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RESEARCH ARTICLE

Intricate but tight coupling of spiracular activity and abdominal ventilation during locust discontinuous gas exchange cycles

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ABSTRACT

Discontinuous gas exchange (DGE) is the best studied among insect gas exchange patterns. DGE cycles comprise three phases, which are defined by their spiracular state: closed, flutter and open. However, spiracle status has rarely been monitored directly; rather, it is often assumed based on CO_2 emission traces. In this study, we directly recorded electromyogram (EMG) signals from the closer muscle of the second thoracic spiracle and from abdominal ventilation muscles in a fully intact locust during DGE. Muscular activity was monitored simultaneously with CO_2 emission, under normoxia and under various experimental oxic conditions. Our findings indicate that locust DGE does not correspond well with the commonly described three-phase cycle. We describe unique DGE-related ventilation motor patterns, coupled to spiracular activity. During the open phase, when CO_2 emission rate is highest, the thoracic spiracles do not remain open; rather, they open and close rapidly. This fast spiracle activity coincides with in-phase abdominal ventilation, while alternating with the abdominal spiracle and thus facilitating a unidirectional air flow along the main trachea. A change in the frequency of rhythmic ventilation during the open phase suggests modulation by intra-tracheal CO_2 levels. A second, slow ventilatory movement pattern probably serves to facilitate gas diffusion during spiracle closure. Two flutter-like patterns are described in association with the different types of ventilatory activity. We offer a modified mechanistic model for DGE in actively ventilating insects, incorporating ventilatory behavior and changes in spiracle state.

KEY WORDS: Tracheal system, Insects, Active ventilation, Spiracles, Electromyogram, Central pattern generator

INTRODUCTION

Discontinuous gas exchange (DGE) has been the most studied gas exchange pattern in insects since it was discovered and described in the 1950s and 1960s (Levy and Schneiderman, 1966a; Punt, 1950; Schneiderman, 1956, 1960). It has been reported in several insect orders (Contreras et al., 2014; Gray and Bradley, 2006; Marais et al., 2005; White et al., 2007), as well as in other tracheated arthropods, such as centipedes, ticks and solifuges (reviewed in Chown, 2011). In insects, the pattern was reported to be limited to periods of quiescence (e.g. diapausing lepidopteran pupae or resting adult insects; see Matthews and White, 2011a) or low metabolic rate (Contreras and Bradley, 2009).

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Based mostly on studies of diapausing lepidopteran pupae, DGE cycles are described as comprising of three phases, defined by the state of the spiracles: the closed (C), flutter (F) and open (O) phases (Levy and Schneiderman, 1966a). However, spiracle behavior has rarely been monitored, but instead was often assumed based on recorded respiratory gas traces. Unlike lepidopteran pupae (and very small insects), which rely on gas diffusion (Krogh, 1920) or passive convection resulting from sub-atmospheric tracheal pressures during DGE (Levy and Schneiderman, 1966b), diffusion alone may be insufficient for larger and more metabolically active insects. Furthermore, insects that rely on diffusion during DGE may also switch to active ventilation (and even to a continuous gas exchange pattern) during higher metabolic demands (e.g. Lighton and Lovegrove, 1990). Hence, many insects actively ventilate their tracheal system during periods of high metabolic demand (e.g. abdominal pumping; see Chown and Nicolson, 2004).

Orthopterans (and also Blattodea to some extent) have been used extensively for studies of the neural control of ventilatory motor activity. Early studies focused on the ventilatory central pattern generator (CPG) and its coordination with spiracle activity in locusts (reviewed in Burrows, 1996; Miller, 1966, 1981). Ample literature is also available on the effects of activity, hemolymph pH and P_{CO_2}, and of tracheal respiratory gas partial pressures, on the control of ventilatory motor patterns in locusts (reviewed in Harrison, 1997). Relatively little attention, however, has been given to ventilatory activity and its control during DGE.

Despite active ventilation having been found in a wide range of insect orders, the O-phase during DGE was often assumed to be diffusive (e.g. Grieshaber and Terblanche, 2015). Kestler (1985) was among the first to report that large insects, such as cockroaches and grasshoppers, actively ventilate their tracheal system during the DGE O-phase. He described consecutive opening and closing events, which he referred to as saw tooth-like, suggesting that shortening the O-phase is a strategy employed to minimize diffusive water loss (Kestler, 1985; but see Talal et al., 2015). Later, it was shown that the beetle *Psammodes striatus* exhibits ventilation throughout the O-phase (Lighton, 1988). Moreover, Lighton (1988) estimated that only ~55% of the total CO_2 emission during the O-phase was caused by diffusion. Additionally, following careful inspection of the F-phase, Hadley and Quinlan (1993) demonstrated that active ventilating grasshoppers lack the true F-phase as was described in lepidopteran pupae. By carrying out separate, simultaneous respirometry from anterior and posterior body parts, Duncan and Byrne (2002) and Byrne and Duncan (2003) demonstrated that wingless dung beetles use unidirectional, posterior to anterior, airflow during DGE. Heinrich et al. (2013) were the first to demonstrate by way of direct spiracle monitoring that cockroaches use unidirectional active ventilation through their bodies during the O-phase. Other studies of actively ventilating insects, examining ventilation during DGE, have been somewhat less attentive to this point (Groenewald et al., 2012; Matthews and White, 2011b).

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Groenewald et al. (2012) monitored endo-tracheal pressure in locusts and found that they exhibit active tracheal system pumping during the interburst phase (C- and F-phases). Subsequently, it was shown that limited oxygen diffusion from the major trachea to the tissues may trigger this ventilatory behavior and the mixing of tracheal gas content during the interburst phase (Huang et al., 2014).

Recently, Slama and Santiago-Blay (2017) demonstrated that different lepidopteran pupae also exhibit active ventilation movements during DGE. Furthermore, they found that pupae of *Cossus cossus*, the species on which the classical 'Krogh's diffusion theory of insect respiration' was based almost 100 years ago, also exhibit abdominal ventilatory movements. Hence, the growing evidence of active ventilation (in some cases unidirectional) throughout the entire DGE cycle in several insect orders may not be explained by the classic DGE model.

The aim of the current research was to study the active ventilation mechanism that underlies DGE in locusts. To this end, we developed a novel setup enabling the use of flow-through respirometry simultaneously with electromyogram (EMG) recordings, in a fully intact locust, during DGE. Recording the activity of the closer muscle of the second thoracic spiracle and that of the abdominal ventilation muscles, simultaneously with the monitoring of CO_2 emission, provided us with novel insights into the control of ventilation motor patterns and their interactions with spiracular activity in insect DGE. We further investigated the effect of P_{O_2} on the different recorded motor patterns by exposing the locusts to different experimental hypoxic levels, as well as to hyperoxia and normoxia.

MATERIALS AND METHODS

Experimental insects

Unless noted otherwise, in this study we used desert locusts, *Schistocerca gregaria* Forsskål 1775, from stock populations at the University of Haifa-Oranim (originated at Tel Aviv University), which were kept at 33.0±3.0°C under a 14 h light:10 h dark photoperiod (supplementary radiant heat was supplied during the daytime by incandescent 40 W electric bulbs). Locusts were fed daily with wheat shoots and dry oats *ad libitum*. All experiments and measurements were carried out on males only, 1–2 weeks after adult eclosion. Locusts were acclimated for at least 3 days to the experimental conditions (MIR-554 incubator, Panasonic, Japan: 30.0±0.5°C, 14 h light:10 h dark) and were denied access to food 12–24 h prior to experiments.

