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SHORT COMMUNICATION

Oxygen diffusion limitation triggers ventilatory movements during spiracle closure when insects breathe discontinuously

Shu-Ping Huang, Roi Sender and Eran Gefen*

ABSTRACT

During discontinuous gas exchange cycles in insects, spiracular opening follows a typical prolonged period of spiracle closure. Gas exchange with the environment occurs mostly during the period of full spiracular opening. In this study we tested the hypothesis that recently reported ventilatory movements during the spiracle closure period serve to mix the tracheal system gaseous contents, and support diffusive exchanges with the tissues. Using heliox (21% O_2, 79% He), we found that by increasing oxygen diffusivity in the gas phase, ventilatory movements of *Schistocerca gregaria* were significantly delayed compared with normoxic conditions. Exposure to hyperoxic conditions (40% O_2, 60% N_2) resulted in a similar delay in forced ventilation. Together, these results indicate that limits to oxygen diffusion to the tissues during spiracle closure trigger ventilatory movements, which in turn support tissue demands. These findings contribute to our understanding of the mechanistic basis of respiratory gas exchange between insect tissues and the environment.

KEY WORDS: DGC, Control, Ventilation, *Schistocerca gregaria*

INTRODUCTION

Insects exchange respiratory gases with their environment through their elaborate network of gas-filled trachea. These are ramified into small-diameter tracheoles whose tips lie in close proximity to individual cells in target tissues and organs. Most insects are able to control gas exchange through muscular control of their spiracles, segmental openings of the tracheal system. The ability to control spiracular opening gives rise to a variety of gas exchange patterns in insects. These have been traditionally categorized as continuous, cyclic and discontinuous gas exchange cycles (DGCs). The latter has been reported to be employed by species of at least five orders (Marais et al., 2005), in states of low metabolic rates (e.g. rest, low temperature). DGCs are typically composed of three consecutive phases characterized by spiracular activity (Chown et al., 2006): in the closed (C) phase, the spiracles are shut and gas exchange between the tracheal system and surrounding environment is negligible, while in the following flutter (F) phase the spiracles open and shut rapidly, with oxygen entering the body but minimal CO_2 released or water vapor lost from the body. In the open (O) phase, the spiracles open substantially, with rapid release of CO_2 and water vapor to the surrounding environment. The intermittent nature of gas exchange with the environment, when tissue respiration is continuous, means that partial pressures of respiratory gases in the tracheal system change during the course of DGCs, and in turn trigger spiracle opening/closure (Förster and Hetz, 2010). Despite decades of extensive research, the adaptive value of DGCs is still contentious (Chown, 2011).

In small insects, such as ants, respiratory gas transport between the tissues and the environment can be diffusive only (Lighton, 1996). However, adequate gas transport in the tracheal system of larger insects depends on active ventilatory movements (VMs) (Chown and Nicholson, 2004). In locusts, VMs include head and thoracic pumping, and the more common dorso-ventral and telescopic abdominal movements (Harrison, 1997). These movements increase the pressure exerted on the compliant air sacs and tracheal walls, and synchronization with spiracular opening results in ventilation of the large tracheal trunks (Harrison et al., 2013). Respiratory gas exchange during the O phase may or may not have to be supported by VMs (Chown et al., 2006). Interestingly, a recent study provided evidence for active VMs during the C phase of DGCs in the desert locust [*Schistocerca gregaria* (Forskål 1775)] (Groenewald et al., 2012). One of the suggested adaptive explanations for this behaviour was that VMs during the C phase in DGCs provide a convective transport mechanism that enhances oxygen transfer to the tissues. The aim of the present study was to test the hypothesis that VMs during the C phase serve to mix the tracheal gaseous contents in order to facilitate respiratory gas transfer between the tissues and major tracheal trunks. We measured the time interval between spiracle closure and the first bout of VMs under nitrox (21% O_2, 79% N_2) and heliox (21% O_2, 79% He) environments in *S. gregaria*. Diffusion rates are doubled in helium compared with nitrogen, and therefore we predicted a delay in VMs if tissue respiratory gas homeostasis was limited by diffusive transport from/to the major tracheal trunks. Both increasing CO_2 and decreasing O_2 partial pressure could trigger increased ventilation through central receptors (Bustami et al., 2002). As enhanced diffusion in heliox could affect oxygen transfer to, and CO_2 removal from, the tissues, we complemented our experiment with an additional set of measurements under hyperoxic conditions (40% O_2, 60% N_2). We hypothesized that increased tissue oxygen availability, under both heliox and hyperoxic environments, would delay the triggering of VMs for mixing the tracheal system content.

