheses incomplete. Why then would the \dot{V}_{CO_2} and ROS production cycles show similar phases and the effects of experimentally induced hyperoxia on minimum ROS production, ROS variance and ROS skewness be so clear? One explanation is that ROS production is not regulated by discontinuous gas exchange, but rather that ROS are involved in signalling during DGC. While little work in this context has been done on insects, the significance of ROS signalling is well documented [7,51]. For example, studies have shown that in response to hypoxia, mitochondrial O_2 sensing induces an increase in mitochondrial ROS signalling (production) to elicit a functional response [52–55]. The increase in ROS may also function to stabilize the 1α subunit of hypoxia-inducible factor (HIF-1)—necessary for the upregulation of hypoxia-responsive genes—that is degraded rapidly in normoxia

[56,57]. Given that similar signalling systems may be present in insects [58,59], during hypoxia and normoxia ROS may act as a signal for oxygen concentration at the cellular level. During hyperoxia, the entire system may be overwhelmed, so leading to increased variance and skewness in ROS.

Our work does not allow us to distinguish fully among these alternatives, although it seems somewhat more consistent with the signalling role for ROS than with the idea that the DGC acts to regulate ROS [17]. In this case, it provides further motivation to investigate the recent suggestions that energy savings and interacting control systems may explain the origin and maintenance of the DGC [21,22,40]. Moreover, it provides an additional, novel approach—live-cell fluorescence microscopy—that can be used to investigate these hypotheses and their competitors, including the oxidative damage hypothesis. These alternatives may be used in combination with other experiments that have been proposed as means to distinguish among current competing hypotheses for the origin and

maintenance of DGC [21]. Measuring the rates of ROS production in both intubated and non-manipulated pupae under 21 per cent O₂, less than 4 per cent O₂ and anoxia, breathing discontinuously and continuously would provide evidence as to whether spiracular regulation (by DGC) is critical to reduce ROS production, as well as testing the oxidative damage hypothesis. Whatever the outcome of such tests, it is clear that ROS and the curious gas exchange pattern known as discontinuous gas exchange are related in unexpected ways, resonating well with other findings about the regulation of oxygen and its role as molecule now critical for much of life (e.g. [1,2]).

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