Simultaneous respirometry and EMG recordings

In order to study the motor patterns underlying DGE, we used custom-built metabolic chambers, enabling simultaneous electrophysiological and respirometry recordings from a fully intact preparation. We modified Hoyle's preparation (Hoyle, 1959) of EMG recording from the closer muscle of the second thoracic spiracle: briefly, instead of cutting a window in the cuticle and exposing the tracheal trunks of the second spiracle, we drilled a tiny hole where the spiracle closer muscle is anchored to the cuticle, using a coated 50 µm diameter tungsten wire, which was also used as an electrode (Fig. 1Ai). In addition, we simultaneously recorded muscle junction potentials from the abdominal expiratory pumping muscles (third abdominal segment) using coated 75 µm silver electrodes (Fig. 1Aii). The electrodes were attached to the cuticle with stamp wax for stability. Following electrode insertion, the locust was placed in a 40 ml cylindrical chamber (made from a 60 ml syringe with the plunger pushed to the 40 ml mark) with two drilled narrow holes for inserting the electrode wires, which were then sealed with Plasticine (Fig. 1B). Flow-through respirometry was carried out at 20°C in the dark, in order to reduce activity and thus increase the likelihood of DGE exhibition. Gas mixtures of different oxygen concentrations (in N_2) were generated by using two mass flow controllers (MC-500SCCM-D; Alicat Scientific, Tucson, AZ, USA) for a total flow rate of 400 ml min^{-1}. Locusts were acclimated to the metabolic chamber, the measurement temperature (20°C) and the flow rate for 1 h prior to initiation of respirometry measurements. CO_2 emission rates ($\dot{V}_{CO_2}$) were measured by passing excurrent air through a LI-7000

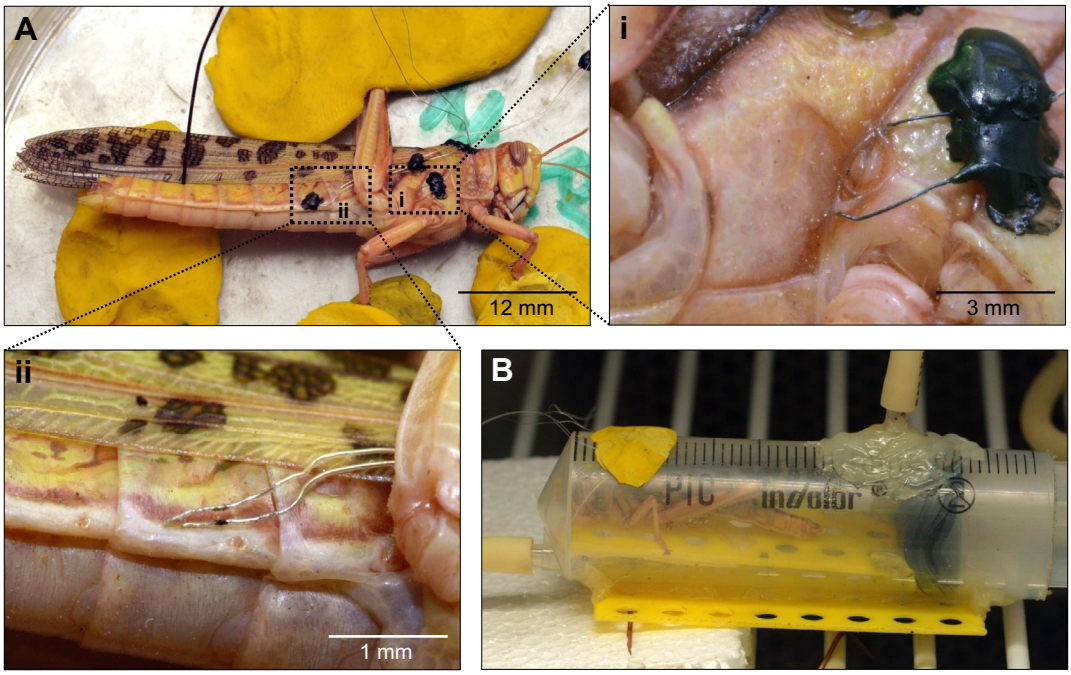

Fig. 1. Preparation of locusts for simultaneous recording of electromyogram (EMG) and respirometry signals. (A) Sites of EMG electrode insertion: (i) closer muscle of the second thoracic spiracle; (ii) expiration muscles of the third abdominal segment. (B) Locust placed in a custom-made metabolic chamber for simultaneous respirometry and EMG recording.

CO_2 analyzer (LiCor, Lincoln, NE, USA) and the O_2 concentration was determined with an Oxzilla II oxygen analyzer (Sable Systems International, Las Vegas, NV, USA). Respirometry data were collected and analyzed using a UI-2 data acquisition interface and Expedata software (Sable Systems International). The EMG signals were amplified by a differential AC amplifier (AM1700, A-M Systems Inc., Sequim, WA, USA) and acquired with a high sample rate digitizer (5000 samples s^{-1} for each channel; NI USB-6211 DAQ, National Instruments, Austin, TX, USA) and LabVIEW acquisition software (National Instruments), and then analyzed offline in DataView10.6 (W. J. Heitler, University of St Andrews, UK).

In order to study the effect of P_{O_2} on the ventilation characteristics during the DGE pattern, we exposed individual locusts to different oxygen partial pressures (40.5, 15.2, 10.1 and 5.1 kPa), in a random order, starting and ending each experiment under normoxia.

For simultaneous recording of the activity of the closer muscle of the last abdominal spiracle, a third EMG electrode was used. For technical reasons (external morphology and small size of the last spiracle), this proved to be extremely challenging in *S. gregaria*, and thus we used several individuals of *Locusta migratoria* (Linnaeus 1758) for these experiments (based on preliminary evidence of the similar overall respiration-related behavior of the two locust species).

Values appear as means±s.e.m. throughout the paper.

RESULTS

DGE, ventilation patterns and spiracle activity under normoxia

We successfully recorded the DGE pattern together with its underlying muscle activity in 19 individuals (Fig. 2A). The spiracle closer muscle was characterized by high-frequency spiking activity relative to the time scale of the overall DGE cycle (based on the respirometry data). However, smoothing the rectified EMG recording (moving average) revealed the tight correlation between the DGE pattern, the spiracle rhythmic activity and the ventilation motor pattern (Fig. 2A).

The smoothed and rectified muscle signals revealed three different spiracle closer muscle activity patterns (defined by the mean level of spiking activity as low, intermediate and high) and two coupled ventilation activity patterns (fast and slow, defined by burst frequency) appearing during the different DGE phases. We found that during the O-phase, when CO_2 emission rate is the highest, the spiracles do not remain open; instead, we observed a lower mean activity of the closure muscle, characterized by rhythmic spiracle openings (Fig. 2B). This spiracular activity pattern coincided with in-phase fast abdominal ventilation bursts and alternated with the activity of the last abdominal spiracle (Fig. 3), thus facilitating a unidirectional flow of air through the body. There was a consistent 0.2±0.06 s delay between thoracic spiracle closure bursts and ventilation bursts, presumably serving to secure sealing of the thoracic spiracles in order to prevent air backflow when abdominal pressure increases. The average ventilation burst frequency during the O-phase was 0.81±0.14 Hz, accompanied by 136.3±12.6 spiracle opening events. However, the ventilation frequency throughout the O-phase did not remain constant but, rather, decreased, usually with some delay after the beginning of ventilation (Fig. 4).