RESULTS AND DISCUSSION

Locust (*S. gregaria*) DGC traces at 20°C were characterized by continuous ventilatory activity during the O phase [or 'burst' of gas exchange (Lighton, 1996)], and intermittent bouts of ventilatory activity during the C and F phases (termed together as 'interburst') (Fig. 1). The metabolic rates of the locusts were not significantly affected by either experimental gaseous environment (heliox, P=0.88; hyperoxia, P=0.07). Mean CO_2 emission rates under normoxic conditions and during exposure to the experimental gaseous treatments ranged from 271.3 to 276.2 µl h^{-1} and 287.9 to 301.1 µl h^{-1} in the heliox and hyperoxia experiments, respectively. Presumably, tissue respiration rate is uninterrupted during the

Department of Biology and Environment, University of Haifa-Oranim, Tivon 36006, Israel.

*Author for correspondence (gefene@research.haifa.ac.il)

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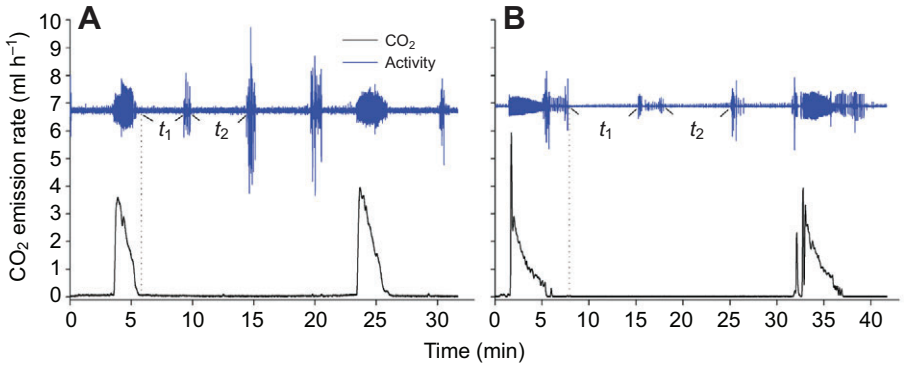

Fig. 1. Simultaneous recording of CO_2 emission and ventilatory activity in *Schistocerca gregaria*. (A) A typical trace of ventilatory activity (blue) and CO_2 emission (black) during discontinuous gas exchange cycles at 20°C (body mass=1.57 g). Amplitudes of the activity trace during ventilatory movements (VMs) are relative to periods of inactivity (horizontal blue line). t_1, time interval between the onset of spiracle closure (marked by vertical dotted line) and the first bout of VMs; t_2, time interval between the first two bouts of VMs. (B) Activity and CO_2 traces showing O-phase VMs persisting into the interburst (locust body mass=1.47 g). Under these circumstances, t_1 was calculated from cessation of that activity (marked by vertical dotted line).

interburst period. Nevertheless, we found significant treatment effects on the time interval from spiracle closure to the first VMs (t_1), and between the first and second VMs (t_2) during the interburst (Fig. 1). The first VMs observed in heliox (228.5±14.9 s) following the onset of the interburst were significantly delayed compared with normoxic conditions (169.0±14.5 s) (Z=–2.73, P=0.006, n=14). Likewise, a significantly longer time interval was measured between the first two bouts of activity in heliox compared with normoxia (Z=–2.85, P=0.004). Upon re-exposure to normoxic conditions, measured t_1 and t_2 values were not significantly different from the original normoxic values (t_1, Z=–1.53, P=0.12; t_2, Z=–0.87, P=0.38; Fig. 2A). This confirms that the delay in VMs was not a result of prolonged exposure to experimental conditions. The above results indicate that VMs are triggered when respiratory gas diffusion rate in the tracheal system during the interburst does not match tissue demands. This supports the hypothesis that VMs serve to mix tracheal gas contents during intermittent gas exchange with the environment (Groenewald et al., 2012).