During the DGE interburst (between bursts of substantial CO_2 emission), a second type of ventilation pattern was observed, which

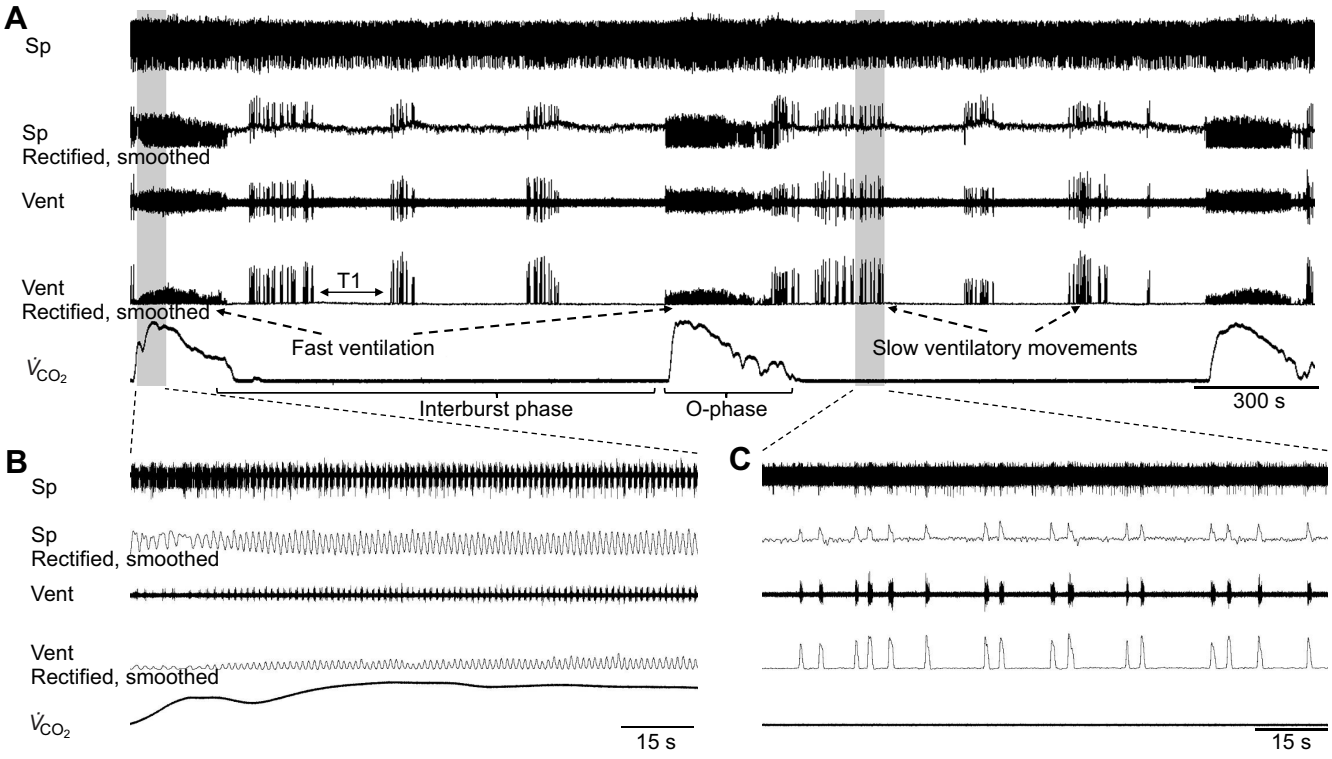

Fig. 2. Simultaneous CO_2 emission and EMG recording during discontinuous gas exchange (DGE). CO_2 emission rate ($\dot{V}_{CO_2}$) is shown without units, for qualitative presentation only. Raw EMG traces show recordings from the closer muscle of the second thoracic spiracle (Sp) and expiratory muscles of the third abdominal segment (Vent). Bursts of spikes of spiracle closer muscle activity indicate spiracle closure whereas bursts of spikes of ventilatory muscle activity indicate abdominal constriction. Positive rectified and smoothed signals are also shown. (A) Two complete DGE cycles. Areas shaded gray are magnified in B [fast ventilation event during the open (O) phase] and C (slow ventilatory movement event during the interburst phase). T1 denotes the time difference between the first two slow ventilatory movement events within the same interburst phase (after Huang et al., 2014).

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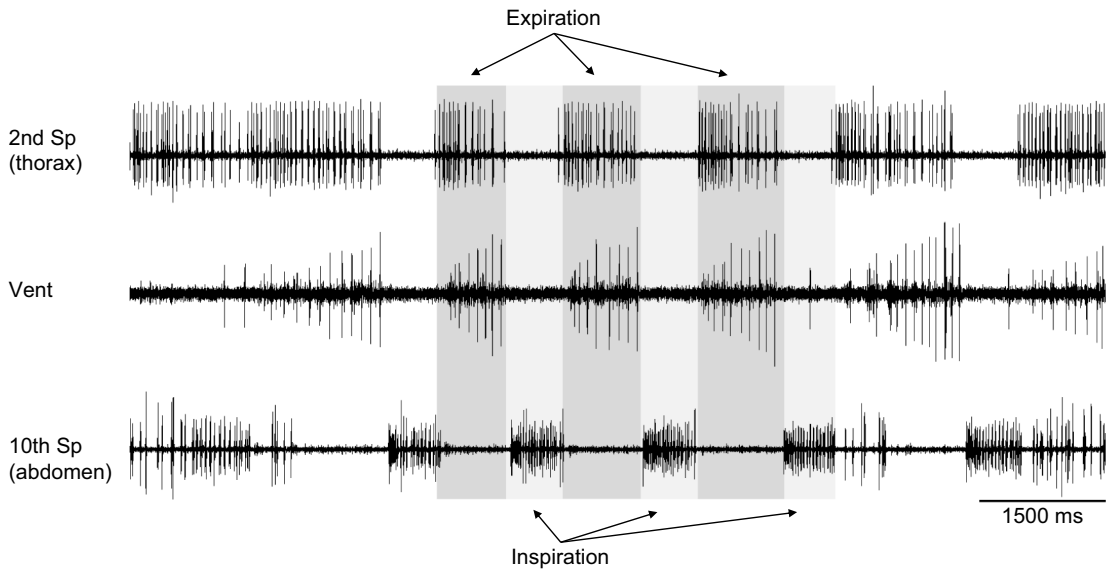

Fig. 3. Muscle activity during unidirectional air-flow ventilation through the locust body. An example of simultaneous EMG recording from the closer muscle of the second thoracic spiracle (2nd Sp), expiratory muscles of the third abdominal segment (Vent) and the closer muscle of the last abdominal spiracle (10th Sp) of *Locusta migratoria* during unidirectional ventilation (see Materials and methods for details). Bursts of spikes of spiracle closer muscle activity indicate spiracle closure whereas bursts of spikes of ventilatory muscle activity in muscles indicate abdominal constriction.

we defined as slow ventilatory movements (Fig. 2A,C). This comprised trains of several dozen to several hundred ventilation bursts, the duration of which varied between individuals, appearing up to three times during the interburst phase. While the regular spiracle closure muscle activity during the interburst phase was characterized by an intermediate spike frequency, each slow ventilatory movement burst was accompanied by much higher activity of the spiracle closer muscles, bringing about tighter spiracle closure (Fig. 2C). In contrast to the O-phase fast ventilation, the ventilatory muscle activity during slow ventilatory movements was characterized by a much higher burst amplitude and lower burst frequency (0.23±0.03 Hz) (Figs 2 and 5). As demonstrated in the example shown in Fig. 5, the slow ventilatory movement events could occur throughout the interburst phase, in the middle (Fig. 5Biii) but also near its end, just prior to the O-phase (and a fast ventilation event; Fig. 5Bii and Biv). These different occurrences had a clear effect on the spiracle closer muscle activity and on CO_2 emission: at the onset of slow ventilatory movement bursts preceding the O-phase, the high spiracle closer muscle activity (tightly closed spiracle) immediately decreased to a lower level (compared with that of the regular closed state), resulting in a low rate of CO_2 emission. We termed this activity slow ventilation flutter (SVF) phase, and it is reminiscent of the classic flutter phase that appears in every cycle in lepidopteran pupae (Levy and Schneiderman, 1966b). Moreover, the duration and shape of these low-emission CO_2 events depended on the number of bursts (or the number of 'small' openings) and burst rate of the slow ventilatory movements (Fig. 5). In contrast, the O-phase (and fast ventilation events) that began without prior slow ventilatory movements was not accompanied by any notable emission of CO_2 (compare the CO_2 trace in Fig. 5Bi with that in Fig. 5Bii and Biv). Slow ventilatory movement events earlier in the interburst were not associated with spiracle closer muscle relaxation, and thus with CO_2 emission (Fig. 5Biii). Further to the described interactions between