Elevated CO_2 and low O_2 partial pressures have both been shown to result in increased VMs via central receptors (Bustami et al., 2002). As the manipulation of gas diffusion rates by heliox enhanced both rates of oxygen transfer from the tracheal trunks to the tissues and CO_2 removal from metabolizing tissues to the tracheal system, delayed VMs in heliox could be a result of changes in tissue oxygen and/or CO_2 levels. In the hyperoxia experiment, t_1 was delayed under hyperoxic treatment to 228.1±23.9 s compared with original normoxia (178.0±20.6 s) (Z=–2.66, P=0.007, n=15). A return to normoxic conditions resulted in t_1 similar to that recorded in the preceding exposure to normoxia (Z=–0.22, P=0.81; Fig. 2B). Together, these results indicate that a decrease in tissue oxygen levels rather than a buildup in CO_2 triggers VMs during spiracle closure. Although we did not find a significant treatment effect on metabolic rate, measured CO_2 emission rates during the hyperoxia exposure were slightly lower than those in normoxic levels (287.9±14.3 µl h^{-1} compared with 301.1±15.7 µl h^{-1}). Nevertheless, t_1 was not significantly correlated to mean metabolic rates in any of the experimental environments (P=0.27, 0.18 and 0.35 for normoxia, hyperoxia and second normoxia exposure, respectively), suggesting that delayed VMs in the hyperoxia treatment were unlikely to have resulted from lower rates of tissue respiration. Delayed t_1 values under both heliox and hyperoxia, compared with normoxic conditions, confirm that VMs during the closed phase of DGC serve to support tissue gas exchange by tracheal gas convection, and are triggered by decreasing oxygen levels in the tissues.

The effect of tracheal gas environment on t_2 was less conclusive in comparison to that on t_1. A treatment effect consistent with that of t_1 was maintained in the heliox experiment, whereas we did not observe a significant effect of the experimental gas on t_2 in the hyperoxia experiment (Fig. 2B). This may be a result of the variability in the intensity and/or duration of ventilatory muscle

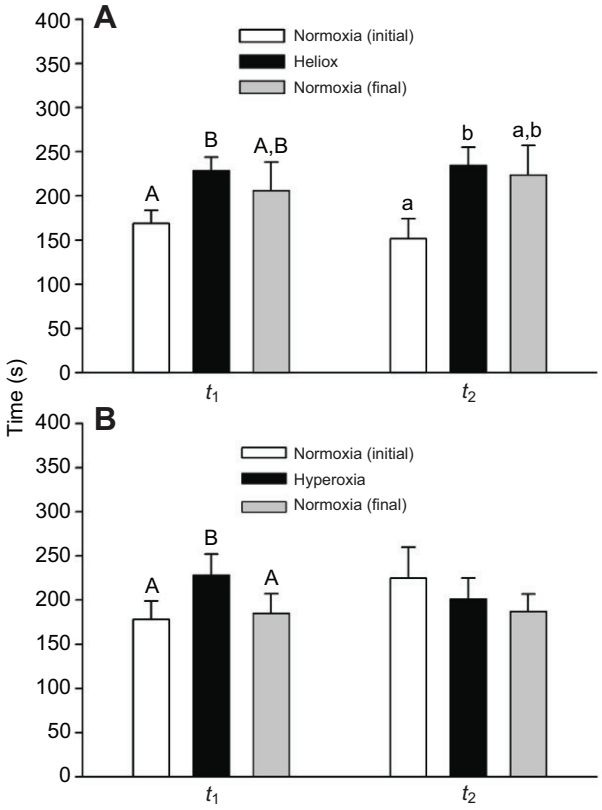

Fig. 2. Experimental gas treatment effects on time intervals between ventilatory movements. Effects of heliox (A; N=14) and hyperoxia (B; N=15) on the time interval (t_1; s) between spiracle closure and the first bout of ventilatory movements, and the following interval (t_2) between the first two bouts of activity (means + s.e.m.). Different letters indicate significant differences between compared groups.

activity. We could not account for the amplitude of muscular activity as the output of our detectors could be influenced by the insect's size and/or its position in the metabolic chamber against the detector. There was no significant treatment effect on VM duration in either experiment (range of median values: heliox experiment, 26.2–32.2 s, all $P>0.49$; hyperoxia experiment, 37.7–42.3 s, all $P>0.75$). However, we observed considerable variability in the duration of the first VMs in hyperoxia experiments (range between median minimal and maximal values: 21.1–63.7 s). This means highly variable extents of tracheal mixing for a given VM intensity, which would affect the time interval leading to a successive bout of VMs. In contrast, the onset of the interburst marks the return of respiratory gas levels in the tracheal system to initial values (Chown and Nicholson, 2004), and therefore provides a more consistent starting point for the subsequent t_1.