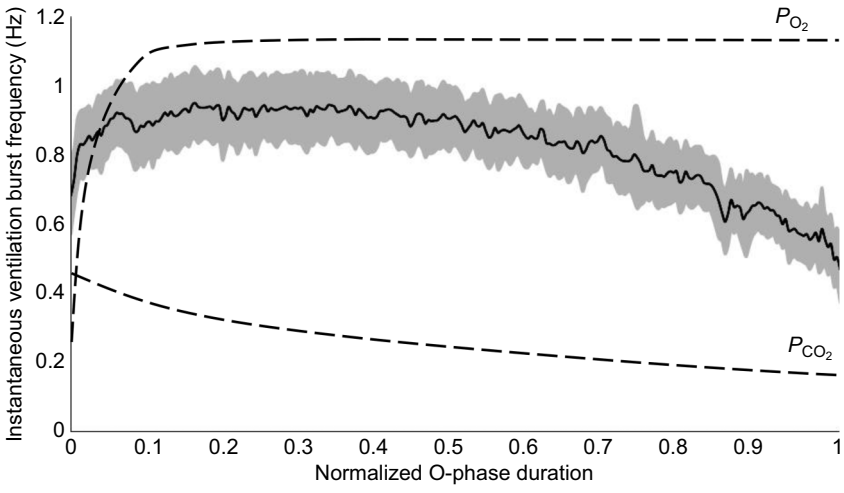

Fig. 4. Modulation of fast ventilation throughout the O-phase. Fast ventilation frequency (±s.d.) as a function of normalized O-phase duration. Dashed lines represent changes in P_{O_2} and P_{CO_2} during the O-phase (after Levy and Schneiderman, 1966a).

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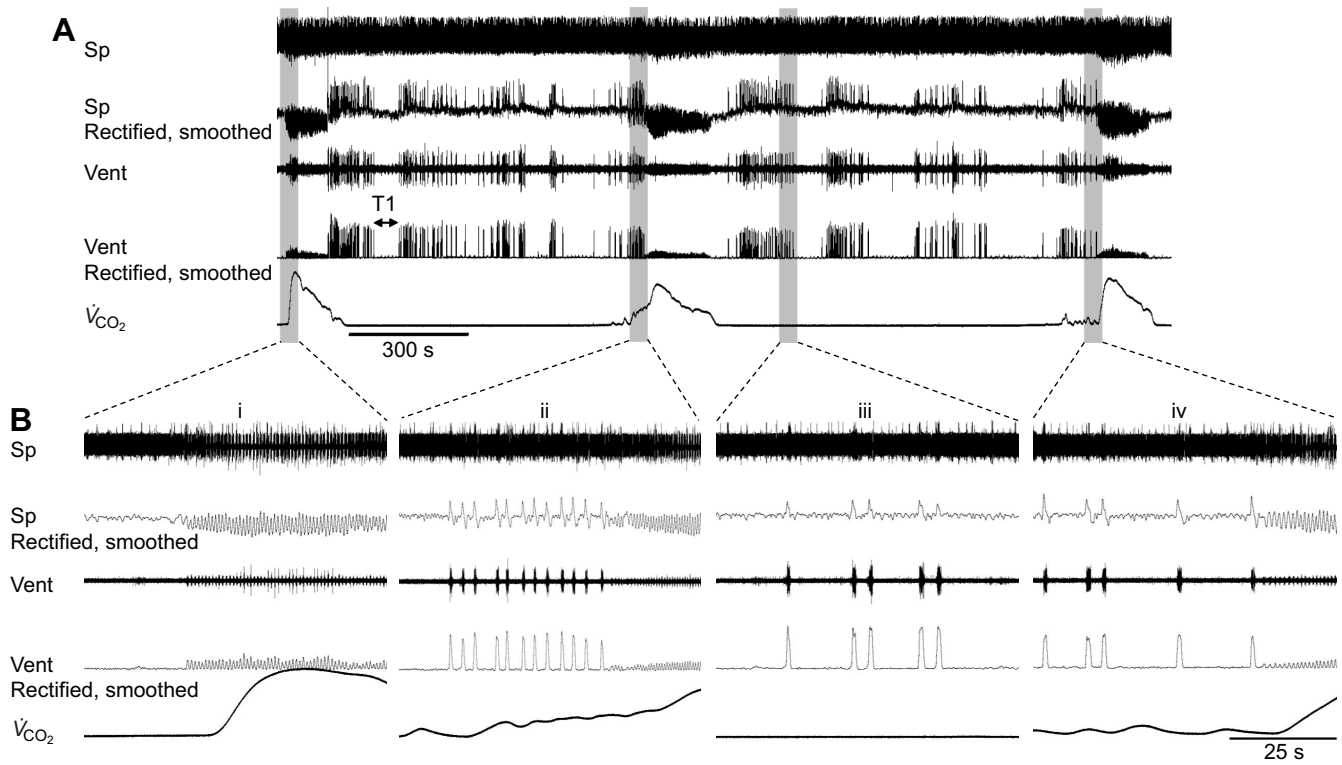

Fig. 5. Slow ventilatory movement events during different phases of the DGE cycle and their effect on CO_2 emission. (A) Two complete DGE cycles. T1 denotes the time difference between the first two slow ventilatory movement events within the same interburst phase (after Huang et al., 2014). (B) Magnification of different DGE cycle events (shaded gray) and the effect of the slow ventilatory movements on CO_2 emission ($\dot{V}_{CO_2}$): (i) beginning of a fast ventilation event with no slow ventilatory movements; (ii and iv) slow ventilation flutter (SVF) observed just prior to fast ventilation events; (iii) a slow ventilatory movement event that occurred earlier during the interburst phase.

the ventilation motor patterns, spiracular muscle activity and the CO_2 emission pattern, the two types of ventilatory rhythms (fast and slow) seemed to be independent, i.e. they occurred in parallel, following one another closely or even overlapping (Fig. S1).

Some interactions, however, could be discerned when monitoring the activity of the spiracular closer muscle during periods of spiracle closure (interbursts), when no ventilation was apparent. Careful inspection revealed that the muscle spiking activity was not constant but, rather, changed from high-frequency spiking to low and vice versa (Fig. S2). These changes demonstrated a rhythm reminiscent of the fast ventilatory rhythm. Moreover, at the very beginning of the O-phase (and sometimes at its termination) when the spiracles open and close rapidly, an intermediate state was seen, where low spiking activity could be seen between closure bursts (Fig. S2).

DGE, ventilation and spiracle activity at different oxygen levels

Changes in oxygen availability affected DGE cycle properties. Fig. 6 presents one example in which the P_{O_2} was gradually and continuously altered during the experiment. In all other experiments

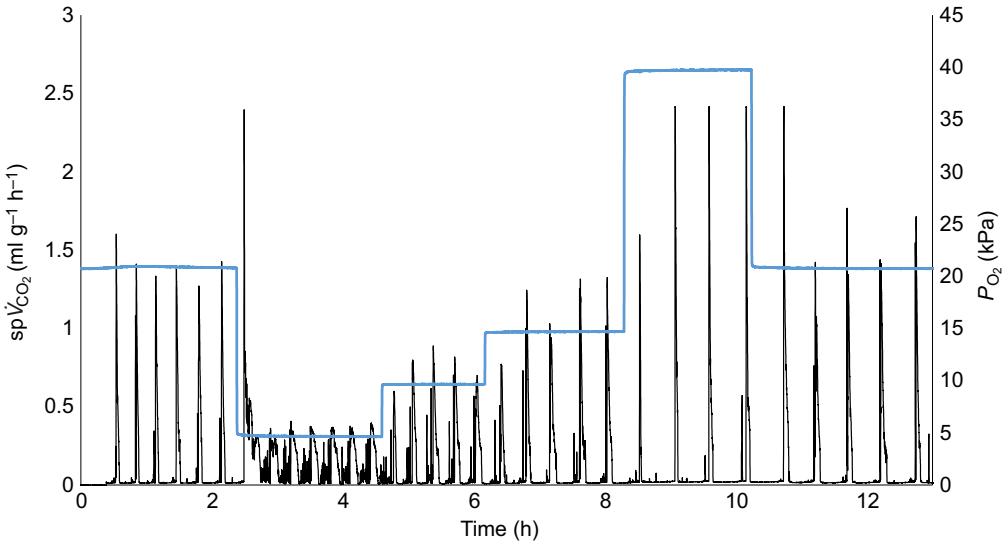

Fig. 6. Effect of O_2 availability on DGE. An example of the effect of ambient P_{O_2} (blue trace) on CO_2 emission ($\dot{V}_{CO_2}$; black trace) and on the DGE pattern in a male *Schistocerca gregaria* (body mass=1.75 g).