We provide the first evidence indicating that VMs mix the gas content in the tracheal system to aid oxygen transport to the tissues during the interburst phase of DGCs. This behaviour could be advantageous in locusts in particular, as a result of the high volume and compliance of their extensive air sacs (Harrison et al., 2013). We cannot rule out convective oxygen transport when spiracles are closed, resulting from gut peristalsis and/or heartbeat. However, our results still indicate diffusive limits to oxygen supply which trigger VMs during the interburst. Triggering VMs is suggested to be mediated by an oxygen-sensitive control mechanism. The location of presumed oxygen sensors and muscles participating in interburst-period VMs, as well as the underlying neural regulation, are not known and merit future investigation.

MATERIALS AND METHODS

Animals

Gregarious *S. gregaria* from stock populations held at the University of Haifa-Oranim were used in this study. Stock populations were fed fresh grass and dry oats *ad libitum* daily, and kept at 33.0±3.0°C, supplemented with light bulbs for thermoregulation. Male locusts were collected from stock population cages within 1–2 days of adult eclosion and were held gregariously in the laboratory at room temperature (~24°C) until experimentation within 1–2 weeks. All animals were isolated and starved for ~12–18 h prior to respirometry. Mean (±s.e.m.) body mass of the experimental animals was 1.48±0.14 g (n=14) and 1.53±0.22 g (n=15) for the heliox (21% O_2, 79% He) and hyperoxia (40% O_2, 60% N_2) experiments, respectively.

Experimental design

Respirometry and activity detection spanned 6 h, consisting of three consecutive 2 h gas–mixture treatments (control, experimental environment, control), where nitrox (21% O_2, 79% N_2) served as the control. A second nitrox–control period served to account for possible effects of prolonged exposure to experimental conditions, e.g. dry environment. In the heliox experiment, the locusts were exposed to normoxic conditions in helium (21% O_2, 79% He). In the hyperoxia experiment, animals were exposed to hyperoxic conditions (40% O_2, 60% N_2). Gas mixtures were achieved by mixing oxygen with either helium or nitrogen to a total of 250 ml min^{-1} using factory-calibrated mass flow controllers (Alicat, Tucson, AZ, USA). Experiments started by placing a locust in a 25 ml glass metabolic chamber that was placed in an activity detector (AD2, Sable Systems International, Las Vegas, NV, USA) consisting of an infrared transmitter (880 nm) and a receiver detecting reflected changes in radiation due to body movements. The chambers were placed on the detectors so that the transmitter and the receiver probes were located ventrally, between the second and third segments of the thorax, and between the second and third segments of the abdomen, respectively. Black cardboard sleeves were placed on both sides of the chambers to keep the animal under dark conditions. Following a 1 h acclimation period in nitrox at the experimental temperature (20.0±0.2°C; MIR-554 incubator, Panasonic, Gunma, Japan), the excurrent air was passed through a CO_2/H_2O dual analyzer (LI-7000, Li-Cor Biosciences, Lincoln, NE, USA).

Data collection and analyses

ExpeData software (Sable Systems International) was used for data acquisition and analysis. Readings of emitted CO_2 and VMs were taken every 0.1 s.

Gas exchange cycle phases were characterized as burst and interburst periods because the F phase could not be distinguished from the C phase using the CO_2 trace (Lighton, 1996). The last three consecutive cycles in each 2 h gas exposure were extracted for analyses. Occasionally, cycles were interrupted by movement and/or sudden changes in gas exchange pattern, leaving only two consistent cycles for analysis. All locusts exhibited DGC at the experimental temperature. Activity data from some experiments were very noisy because of animal movement within the chamber. These data were discarded and are not included in the analysis. The initiation of the interburst was determined by the return of the CO_2 trace to near-zero values (Fig. 1A). Occasionally, when O-phase VMs persisted into the interburst period (n=16 out of 243 cycles), t_1 was calculated from cessation of that activity (Fig. 1B).

Statistics

The VMs data from our heliox experiment did not satisfy the assumption of equal variance among treatments (Bartlett's test, P=0.001) required for parametric statistical analysis. Instead, we used Wilcoxon signed-rank tests to determine treatment effects (i.e. three gas–mixture exposures) on dependent variables, including t_1, t_2 and CO_2 emission rates. The significance level (α) was adjusted to 0.017 (=0.05/3) using Bonferroni correction to account for multiple comparisons. The effect of experimental gaseous environments on metabolic rates was tested using a linear mixed model with individual treated as a random variable and treatment as a fixed variable. All values are shown as means ± 1 s.e.m. All tests were performed using JMP software (SAS Institute, Cary, NC, USA).

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Competing interests

The authors declare no competing financial interests.

Author contributions

E.G. attracted funds, and conceived and designed the experiments. S.P.H. and R.S. performed the experiments. S.P.H. analyzed the data. E.G. and S.P.H. wrote the paper.

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