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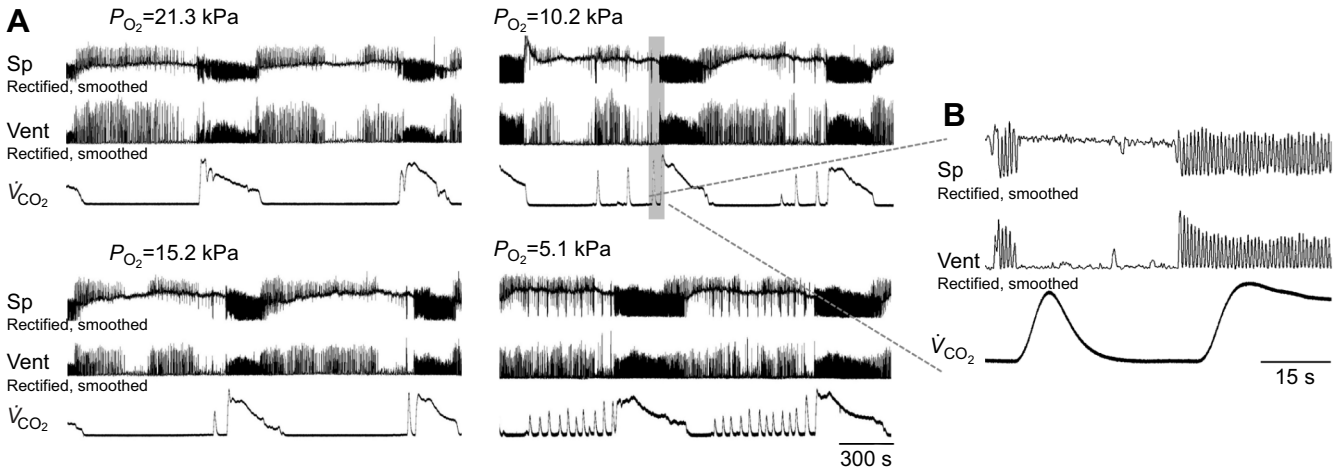

Fig. 7. Effect of O_2 availability on the appearance of the fast ventilation flutter (FVF) phase. (A) The effect of hypoxic conditions on FVF, appearing as short fast ventilation events followed by short bursts of CO_2 emission prior to the O-phase (magnified in B).

(N=19 preparations), the order of changes was random. The outcome of exposure to hypoxic levels was the appearance of short, high-rate CO_2 bursts prior to the O-phase. We termed this flutter-like activity, rarely seen in normoxic animals, fast ventilation flutter (FVF) phase (Fig. 7). Lowering ambient oxygen availability resulted in an increase in the number and frequency of these CO_2 bursts (Figs 7A and 8C), as well as in the overall duration of the FVF phase (Fig. 8A). In contrast to the SVF phase described above (Fig. 5; and that of lepidopteran pupae), FVF events were characterized by several fast ventilation cycles, similar in amplitude and frequency to the O-phase fast ventilation (from 3 to 10 ventilation cycles/spiracle openings and closings; Fig. 7B).

Unlike the fast ventilation events, which were always coupled with spiracle openings, the coupling of slow ventilatory movements with spiracle muscle activity varied during the DGE cycle. Whereas the spiracles opened and closed during slow ventilatory movement events just prior to the O-phase (leading to SVF), they remained tightly closed during slow ventilatory movement events earlier in the interburst phase. The time between the first two such slow ventilatory movement events during the interburst (T1 in Figs 2A

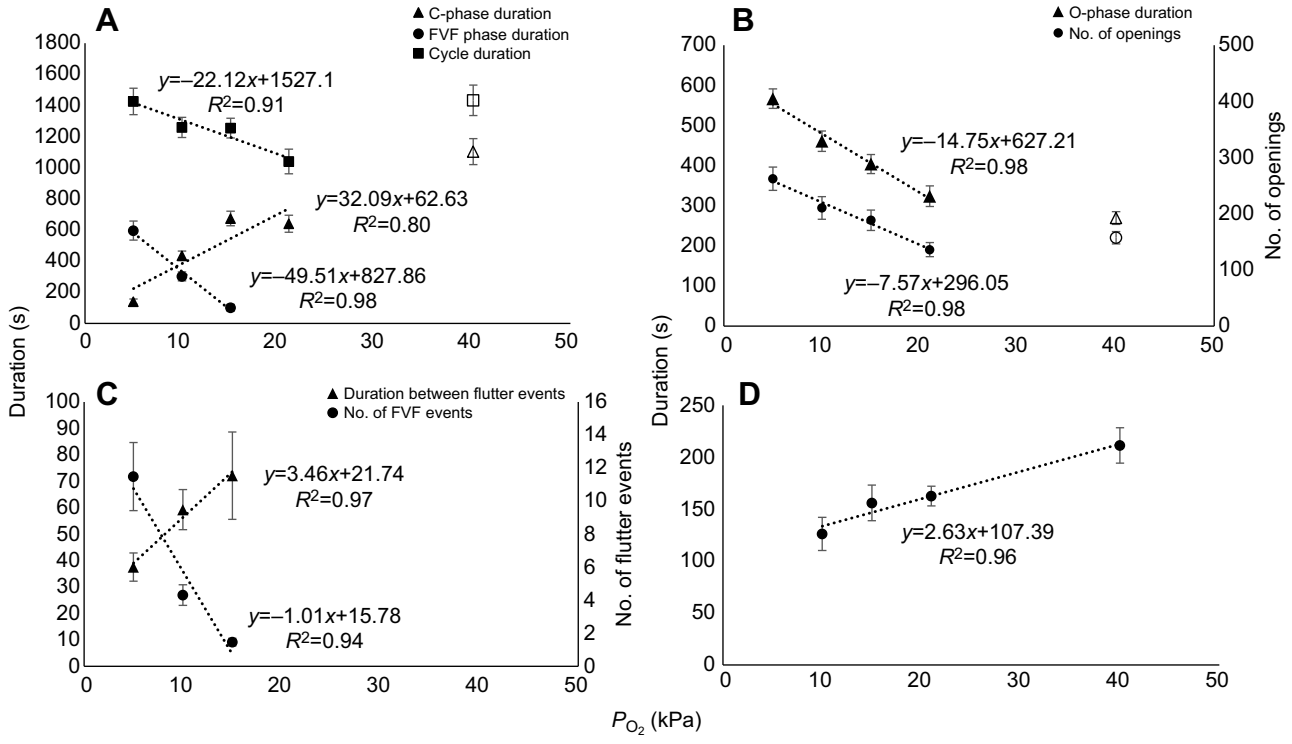

Fig. 8. Effect of P_{O_2} level on different DGE and ventilation properties. (A) The duration of the closed (C) phase (triangles), FVF phase (circles) and overall cycle (squares). (B) The O-phase duration (triangles) and the number of spiracle opening/ventilation movements during the O-phase (circles). (C) Time difference between FVF ventilation events (triangles) and the number of FVF ventilation events during the flutter (F) phase (circles). (D) The time difference between the first two slow ventilatory movement events (T1) (see Results for detailed description). The open symbols signify the same parameters during hyperoxic conditions. Values are means±s.e.m.

and 5A; after Huang et al., 2014) was positively correlated with P_{O_2} (Fig. 8D).

The duration of the C-phase was positively correlated with P_{O_2}, whereas the overall cycle duration as well as the duration of the F- and the O-phases were negatively correlated with P_{O_2} (Fig. 8A, B). In addition, the number of spiracular openings during the O-phase (fast ventilation) showed a negative correlation with P_{O_2} (Fig. 8B), while the mean opening frequency remained almost unaffected [opening frequency (Hz)=−0.001×P_{O_2}+0.85; R^2=0.33]. The DGE cycle characteristics were affected by hyperoxia but to a lesser degree compared with other oxygen environments (Fig. 8A,B).

It should be noted that the prolonged recordings, which sometimes lasted more than 12 h, had a significant effect on the metabolic rate. The metabolic rate (as expressed by mass-specific $\dot{V}_{CO_2}$) was 154±7 µl h^{-1} g^{-1} at the beginning of the measurements (normoxic conditions) and decreased by ∼7% when the locusts were re-exposed to normoxia (143±7 µl h^{-1} g^{-1}) at the end of each experiment (paired *t*-test, t_{15}=3.26; P=0.005). However, the random order of exposure to the various oxygen levels throughout the experiments suggests that the changes in DGE properties reflected a response to changing oxygen availability, and are unlikely to have been systematically skewed by measurement duration effects. The difference in metabolic rate between the normoxic conditions (beginning and end of each experiment) resulted in an increase in C-phase duration (671.5±66.3 and 903.2±79.1 s, respectively; paired *t*-test, t_{15}=3.21; P=0.006), but did not affect the O-phase duration (298.4±23.1 and 308.5±15.6 s, respectively; paired *t*-test, t_{15}=0.48; P=0.641). Ambient oxygen levels were altered mostly during interbursts, and resulted in significantly higher CO_2 emission during the subsequent first O-phase when switching to more hypoxic conditions. Switching from normoxia to 15.2 kPa, 15.2 kPa to 10.1 kPa, and 10.1 kPa to 5.1 kPa, transiently elevated CO_2 emission by 30%, 24% and 38%, respectively (paired *t*-test, t_5=11.02, t_8=4.13, t_7=10.44, respectively; P<0.001). The response to changes towards higher P_{O_2} values had the opposite effect on CO_2 emission.

DISCUSSION

We report here our experimental results from utilizing a novel preparation enabling the combined use of respirometry and electrophysiology recording techniques. This preparation allowed us to investigate and correlate ventilatory and spiracular activity during DGE, and to determine their combined effect on gas exchange between the insect tissues and the environment. Under normoxic conditions, locust DGE is characterized by intermittent bursts of gas exchange (O-phase) separated by periods during which there is practically no gas exchange with the environment (interburst phase). During the O-phase, locusts quickly ventilate their main tracheal trunks, and this ventilation motor pattern is strongly coupled with fast opening and closing of the spiracles to create unidirectional air flow through the body. In contrast, the interburst phase is characterized by bouts of slow ventilatory movements, but these are typically coupled with tight spiracular closure. This probably facilitates efficient diffusion from the main tracheal trunks and air sacs and through the finest tracheoles by mixing tracheal gases (Huang et al., 2014). Occasionally, slow ventilatory movement events, immediately prior to the O-phase, were coupled with spiracle muscle relaxation and detectable CO_2 emission (SVF; Fig. 5Bii, Biv).

The classical/lepidopteran O-phase is defined as one prolonged spiracle opening event, during which gas exchange with the environment occurs almost exclusively by diffusion (reviewed in Chown et al., 2006; Lighton, 1996; Matthews, 2017; Quinlan and Gibbs, 2006). The lepidopteran O-phase is triggered when rising endo-tracheal/hemolymph P_{CO_2} (or a decreasing pH) levels reach a threshold (Förster and Hetz, 2010; Levy and Schneiderman, 1966c). In contrast, in the locust, spiracles may open at a P_{CO_2} threshold, but continue to close and open at lower P_{CO_2} values (as CO_2 is being washed out during the O-phase), and despite the elevated tracheal P_{O_2} levels resulting from efficient fast ventilation (Matthews et al., 2012). A unidirectional air flow during the O-phase accompanied by alternated spiracle opening and closing was also reported in cockroaches (Heinrich et al., 2013). These observations are somewhat inconsistent with the DGE mechanistic/gas-sensing model (Burkett and Schneiderman, 1974; Förster and Hetz, 2010; Levy and Schneiderman, 1966c) that is often used in reference to DGE of active ventilating insects too (e.g. Grieshaber and Terblanche, 2015).

Unlike that in lepidopteran pupae (but see Slama and Santiago-Blay, 2017), the locust's O-phase is characterized by a fast rhythmic abdominal pumping motor pattern recorded from the ventilatory muscles. This rhythmic motor pattern is tightly coupled to the spiracle muscle activity. Throughout our experiments, this full synchrony was never broken (i.e. there was no uncoupling of ventilation and spiracle movements). Hence, we assume that the two behaviors are controlled by a common rhythmic input – the ventilation central pattern generator (CPG), which has been extensively studied in locusts (reviewed by Burrows, 1996; Miller, 1966, 1981). The presence and role of this CPG was also demonstrated in *in vitro* preparations, by way of alternation between activities recorded in the nerves innervating inspiration and expiration muscles, as well as between those innervating the anterior and posterior spiracles (Burrows, 1975a,b; Lewis et al., 1973; S.T., E.G. and A.A., unpublished).

Hence, the observed unidirectional air flow during ventilation (involving abdominal pumping and spiracle activity) seems to be hard wired in the system. However, the ventilation-controlling circuits have been shown to also have complex interactions with other motor systems (Hoyle, 1964; Kutsch, 1969; Miller and Mills, 1976; Paripovic et al., 1996; Ramirez, 1998; Zilberstein and Ayali, 2002). Studies of ventilation in other physiological contexts (e.g. flight: Miller, 1981; molting: Ramirez and Pearson, 1989a) suggest that the above-reported synchrony can be modulated, or even switched off, in accordance with a hierarchical relationship between the ventilation and spiracular motor patterns. Also of interest in this respect is Miller and Mills's (1976) account of a lack of synchrony between ventilatory movements of the abdomen and the spiracular motor pattern during early development (1st, 2nd and even 3rd instar hoppers). Spiracular opening at these stages is controlled by CO_2 levels. In the 3rd instar, the spiracles may alternate between coupled (with ventilation) and uncoupled states (Miller and Mills, 1976).

Our findings indicate two different ventilation-related motor patterns that occur in parallel, at different amplitudes and frequencies, and with different effects on spiracle state, suggesting their differential control, i.e. two CPGs differing in function and properties. This is again reminiscent of the work of Miller and Mills (1976), who suggested the existence of a pacemaker system in the locust metathoracic ganglion that controls a ventilation motor pattern that is slower than that described elsewhere. The motor output of this second CPG was revealed via two ventilatory rhythms displaying different periodicities during locust ecdysis. Additional studies of molting and post-molting behavior control in locusts

revealed two types of abdominal pumping: the first is described as regular fast ventilation, and the second as slow, with prolonged ventilation bursts (Hughes, 1980a,b). This slow motor pattern causes an internal pressure build up, expanding the body after molting (reviewed in Miller, 1981). Based on extracellular and intracellular recordings, Elliott (1982) suggested that the control of the two motor patterns is independent and that they originate in separate pacemakers. The slow ventilation CPG and the slow rhythm used to increase endotracheal pressure during post-molting body expansion at ecdysis may well be similar to the slow motor pattern observed in the current study, again increasing endotracheal pressure in order to mix respiratory gases and facilitate diffusion during the interburst phase (Groenewald et al., 2012; Huang et al., 2014).

What are the sensory inputs to the ventilatory CPGs and how are they modulated? Our results are consistent with previous studies in suggesting a dominant role for CO_2 sensing. We have shown that the frequency of the fast ventilation CPG starts to decrease during the O-phase, as was also demonstrated in cockroaches (Matthews and White, 2011b), but that this usually happens with some delay (Fig. 4). Tracheal oxygen pressure reaches near-atmospheric levels early on in the O-phase (Matthews et al., 2012), whereas hemolymph pH level increases slowly, which is an indication for slow CO_2 washout during the O-phase (Matthews and White, 2011b). It was suggested previously that modulation of locust ventilation is mediated by CO_2 sensing in the thoracic ganglia (reviewed in Harrison, 1997; Miller, 1966). Miller (1960) found that ventilation frequency could be dramatically increased by perfusion of CO_2 separately to each ganglion in the head and the thorax. It is possible that CO_2 has a direct effect on the CPG interneurons extending within each ganglion (Pearson, 1980; Ramirez and Pearson, 1989b).

Yet another aspect of the modulation of ventilation-related motor patterns is related to the inputs to the spiracular muscles, as reported by Burrows (1975a,b, 1982). Burrows found two pairs of interneurons that are innervated by the ventilation CPG: two interneurons that cause an excitatory postsynaptic potential (EPSP) in the closer motor neurons (of the four most anterior spiracles) during expiration, and two interneurons that cause an inhibitory postsynaptic potential (IPSP) in the closer motor neurons during inspiration. In addition to this mechanism resulting in opening and closing of the spiracles, Burrows found that some of these interneurons extend to the first unfused abdominal ganglion and take part in coordinating abdominal pumping (Burrows, 1975a,b, 1982). When the membrane potential of the inhibitory interneurons was manipulated by current injection, the inhibition level of the closer motor neuron was altered, resulting in changes in the burst activity (Burrows, 1982). These results (an inhibitory neuron modulating input) are in accord with our observation of the spiracle closer muscle activity in the different ventilation-related events during DGE (Fig. S2). Monitoring muscle activity during periods of spiracular closure (interburst) revealed that its spiking activity was also not constant but, rather, showed changes in a rhythmic manner reminiscent of the fast ventilation rhythm (Fig. S2). Hence, the fast CPG is constantly active, but during the interburst phase there are inhibitory mechanisms preventing the execution of a ventilation rhythm and spiracle openings. These inhibitory interneurons, in order to enable/disable the ventilation function, need in turn to receive gas sensory inputs. Such a mechanism would also explain the higher closer muscle activity during earlier slow ventilatory movement bursts (high level of EPSP on spiracle motor neurons, when low O_2 and intermediate CO_2 levels are detected in the tracheae), as well as the reduced activity of the closer muscle between slow ventilatory movement bursts (high level of IPSP on spiracle motor neurons, when low O_2 and high CO_2 levels are detected in the tracheae), which resulted in a low rate of CO_2 emission just prior to the O-phase.

Förster and Hetz (2010) proposed an integrated DGE model based on spiracle states, which are controlled by the interactions of two endo-tracheal gas sensory loops. Their model explains the time course of endo-tracheal P_{CO_2} and P_{O_2} during DGE and the alternation between C-, F- and O-phases, as exhibited by lepidopteran pupae and other non-active ventilating insects (Levy and Schneiderman, 1966a; Lighton and Garrigan, 1995; but see Slama and Santiago-Blay, 2017). The vast majority of published DGE-related studies have distinguished between these cycle phases based on the respective alleged three spiracular states. However, our recent findings clearly demonstrate that this model cannot apply to DGE phases in locusts, and perhaps in actively ventilating insects in general. During the O-phase, which is typically portrayed as a period of continuous spiracle opening, spiracles actually open and close at high frequency, as our EMG traces indicate. Moreover, any tracheal gas composition that may be responsible for triggering the O-phase is quickly altered as a result of vigorous active ventilation. As CO_2 is washed out to the environment, although tracheal P_{CO_2} drops below the threshold values responsible for triggering spiracle opening and the O-phase, intermittent closure and opening of the spiracles nonetheless continues, which has often been associated in the literature with the preceding 'flutter' phase.

Here, we propose a modified model for DGE in actively ventilating insects, presented in Fig. 9, showing hypothetical trajectories of endo-tracheal P_{CO_2} and P_{O_2} during DGE cycles in our different experimental oxygen environments. Crossing one of the thresholds causes a fast ventilation event and changes in tracheal gas partial pressures. The slow ventilatory movements, exhibited only during the C-phase, typically do not result in gas exchange with the environment. However, it affects the oxygen transport rate from the main tracheal trunks and can thus affect C-phase (spiracle closure) duration. An interaction has been shown between CO_2 and O_2 sensing in both directions of endo-tracheal O_2 concentration. Studying spiracle control in moth pupae, Schneiderman (1960) found that exposure to hyperoxia elevated spiracle CO_2 threshold level; a higher CO_2 level would thus cause the spiracles to open after a longer C-phase. It has also been found that hypoxia decreased the minimum endo-tracheal P_{O_2} level, which was monitored just prior to initiation of the F-phase in locusts (Matthews et al., 2012). Thus, based on the above and on our own results, the threshold lines in our DGE model are not parallel to the axes (Fig. 9). For example, during hyperoxic conditions, locusts exhibited a 60% longer C-phase, despite a similar rate of CO_2 production. This could be a result of an interaction between P_{O_2} and P_{CO_2} set points following the elevation of spiracle CO_2 threshold level at high P_{O_2} levels (Levy and Schneiderman, 1966c).

Because of efficient O-phase ventilation, we assume that in the early stages of the C-phase (Fig. 9; upper left side of each trajectory line), tracheal P_{O_2} is similar to atmospheric levels (Matthews et al., 2012). During the C-phase, the oxygen consumption results in a decrease in tracheal P_{O_2} while P_{CO_2} increases. In contrast to lepidopteran pupae, locusts rarely exhibit F-phase in normoxia, as the CO_2 (or pH in different insect orders; Farley et al., 1967; Snyder et al., 1980) threshold is reached before the O_2 threshold, thus initiating a prolonged fast ventilation event (O-phase) (see Huang et al., 2015; Matthews et al., 2012). The difference in endo-tracheal dynamics between locusts and lepidopteran pupae could be explained by the difference in tracheal system volume (2-fold higher in locusts

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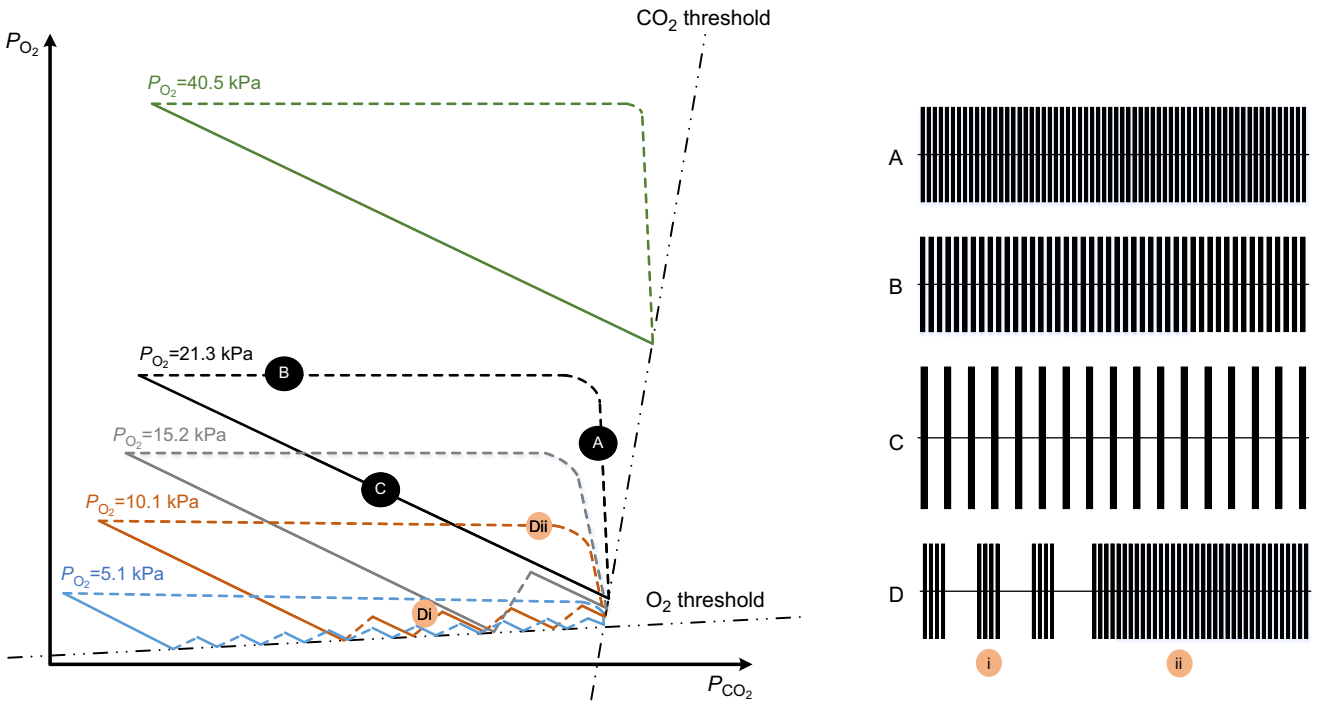

Fig. 9. A model for the control of ventilatory movements and endo-tracheal gas sensing during DGE in actively ventilating insects. Modified from Förster and Hetz (2010). Different trajectories describe the effect of different oxygen levels on the DGE properties. Solid lines represent periods of spiracle closure and dashed lines correspond to periods of simultaneous activity of spiracular muscles and fast ventilation. Schematically drawn ventilatory activity patterns (right) describe: (A) higher frequency fast ventilation in the first part of the O-phase; (B) lower frequency fast ventilation in the second part of the O-phase; (C) slow ventilatory movements with higher amplitude which occurs during the interburst (closed spiracles); (D) the FVF phase that occurs in hypoxia and is characterized by short fast ventilation (i) events prior to the prolonged fast ventilation event (ii) (O-phase).

per gram) and whole-body buffer capacity (2- to 3-fold higher in pupae) (see supplementary material in Matthews et al., 2012).

Unlike lepidopteran pupae, in which passive suction ventilation occurs during the F-phase (Levy and Schneiderman, 1966b; Lighton, 1996), locusts and other insect taxa, such as cockroaches and beetles, exhibit active tracheal ventilation. We found two different types of CO_2 emission prior to the O-phase that were caused by either slow or fast ventilation (SVF and FVF, respectively). Our results suggest that tracheal oxygen levels can trigger both the slow ventilatory movements (Fig. 8D; see also Huang et al., 2014) and the short and fast ventilation events that occur only under hypoxic conditions (Fig. 7) prior to the O-phase. This may require the presence of more than one oxygen sensor (to trigger the different ventilation motor patterns), and is in accordance with evidence of several internal CO_2-sensor locations (Miller, 1960). Slow ventilatory movement events are typically associated with tight spiracle closure, although when occurring near the end of the interburst (SVF) they result in gas exchange with the external environment, indicating an interaction between low P_{O_2} and high P_{CO_2} thresholds. However, only the CO_2 emission that was coupled to the FVF (which appear in experimental hypoxia) showed a clear and consistent dependence on ambient oxygen availability (Figs 7 and 9, lower trajectories; see also Matthews and White, 2011b). Our data may indicate that the short fast ventilation events (FVF) are caused by oscillations around the P_{O_2} set point. Following spiracle closure, P_{O_2} gradually decreases as oxygen is consumed, followed by an effective fast ventilation that quickly elevates the endo-tracheal P_{O_2} above the threshold, with a reduced effect on the levels of the mostly hemolymph-dissolved CO_2. The oscillation period (and the number of short fast ventilation events) is affected by a different offset of the initial oxygen (at the beginning of the C-phase) level from the normoxic environment conditions. Only when the P_{CO_2} threshold is reached is the prolonged fast ventilation event (O-phase) initiated (Fig. 9). Importantly, our findings demonstrate a clear distinction between two types of 'flutter' events, varying in frequency, which are likely to originate in separate CPGs and respond to different gaseous set points. Such a distinction is missing in the current literature, in which any sporadic CO_2 emission prior to the O-phase is referred to as a 'flutter' phase.

Despite the strong negative correlation between the O-phase duration and the atmospheric oxygen availability, also shown for other grasshopper species (Groenewald et al., 2014; Matthews et al., 2012), it is not clear whether the increase in endotracheal P_{O_2} is directly responsible for the termination of the O-phase. Experimental hyperoxia shortened the O-phase of locusts (Fig. 8B; Matthews et al., 2012; but see Groenewald et al., 2014), suggesting that an appropriate endotracheal oxygen level terminates the O-phase. Interestingly, the O-phase duration was shortened under hyperoxia (40% O_2 in N_2) but not affected when locusts were treated with heliox (21% O_2 in He), when the oxygen diffusion rate was doubled compared with nitrox (21% O_2 in N_2) (E.G., unpublished data). This suggests that convection is dominant in gas exchange between the environment and the tracheal gas sensors involved in DGE cycle dynamics. In contrast to hyperoxia, hypoxic conditions resulted in a longer O-phase duration, presumably as the increase in tracheal P_{O_2} is limited by ambient oxygen availability. Eventually, O-phase termination under these conditions could result from persistent CO_2 washout through the open spiracles, as a threshold P_{O_2} would not be reached, with a resulting decrease in tracheal/hemolymph P_{CO_2} and or increase in hemolymph pH (Matthews et al., 2012).

In conclusion, we employed a novel experimental setup that combined simultaneous electro-physiological and respirometry

recordings to depict the motor activity patterns that underlie DGE in actively ventilating insects. Our observations are in contrast with the classic model of DGE typical of lepidopteran pupae, and we show that the three cycle phases, defined by spiracle states, could not describe respiration-related behavior in actively ventilating locusts. The typical lepidopteran F-phase, fundamental to various adaptive hypotheses for the evolution of DGE (reviewed in Chown et al., 2006), does not appear during locust DGE. Instead, we describe two different CO_2 emission phenomena prior to the O-phase, which are tightly coupled to two different ventilation motor patterns. Using experimental work and the literature, we propose a modified DGE model for actively ventilating insects, which could explain this gas exchange pattern by means of ventilation and internal (endotracheal/hemolymph) gas-sensing control. The exact gas-sensing thresholds and sensor locations are still unknown, however, as are the dynamics of CO_2- and O_2-sensing interactions. In order to further elucidate the CO_2/O_2 sensory control of ventilation during DGE, future studies should include manipulation of environmental CO_2/O_2 while monitoring endotracheal CO_2/O_2 simultaneously with ventilation patterns. Alternatively (or in addition), the study of CO_2/O_2 sensory control could be applied to the more controlled isolated ganglia/*in vitro* level, where fictive ventilatory motor patterns could be recorded while manipulating respiratory gases.

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Author contributions
Conceptualization: E.G., A.A.; Methodology: S.T., E.G., A.A.; Software: S.T.; Validation: S.T.; Formal analysis: S.T.; Investigation: E.G., A.A.; Resources: E.G., A.A.; Data curation: S.T.; Writing - original draft: S.T.; Writing - review & editing: E.G., A.A.; Supervision: E.G., A.A.; Funding acquisition: E.G., A.A.

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